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J. Michael Lord • Martin R. Hartley
Editors

Toxic Plant Proteins

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Editors
Dr. J. Michael Lord
University of Warwick
Dept. Biological Sciences
CV4 7AL Coventry
United Kingdom
Mike.Lord@warwick.ac.uk

Dr. Martin R. Hartley
University of Warwick
Dept. Biological Sciences
CV4 7AL Coventry
United Kingdom
Martin.Hartley@warwick.ac.uk

Series Editor
Professor Dr. David G. Robinson
Ruprecht-Karls-University of Heidelberg
Heidelberger Institute for Plant Sciences (HIP)
Department Cell Biology
Im Neuenheimer Feld 230
69120 Heidelberg
Germany

ISSN 1861-1370 e-ISSN 1861-1362
ISBN 978-3-642-12175-3 e-ISBN 978-3-642-12176-0
DOI 10.1007/978-3-642-12176-0
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010929802

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Editors

Mike Lord studied Biochemistry at the University of Salford, UK, and completed his PhD at the University of Bradford, UK, in 1970. From 1970 to 1972 he worked at the University of California at Santa Cruz as a postdoctoral researcher. He returned to the UK in 1972 as a postdoctoral researcher at the University of Leicester, from where he moved to the University of Bradford as a Lecturer in 1973. He transferred to the University of Warwick as a Reader in Cell Biology in 1982, and he was appointed as Full Professor in Molecular Cell Biology there in 1988. His research interests have focussed on how protein toxins, such as the ribosome-inactivating protein ricin, enter and intoxicate mammalian cells.

Martin Hartley studied Botany at Imperial College, London, and completed his PhD there in 1970 in Plant Molecular Biology. He was appointed to the University of Warwick in 1970 where he worked on various molecular aspects of chloroplast development, including the biogenesis of chloroplast ribosomes. In 1987, he became interested in the action of ribosome-inactivating proteins on ribosomes and the physiological rationale for the production of ribosome-inactivating proteins by plants.

Preface

Ribosome-inactivating proteins (RIPs) are a diverse group of proteins with an RNA *N*-glycosidase activity that irreversibly inactivates ribosomes through an active site – the RIP domain – that is unique. They are widely distributed among higher plants and a few species of bacteria, and in both kingdoms, the enzymatically active protein (the A chain) has become fused to a lectin or ceramide-binding B-chain giving rise to A–B toxins that are among the most potent cytotoxic agents known. Although there is very good evidence that these AB toxins have evolved to fulfil their toxic roles, it is only for the bacterial toxins Shiga toxin (from *Shigella dysenteriae*, responsible for outbreaks of bacillary dysentry) and the related Shiga-like toxin (from certain enterohemorrhagic strains of *Escherichia coli*) that their biological roles are known with certainty. For plant toxic lectins, such as ricin from the seeds of *Ricinus communis*, the biological role is less clearly understood. Many plants produce single-chain RIPs equivalent to the A chain of the toxic lectins. These are mainly active on their own (conspecific) ribosomes, are secreted into the apoplast, and have been postulated to have an anti-viral role, possibly by depurinating capped viral RNAs. In the Poaceae, they are cytosolic proteins and could be involved in the senescence of tissues/organs, as in the case of the wheat coleoptile.

RIPs have attracted the attention of researchers from several different backgrounds. Those interested in ribosome structure and function have studied the role played by the domain in 28S rRNA upon which RIPs act. Cell biologists have used ricin as a model for understanding retrograde transport and membrane dislocation processes in animal cells, which has led to the possibility of using disarmed versions of ricin to deliver fused peptides into cells, leading to MHC class 1-restricted antigen presentation and the development of novel vaccination strategies. There has been considerable interest in using the catalytic chains of RIPs as the toxic moiety of immunotoxins directed against cancer cells. Neurobiologists have used the specificity of uptake afforded by certain neuropeptides to use neuropeptide-RIP fusions to ablate cells of the CNS. Plant biotechnologists are attempting to use RIPs to engineer plants to resist attack by pathogens.

The last publication of a collection of articles on RIPs was in 2004 to mark the retirement of Fiorenzo Stirpe, one of pioneers in RIP research (Mini Reviews in Medicinal Chemistry 4:461–595). Significant advances have been made in the past 6 years and we feel that it is both timely and appropriate that this monograph should convey some of the developments that have taken place on this interesting, but to some extent enigmatic, class of proteins.

January 2010

J. Michael Lord
Martin R. Hartley

Contents

Evolution of Plant Ribosome-Inactivating Proteins

Willy J. Peumans and Els J.M. Van Damme

Abstract This contribution presents an updated analysis of the evolution of ribosome-inactivating proteins (RIPs) in plants. All evidence suggests that an ancestor of modern seed plants developed the RIP domain at least 300 million years ago. This ancestral RIP domain gave rise to a direct lineage of type 1 RIPs (i.e., primary type 1 RIPs) still present today in many monocots and at least one dicot. In a later stage, a plant succeeded in fusing the RIP domain to a duplicated ricin-B domain acquired from a bacterium. The resulting ancestral type 2 RIP gave rise to all modern type 2 RIPs and by domain deletion, to different lines of "secondary" type 1 RIPs and ricin-B type lectins. In the recent past, at least three other domain fusions took place in the Poaceae family, whereby type AC1 (type 3), type AC2, and type AD chimeric forms were generated.

1 Introduction

Plant ribosome-inactivating proteins (RIPs) are a fairly extended and heterogeneous family of proteins characterized by the presence of a domain equivalent to the toxic A-chain of ricin (or A-subunit of the bacterial Shiga toxins). Basically, the plant RIPs can be subdivided into holoenzymes and chimero-enzymes. Holoenzymes or type 1 RIPs consist solely of a RIP domain whereas the chimero-enzymes are built up of an N-terminal RIP domain linked (at least in the gene) to an unrelated C-terminal domain. Depending on the nature of the latter chain, the chimeric forms are referred to as type 2 RIPs (with a lectinic B-chain) and type 3 RIPs

W.J. Peumans and E.J.M. Van Damme (✉)
Laboratory of Biochemistry and Glycobiology, Department of Molecular Biotechnology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium
e-mail: ElsJM.VanDamme@UGent.be

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_1,

(with an unidentified C-terminal domain). Both type 1 and type 2 RIPs are quite common in plants whereas hitherto only a single type 3 RIP has been isolated and characterized, namely the barley JIP60 (Chaudhry et al. 1994). However, recent genome and transcriptome data revealed the occurrence of homologs in some other Poaceae. Moreover, there is also evidence for yet another chimeric form in rice and *Brachypodium distachyon*. Since none of these "putative novel" RIPs has been studied at the biochemical level, it is precocious to introduce a new nomenclature. Therefore, JIP60 and its homologs will be referred to as type AC proteins, and the additional form found in rice and *Brachypodium* as type AD proteins, to emphasize the fact that they possess a different C-terminal domain.

The issue of the molecular evolution of plant RIPs was already discussed in numerous research and review papers (Barbieri et al. 1993; Peumans et al. 2001; Van Damme et al. 2001; Stirpe and Battelli 2006). Though several aspects of the overall evolution are fairly well understood, some important questions remain to be answered especially with respect to the origin of the RIP domain, the relationships between type 1 and type 2 RIPs, and the origin of the type 3 RIP. One of the major problems encountered in the study of the evolution and phylogeny concerns the limited number of sequences and the patchy taxonomic distribution of plant RIPs. Fortunately, the wealth of information provided by genome and transcriptome sequencing programs allows composing a more detailed overview of the occurrence of RIPs in plants and reassessing the interrelationships between the different subgroups. Moreover, the eventual origin of the RIP domain itself as well as the B-chain of type 2 RIPs could also be revised using the sequence information made available for bacteria and eukaryotes other than plants.

This contribution aims to make an updated comprehensive analysis of the overall evolution of plant RIPs. Therefore, an as-complete-as-possible set of sequences was retrieved from the publicly accessible databases and subsequently subjected to a preliminary phylogenetic analysis (using CLUSTALW). Considering the limitations of this method, the results should be interpreted with care. However, the outlines of the analyses give a fairly accurate idea and place the overall evolution of RIPs in a novel perspective. Moreover, the data generated here provide a firm basis for an in-depth phylogenetic analysis with a more performing program.

2 General Overview of the Taxonomic Distribution of A and B Domains within the Viridiplantae

According to the data published in previous research and review papers, the occurrence within the Viridiplantae of both the RIP and the ricin-B domain is confined to the Magnoliophyta (flowering plants) (Van Damme et al. 1998; 2001). To check whether these domains possibly occur in other taxa, a comprehensive analysis of the publicly accessible databases was made. BLAST searches (using

different type 1 and type 2 RIP sequences[1] as queries) in the completed genomes of *Chlamydomonas reinhardtii*, *Chlorella* sp., *Micromonas* sp., *Ostreococcus* sp., and *Volvox carteri* yielded no positive hits indicating, though not proving, that Chlorophyta (green algae) genomes acquired neither the RIP nor the ricin-B domain. Within the Embryophyta, proteins/genes with a RIP domain are apparently confined to the Spermatophyta (seed plants). No A domain could be identified, indeed, in any member of the Anthocerotophyta, Bryophyta, Marchantiophyta, or Euphyllophyta other than Spermatophyta. Though, due to the limited sequence information, one cannot draw definitive conclusions regarding the possible occurrence of this domain in these major taxonomic groups, the apparent absence of the RIP domain in the completed genomes of the moss *Physcomitrella patens* and the club moss *Selaginella moellendorffii* is certainly indicative. Contrary to the A domain, there is compelling evidence of the expression of proteins with a typical ricin-B domain in the liverwort *Marchantia polymorpha*. Analysis of the transcriptome database revealed that thalli and sexual organs of *M. polymorpha* express a set of at least three different proteins comprising two in tandem arrayed ricin-B domains (and hence can be considered the equivalent of the B-chain of a type 2 RIP). One of these expressed proteins has -apart from the N-terminal Met residue- exactly the same sequence as the N-terminus of a galactose-binding lectin isolated from thallus tissue (EVD unpublished results), which leaves no doubt that this liverwort actually expresses a carbohydrate-binding protein of the ricin-B family. It should be noted here that the purified lectin is synthesized without signal peptide and undergoes, apart from the removal of the methionine, no processing at its N-terminus. This implies that the *Marchantia* lectins are unlike all other documented plant lectins of the ricin-B family (which are synthesized with a signal peptide and follow the secretory pathway) (Van Damme et al. 2001). *Marchantia* lectins are synthesized on free ribosomes in the cytoplasm and accordingly destined to reside in the cytoplasmic and/or nuclear compartment.

Hitherto, all purified plant RIPs and cloned plant RIP genes were obtained from Magnoliophyta (flowering plants). No homologs were isolated from or identified in any other seed plant. Transcriptome analyses also yielded no evidence of the expression of RIP genes in Coniferophyta (approximately 800,000 entries), Cycadophyta (22,000 entries), and Ginkgophyta (21,000 entries). In contrast, a recently deposited transcriptome database of *Gnetum gnemon* (10,700 entries) contains a set of three expressed sequence tags (ESTs) encoding two different type 2 RIPs. The latter finding is important because it demonstrates for the first time the occurrence of RIP genes in a seed plant outside the flowering plants. Taking into consideration the very large number of deposited EST sequences (about 800,000 in total) of different *Pinus* and *Picea* species, it seems unlikely that RIP genes are present in the genome of most modern Coniferophyta. Due to the

[1]Sequences of all RIPs used in this study can be retrieved from: http://www.molecularbiotechnology.ugent.be/publications/VanDamme2010A/.

relatively small number of entries no such conclusion can be drawn yet for the Cycadophyta (22,000 entries) and Ginkgophyta (21,000 entries).

Comprehensive BLAST searches of plant transcriptome databases yielded several sequences encoding proteins consisting of a single ricin-B domain (i.e., corresponding to one half of the B-chain of type 2 RIPs). At first sight, the identification of these proteins was exciting because it could give valuable hints with regard to the origin of the B-chain of type 2 RIPs. However, a closer examination indicated that these sequences are not encoded by the plant genome but by a contaminating fungus or other eukaryotic symbiont/parasite. For example, a strongly conserved protein expressed in roots of wheat and poplar, and stolons of potato turns out to be 94% identical to a large set (>250) of ESTs present in the transcriptome of *Hartmannella vermiformis* (a protozoan belonging to the Euamoebida). Hence, it is almost certain that the sequences encoding these "root-specific" proteins are derived from a contaminating amoeba.

3 Overview of the Taxonomic Distribution of A and B Domains within the Magnoliophyta (Flowering Plants)

3.1 "Classical" Type 2 RIPs (AB proteins)

Hitherto, only a relatively small set of type 2 RIPs (<40) has been purified and characterized. Moreover, since all these proteins were isolated from a rather limited number of seed plants (*Ricinus communis*, *Abrus* sp., *Adenia* sp., *Cinnamomum camphora*, *Sambucus* sp., *Viscum* sp., *Momordica charantia*, *Trichosanthes* sp., *Bryonia dioica*, *Panax ginseng*, *Ximenia americana*, *Iris hollandica*, and *Polygonatum multiflorum*) belonging to only 13 genera, it is generally believed that type 2 RIPs are scarcely distributed among flowering plants. However, taking this into consideration, by analogy to other plant lectins, the currently documented taxonomical distribution might well be underestimated. This is because only highly expressed type 2 RIPs have a reasonable chance of being discovered by routinely applied techniques. To address this issue, we searched the publicly accessible databases (mostly transcriptomes) for the occurrence of type 2 RIPs and related proteins with ricin-B domain(s). Evidence was obtained for the expression of one or more genuine type 2 RIP homologs in several species with no "RIP history." The list of novel species includes flowering plants from most major taxa: Ranunculaceae (*Adonis aestivalis*); Asteraceae (*Helianthus* sp., *Centaurea* sp., *Artemisia annua*), Ericales (Actinidiaceae: *Actinidia deliciosa* or kiwi fruit; Polemoniaceae: *Ipomopsis aggregata*; Theaceae: *Camellia sinensis* or tea plant); Cucurbitaceae (*Cucumis sativus*), Rosaceae (*Malus domestica* or apple); Malvaceae (*Gossypium* sp.), Sapindaceae (*Paullinia cupana* or guarana), Poaceae (*Triticum aestivum*, *Saccharum officinarum*, *Sorghum* sp., *Zea mays*, *Panicum virgatum*), and Arecaceae (*Elaeis guineensis* or oil palm).

3.2 Other Proteins with Ricin-B Domains

Proteins consisting exclusively of polypeptides equivalent to the B-chain of type 2 RIPs have been isolated from several *Sambucus* species (e.g., fruit lectin SNA-IV) (Girbés et al. 2004; Van Damme et al. 1997b) and from cucumber (*C. sativus*) roots (XSP30) (Masuda et al. 1999). Moreover, it seems quite likely that several lectins previously isolated from different *Euphorbia* species are built up of B-chains only (Stirpe et al. 1993). The searching of databases yielded only a few novel B-type proteins. Transcriptome analysis confirmed that *Euphorbia esula* expresses several proteins consisting of B-chains only. In addition, a set of five different but closely related B-chain proteins (most of which are expressed) could be identified in the genome of *Populus trichocarpa*. The same genome also contains a gene encoding a protein consisting of a single ricin-B domain, but no corresponding ESTs could be retrieved.

4 Molecular Evolution of Type 2 RIPs

4.1 General Observations Concerning the Taxonomic Distribution of Type 2 RIPs and the Occurrence of Multiple Paralogs

In spite of the tremendous amount of (multidisciplinary) research devoted to ricin and related proteins, the molecular evolution and especially the evolutionary origin of type 2 RIPs is still far from understood. At present, no direct evolutionary link can be made between type 2 RIPs from modern seed plants and any protein from lower Viridiplantae (or any other organism). It seems likely, therefore, that type 2 RIPs were developed by a direct ancestor of modern seed plants. Until recently type 2 RIPs were believed to occur exclusively in flowering plants (Van Damme et al. 2008). However, novel data leave no doubt that genuine homologs are also expressed in at least one species of the Gnetophyta. On the basis of this updated taxonomic distribution (summarized in Tables 1 and 2) one can reasonably conclude that type 2 RIP gene(s) were already present in the common ancestor of the Gnetophyta and Magnoliophyta, and accordingly exist for over 300 million years (Palmer et al. 2004). The reasonably high sequence identity between the homologs from *G. gnemon* and those from flowering plants can be perfectly explained by a classical vertical inheritance along the main evolutionary lineages of the seed plants. However, there are a few obvious peculiarities. First, the type 2 RIP genes were apparently not transferred into all daughter lineages of the common ancestor of all modern seed plants. Second, type 2 RIP genes are certainly not ubiquitous in all taxa of flowering plants but seem to be patchly distributed. It is certainly true that (many) other homologs remain to be discovered. However, genome sequencing clearly demonstrated that they are absent from, e.g., *Arabidopsis thaliana*,

Table 1 Summary of the documented occurrence of the four different types of ribosome inactivating proteins within the Embryophyta (terrestrial plants)

Anthocerotophyta (hornworts)		No sequences found
Bryophyta (mosses)		No sequences found; A and B domain are absent from the genome of *Physcomitrella patens*
Marchantiophyta (liverworts)		Several proteins comprising two in tandem arrayed ricin B domains are expressed in *Marchantia polymorpha*. No expressed protein with a RIP domain could be retrieved.
Tracheophyta (vascular plants)	Lycopodiophyta (club mosses)	No sequences found; A and B domain are absent from the genome of *Selaginella moellendorffii*
	Euphyllophyta	Moniliformopses Equisetophyta (horsetails): no sequences found Filicophyta (ferns): no sequences found Spermatophyta (seed plants) Coniferophyta: no sequences found Ginkgophyta: no sequences found Cycadophyta: no sequences found Gnetophyta: type AB from *Gnetum gnemon* Magnoliophyta (flowering plants): A, AB, B, AC and AD*

*See Table 2 for a detailed overview

P. trichocarpa, *Medicago truncatula*, *Glycine max*, *Vitis vinifera*, *Carica papaya*, *Oryza sativa,* and *B. distachyon*. Third, within a given taxon/family, type 2 RIPs might be confined to a single species/genus. For example, apart from two *Abrus* species, type 2 RIPs were not found in any other legume species (despite the fact that the legume family has been extensively explored for the occurrence of lectins). Fourth, in some species, type 2 RIP genes were (strongly) amplified. For example, genome analysis revealed that the genome of *R. communis* contains at least eight type 2 RIP genes. Similarly, the expression of complex mixtures of type 2 RIP in *Sambucus* and *Viscum* species can only be explained by the occurrence of multiple genes.

4.2 Overall Phylogeny of Type 2 RIPs

To further corroborate the evolutionary relationships a phylogenetic analysis was made of all type 2 RIPs for which a (near) complete sequence is available or can be assembled from EST sequences. As shown in Fig. 1 the dendrogram of the type 2 RIPs does not reflect the phylogeny of the species in which they occur but exhibits several obvious anomalies. Only the proteins found in *Sorghum* sp., *S. officinarum*, *Z. mays,* and *P. virgatum* form a distinct clade with a "normal" phylogeny. All other Liliopsida proteins (i.e., those from *I. hollandica*, *E. guineensis,* and *P. multiflorum*) are placed in two different branches together with homologs from unrelated Eudicots/Magnoliids. The most striking anomaly concerns the type

Table 2 Summary of the documented occurrence of the four different types of ribosome inactivating proteins in Magnoliophyta (flowering plants)

Eudicotyledons	Stem Eudicotyledons	Ranunculales			Ranunculaceae	AB*
	Core Eudicotyledons	Asterids		Campanulids	Araliaceae Apiaceae Asteraceae Adoxaceae	AB A AB AB, B
				Ericales	Actinidiaceae Ebenaceae Polemoniaceae Theaceae	AB, B B AB AB, B
				Lamiids	Lamiaceae	A
		Caryophyllales			Aizoaceae Amaranthaceae Caryophyllaceae Nyctaginaceae Phytolaccaceae	A A A A A
		Santalales			Loranthaceae Olacaceae	AB AB
		Rosids	Eurosids I	Cucurbitales	Cucurbitaceae	AB, A
				Fabales	Fabaceae	AB
				Malpighiales	Euphorbiaceae Passifloraceae Salicaceae	AB, A, B AB A, B
				Rosales	Cannabaceae Rosaceae	A A, AB
			Eurosids II	Malvales	Malvaceae	AB
				Sapindales	Sapindaceae	AB
Liliopsida	Asparagales				Agavaceae Asparagaceae Iridaceae Ruscaceae	A A AB, A AB, A
	Commelinids	Arecales			Arecaceae	AB
		Poales			Bromeliaceae	A
			Poaceae	BEP clade	Bambuseae Oryzeae Pooideae	A A, AD A, AB, AC, AD
				PACCAD clade	Panicoideae	A, AB, AC
Magnoliids	Laurales				Lauraceae	AB

*A: type 1; AB: type 2; AC: type 3; AD: new chimeric RIP; B: lectinic B chain
For more details on taxonomic distribution of ribosome-inactivating proteins, please see Supplementary data 2 on http://www.molecularbiotechnology.ugent.be/publications/VanDamme2010A/

2 RIPs from *P. multiflorum* (Van Damme et al. 2000), which are placed in the same cluster as the *Sambucus nigra* proteins. Evidently, the anomalous phylogeny raises some questions with regard to the evolution of type 2 RIPs in flowering plants. To check whether the anomalies might be due to pronounced differences between the evolution of the RIP and the lectin domain, the same phylogenetic analysis was made using sequences of the respective A and B-chains. Though these analyses yielded slightly different results, major anomalies persisted (results not shown). For example, both the A and B-chain of the *P. multiflorum* proteins were invariantly placed in the *Sambucus* cluster.

Summarizing one can conclude that the origin of the type 2 RIP family predates the common ancestor of the Gnetophyta and the Magnoliophyta. The ancestral gene

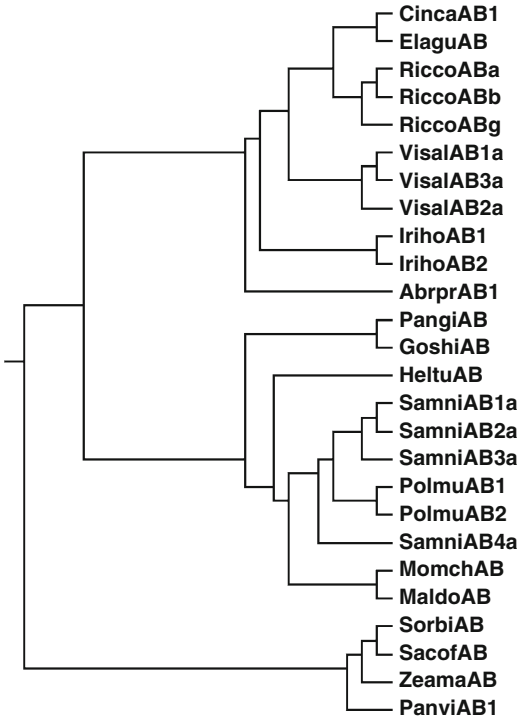

Fig. 1 Phylogenetic analysis of the currently identified type 2 RIPs. Only entries for which complete sequences are available are included. To reduce the complexity of the dendrogram a number of highly similar paralogs from a single species or highly similar orthologs from related species are not included. For more details on the sequences of the ribosome-inactivating proteins and the codes used for each RIP, please see Supplementary data 1 and 3 on http://www.molecularbiotechnology.ugent.be/publications/VanDamme2010A/

was vertically transmitted into the daughter lineages but during further evolution it purged from the genome of most Spermatophyta. This process of gene loss still occurred in evolutionary recent terms and might possibly continue today (as is suggested by the patchy distribution in, e.g., the Poaceae family). The retained genes evolved along with the evolution of the seed plants but it seems that some events took place that eventually resulted in a few obvious but unexplained phylogenetic anomalies.

4.3 Special Evolutionary Events: Gene Amplification and Generation of Type A and Type B Proteins from Genuine Type 2 RIPs

Apart from the general scheme described above, the molecular evolution of type 2 RIPs includes some peculiar events. In several unrelated species gene amplification

has taken place. The occurrence of multiple genes was already inferred from the fact that some plants like, e.g., *Sambucus* sp and *Viscum album* express complex natural mixtures of "isoforms" (Girbés et al. 2004; Mishra et al. 2004). Full details about such a gene family were provided by complete sequencing of the castor bean genome. This genome contains at least eight genuine type 2 RIP genes and several (at least eight) pseudogenes (with an incomplete or interrupted ORF). In addition, a type 2 RIP gene could be retrieved that encodes a protein with a heavily truncated B-chain (only the first 44 amino acid residues are left). Interestingly, a perfectly matching EST sequence is deposited, which indicates that the truncated protein might be expressed. Though there is no experimental evidence yet for the presence of the corresponding protein in castor bean tissue, the truncated AB gene illustrates that a type 1 RIP can be generated from a genuine type 2 RIP through the deletion of the B domain. This observation is not merely anecdotal but has important consequences for what concerns the molecular evolution of type 1 RIPs. As was already suggested on several occasions (Peumans et al. 2001; Van Damme et al. 2001) some type 1 RIPs (e.g., from *I. hollandica*) (Van Damme et al. 1997a) are more closely related, indeed, to type 2 RIPs from the same or a related species than to any other (genuine) type 1 RIP. Analysis of the castor bean genome now provides a firm basis for the formerly predicted "domain-deletion" origin of some type 1 RIPs and as such urges the reassessment of the evolutionary origin of the whole group of type A proteins (which is discussed in detail in a separate section).

Comprehensive biochemical and molecular analyses demonstrated that *Sambucus* sp. also express a very complex set of both genuine and truncated type 2 RIP genes and revealed that within this taxonomic group an evolutionary event took place whereby a genuine type 2 RIP (called SNA-V) was converted in a type B protein (SNA-II) through a deletion of the RIP domain (Van Damme et al. 1996, 1997b). To check whether the origin of the other identified type B proteins relies on a similar mechanism, a phylogenetic analysis was made of the sequences of these proteins and the corresponding sequences of the B-chain of type 2 RIPs. According to the dendrogram shown in Fig. 2, the B-type proteins found in *P. trichocarpa* cluster with the type 2 RIP from *Adenia volkensii* (which belongs to the same order Malpighiales as poplar) indicated that they are derived, indeed, from a genuine type 2 RIP through deletion of the A domain – an event that most probably took place after the Passifloraceae and Adoxaceae diverged from the Euphorbiaceae. Since the parent type 2 RIP gene is no longer present in poplar, it must have been purged from the genome during the evolution of the lineage Malpighiales that led to modern poplars. At the same time, the original gene encoding a B-type protein was amplified by two in tandem duplications followed by a region/chromosome or genome wide duplication. In addition to B-type genes,the poplar genome contains a gene encoding a protein consisting of a single ricin-B domain (or a half B-chain). The origin of this gene is not clear. It exhibits the highest similarity with the C-terminal domain of the B-type proteins in poplar but the sequence identity is relatively low (approximately 35% within the ricin-B domain) indicating that there is most likely no direct evolutionary link. Possibly the single ricin-B domain gene

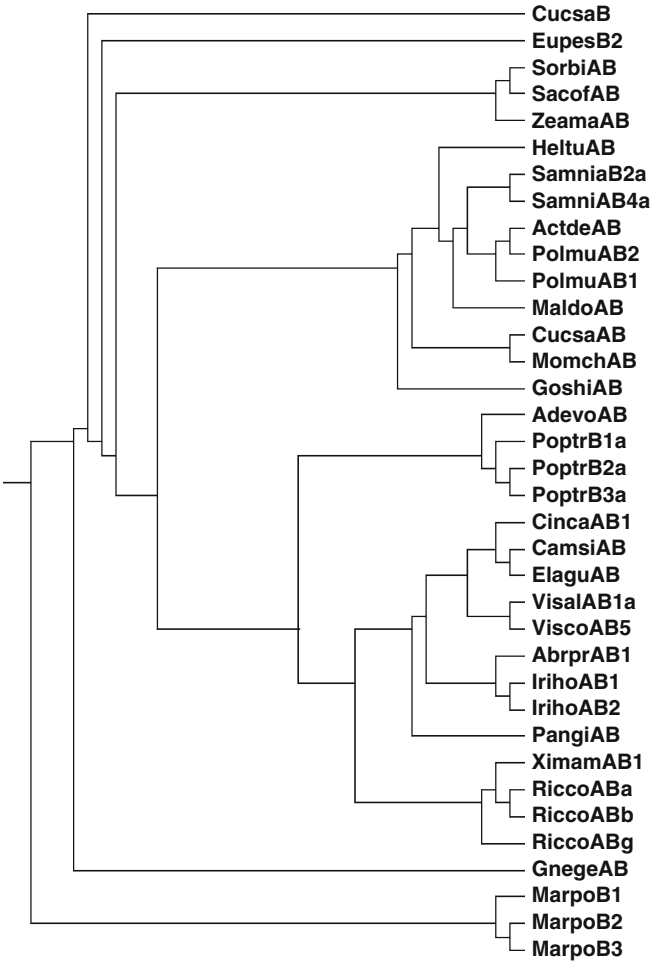

Fig. 2 Phylogenetic analysis of all identified plant B-chain proteins and a selected set of the B-chains of type 2 RIPs. To reduce the complexity of the dendrogram a number of highly similar type 2 RIP paralogs from a single species or highly similar orthologs from related species are not included

results from a different evolutionary event whereby both the A domain and the first half of the B domain were deleted from a genuine type 2 RIP gene.

Taking into consideration the obvious evolutionary origin of the *Sambucus* and *Populus* B-chain proteins, the position in the dendrogram of the homologs from *C. sativus* and *E. esula* also can be reconciled with a similar domain-deletion event. However, in these two cases the respective evolutionary events took place most likely in a distant past.

A final important conclusion to be drawn from the dendrogram shown in Fig. 2 concerns the cytoplasmic B-chain proteins expressed in *M. polymorpha*. These proteins are apparently unrelated to any other ricin-B protein found in plants and

accordingly are not likely candidate ancestors of the ricin-B domain found in type 2 RIPs and related proteins. As matter of fact, the liverwort proteins share little sequence similarity with any known protein (the best match is an α-galactosidase from the Actinomycete bacterium *Catenulispora acidiphila* DSM (gb|EEN35459.1|) and hence might represent a separate subgroup of the ricin-B family).

4.4 What is the Origin of Type 2 RIP Genes?

Hitherto, no homologs of the type 2 RIPs have been identified outside the seed plants, which strongly indicates that this chimeric protein was developed within the lineage leading to the modern Spermatophyta (or alternatively was acquired by non vertical inheritance from an unidentified organism that lost the corresponding gene or became extinct). All that can be stated with certainty is that the ancestor of today's type 2 RIPs already existed before the Gnetophyta and Magnoliophyta lineages diverged from each other. Evidently, the chimeric structure must result from an evolutionary event whereby a RIP domain was fused to a duplicated ricin-B domain.

4.4.1 Origin of the B-Chain

On the basis of the widespread distribution over all major prokaryotic and eukaryotic taxa, one can reasonably assume that the ricin-B fold was developed by bacteria very early in evolution and transmitted into all major eukaryotic lineages. Unfortunately, because of the apparent absence of the (sequenced) genomes of Chlorophyta, it is impossible to trace the origin of the plant ricin-B domain back to the common ancestor of plants and other eukaryotes. Sequence comparisons revealed that the B-chain of the type 2 RIPs shares the highest similarity with the (double ricin-B domain) carbohydrate-binding part of an (extracellular or secreted) β-glycosidase-like glycosyl hydrolase (gb|EEN27866.1|) and an α-L-arabinofuranosidase B family protein (gb|EEN23780.1|) from the Actinomycete bacterium *C. acidiphila*. Moreover, all the cysteine (Cys) residues that stabilize (by four disulfide bonds) the rigid fold of the B-chain of the plant type 2 RIPs are also present in these bacterial sequences whereas virtually all other eukaryotic ricin-B domains lack disulfide bonds. The latter fact is not surprising because apart from type 2 RIPs and related proteins most eukaryotic proteins with ricin-B domains are synthesized on free ribosomes in the reducing cytoplasm where disulfide bonds are not usually formed. Hence, all evidence suggests that at a given time (at least predating Gnetophyta and Magnoliophyta lineages) in the evolution of the lineage Spermatophyta, a plant acquired a gene encoding a protein consisting of a duplicated Cys-rich ricin-B domain by lateral transfer from a bacterium. Possibly, the cytoplasmic ricin-B proteins found in *M. polymorpha* were also acquired by a similar lateral transfer from a bacterium, but in this case the bacterial gene encoded a cytoplasmic (Cys-poor) protein.

4.4.2 Origin of the A-Chain

The A domain is far less common than the ricin-B domain. Apart from plants, it seems to be confined to bacteria and viruses of the (entero) bacteriophage group. Moreover, within the bacteria the prokaryotic homolog of the type 1 RIP (called Shiga and Shiga-like toxins A component) is rather rare as it is found only in a small number of species. Proteins were identified in not more than 13 species, including *Streptomyces coelicolor*, 11 Enterobacteriaceae sp., and *Acinetobacter haemolyticus*. This very narrow taxonomic distribution (especially when compared to the widespread occurrence in plants) raises some questions with regard to the presumed bacterial origin. An alternative explanation might be that the RIP domain was developed by plants and acquired by some bacteria through lateral gene transfer. The latter (admittedly speculative) hypothesis is supported by the fact that the target of the Shiga toxins (globotriaosylceramide, a typical animal glycolipid), was developed later in evolution than the RIP domain, which implies that there was no selective pressure for bacteria to develop the RIP domain before it was already present in plants. Irrespective of the true origin, the present taxonomic distribution indicates that the A domain was developed or acquired by an early seed plant before the Gnetophyta and Magnoliophyta lineages diverged from each other. No type 1 RIP could be found in the transcriptome of *G. gnemon* but the limited number of sequences does not allow the conclusion that the corresponding gene is absent. Unfortunately, this implies that no direct link can be made between the A chain of the *G. gnemon* type 2 RIPs and a possible type 1 RIP from the same species.

5 Molecular Evolution of Type 1 RIPs

Type 1 RIPs are not only more common in seed plants they also exhibit a much higher (sequence) heterogeneity than type 2 RIPs. In the past, type 1 RIPs were usually subdivided in three groups (Van Damme et al. 2001). The first group comprises the "classical" type 1 RIPs found in numerous dicotyledons (e.g., in Cucurbitaceae, Phytolaccaceae, and Amaranthaceae species). All these RIPs are synthesized on the ER and follow the secretory pathway to their final subcellular destination (vacuole/extracellular space). The second group is exemplified by the RIPs that were isolated from wheat, barley, and some other grasses. These Poaceae RIPs are synthesized without signal peptide and presumably reside in the cytoplasm. Members of the third group are also found in Poaceae and more precisely in *Z. mays* and related (Panicoideae) species (Walsh et al. 1991; Hey et al. 1995). What is special about this group is the fact that they are synthesized (on free ribosomes) as inactive precursors that are converted into an enzymatically active form through the proteolytic removal of a short peptide at both the N- and C-terminal end, and the excision of an internal peptide. As a result the active RIP consists of two different polypeptides and accordingly is also referred to as a "two-chain" type 1 RIP.

Most attempts to elucidate the phylogenetic relationships were based on the assumption that the family of type 1 RIPs is, notwithstanding the obvious heterogeneity, monophyletic. However, as earlier work with type 1 and type 2 RIPs from *I. hollandica* (Van Damme et al. 1997a) and phylogenetic analyses of type 1 and type 2 RIPs already indicated (Van Damme et al. 2001), sequencing of the castor bean genome provided firm evidence that type 1 RIPs are generated from parent type 2 RIP genes. Since these two examples might be indicative of a more general evolutionary mechanism the phylogeny of type 1 RIPs was reassessed using a novel approach. At the same time the phylogeny was refined by incorporating recent sequence data. Taking into account that the type 1 RIPs from Poaceae differ in several aspects from those found in dicots and monocots other than Poaceae, the two groups were first analyzed individually. After establishing the phylogenetic relationships within each group, a comprehensive analysis was made of all plant RIPs using a selected set of sequences of both groups. Finally, to corroborate the possible link with bacterial RIPs, a selection of bacterial sequences was included in the final analysis.

5.1 Dicots and Monocots Other Than Poaceae

Due to the large number of sequences, it was virtually impossible to make a phylogenetic analysis in a single step. Therefore, a reiterative process was followed whereby smaller sets of sequences from taxonomically related species were analyzed. To reduce the complexity, highly similar paralogs from single species and/or orthologs from related species were omitted and the limited set of sequences combined with similarly reduced sets from other taxonomic groups. Using this approach, a fairly accurate dendrogram could be generated of the sequences of all type 1 RIPs except those from the Poaceae species. As shown in Fig. 3, the resulting dendrogram comprises three distinct clades. The first clade, which groups all Caryophyllales type 1 RIPs comprises two side branches corresponding to two distinct forms. Since both forms are found in *Beta vulgaris* (Amaranthaceae) and *Mesembryanthemum crystallinum* (Aizoaceae), they most probably result from a gene or genome duplication that predates the separation of the different Caryophyllales families. The second major clade comprises all Eurosids I sequences and consists of two-side branches with a cluster of Euphorbiaceae and Cucurbitaceae proteins, respectively. The only documented type 1 RIPs from Rosaceae (from *Prunus* sp.) and Ericales (from *C. sinensis*) are also placed in the Cucurbitaceae cluster. The third clade is well separated from the two other groups – the type 1 RIPs from the dicot *P. trichocarpa* and the monocots *Muscaria*, *Asparagus,* and *Ophiopogon* – and thus covers a broader taxonomic range than the other clades. The main conclusion that can be drawn is that apart from a few exceptions the dendrogram of the type 1 RIPs reflects the taxonomy of the dicots. However, the dendrogram also reveals the occurrence of a second more distant group found in both dicots and monocots.

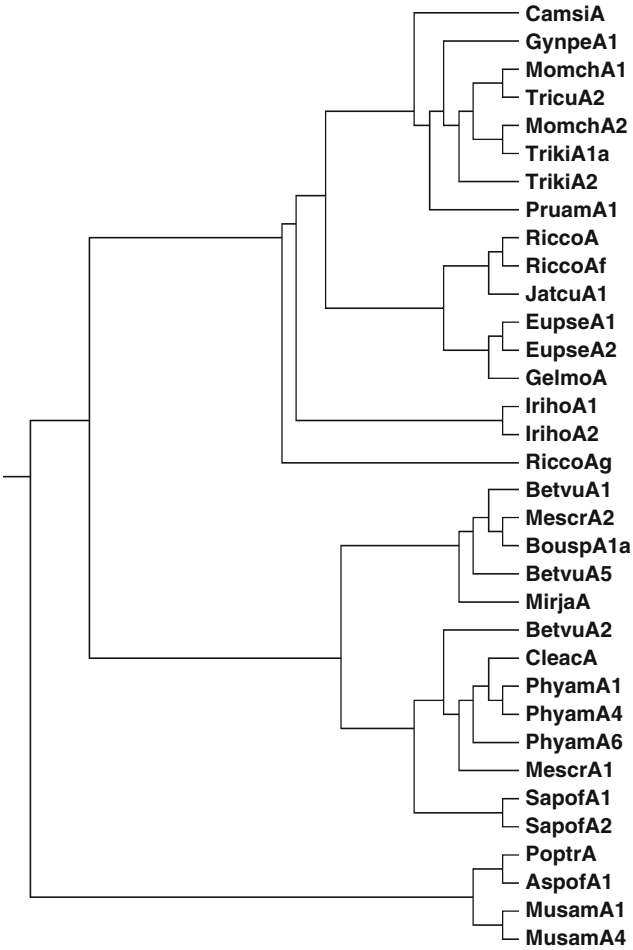

Fig. 3 Phylogenetic analysis of type 1 RIPs from dicots and monocots other than Poaceae. Highly similar paralogs from a single species or highly similar orthologs from related species are not included

To corroborate which groups of type 1 RIPs are the products of domain-deletion (of AB genes), the phylogenetic analysis was extended by incorporating the A domain (including signal peptide) of type 2 RIPs (Fig. 4). The resulting dendrogram leaves no doubt that the type 1 RIPs from iris originated from a conspecific type 2 RIP in an evolutionary recent past. The same applies to the type 1 RIPs from Rosids (*Malus* and *Momordica*), but in this case the domain loss apparently predates the divergence of the Cucurbitales and Rosales. Reasoning along the same line, it seems likely that the Euphorbiaceae type 1 RIPs also evolved from type 2 RIPs in a more distant past. The dendrogram leaves some

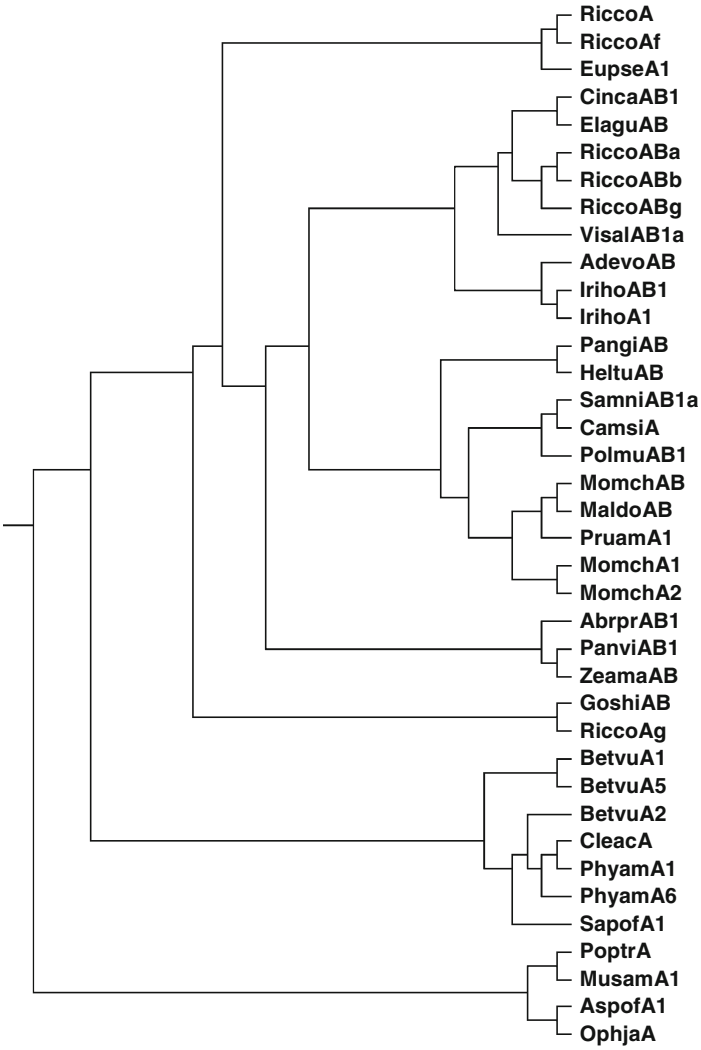

Fig. 4 Preliminary phylogenetic analysis of a selected set of sequences of type 1 RIPs from dicots and monocots other than Poaceae and the N-terminal domain (including signal peptide) of type 2 RIPs. Only sequences relevant for the issue of the B domain loss origin of most dicot type 1 RIPs are included

uncertainty with regard to the origin of the homologs from Caryophyllales and *Clerodendron* (but as discussed below, they were also derived in a more distant past from a type 2 RIP gene). This implies that only the sequences in the "aberrant" clade with *Populus*, *Muscaria,* and *Ophiopogon* are derived from a genuine type 1 RIP.

5.2 *Poaceae Type 1 RIPs*

It has been known for more than a decade that many Poaceae species express complex mixtures of type 1 RIPs. However, recent genome and transcriptome sequencing data revealed that the complexity inferred from biochemical and molecular analyses is still an underestimation of the total RIP gene complement. The following examples illustrate the complexity of the RIP gene family in Poaceae.

5.2.1 *O. sativa*

In a recent study, Jiang et al. (2008) reported that the "RIP domain family" in *O. sativa* comprises 31 different members. A careful reanalysis of the genome indicated that six presumed RIP genes are pseudogenes or part of a transposon. Moreover, at least three additional genes could be identified (which are not annotated yet) yielding a total number of 28 genes. Phylogenetic analyses indicated that the rice genes cluster in four major clades (Fig. 5) and confirmed the conclusion drawn by Jiang et al. (2008) that the expansion of the RIP gene family is primarily based on genome-wide duplications and to a lesser extent on tandem duplications.

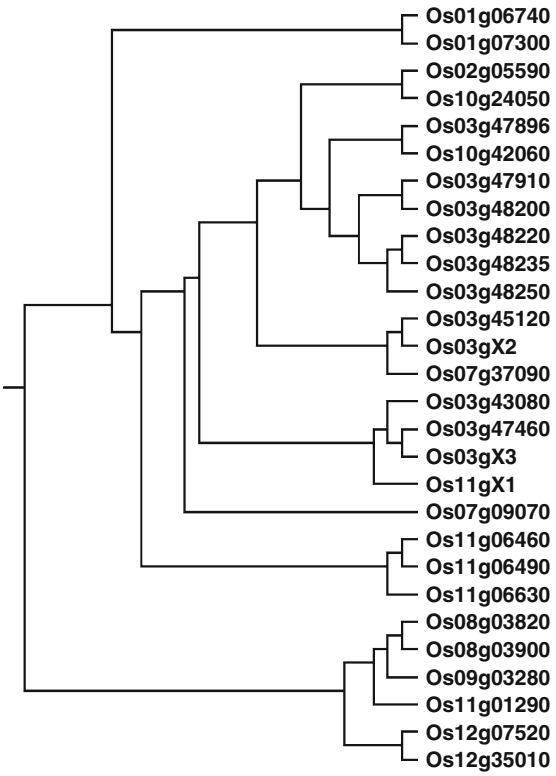

Fig. 5 Phylogenetic analysis of the *Oryza sativa* RIP family. Several annotated sequences have been corrected. For Os11g06460 and Os11g06490 (which are AD type chimers) sequences corresponding to the A domain were used

5.2.2 Andropogoneae: *Z. mays* and *Sorghum bicolor*

Similar analyses indicated that the RIP gene family in the genomes of *Z. mays* and *S. bicolor* is less extended than in *O. sativa,* but is still fairly complex. However, unlike rice these two Andropogoneae species possess a genuine type 2 RIP gene (Figs. 6–9). In addition, a set of two type AC chimeric genes occurs in the genome of maize but not in that of sorghum. The dendrogram of the combined maize and sorghum gene families consists of two major clades one of which comprises three side branches. Apart from the maize AC type gene, all other types of genes are present in both species but there are obvious differences in the number of genes in

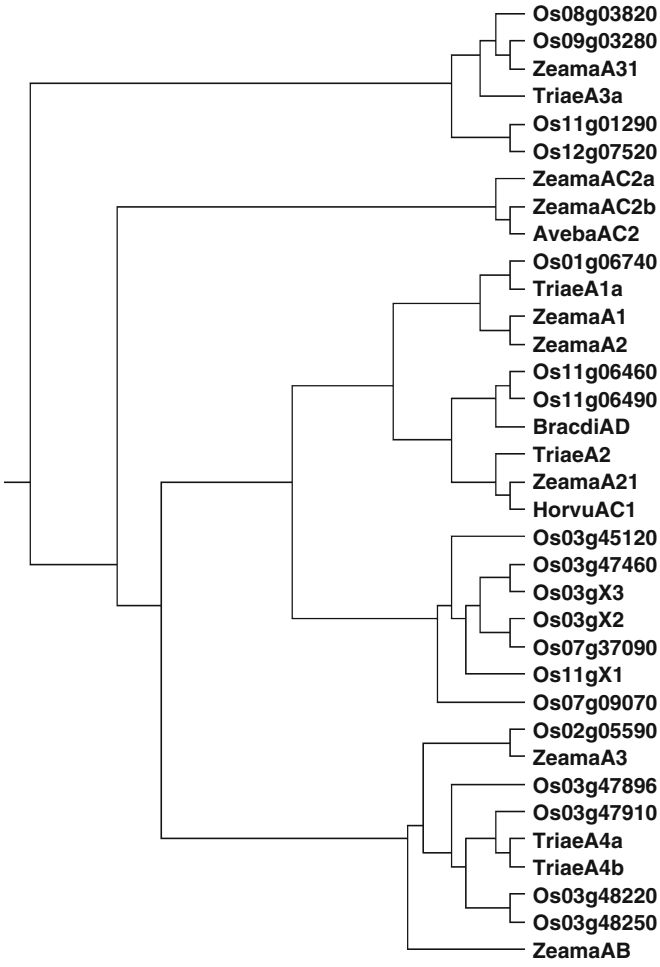

Fig. 6 Phylogenetic analysis of the RIP gene family within the family Poaceae. To reduce the complexity of the dendrogram three limited sets of sequences from *Triticum aestivum*, *Zea mays,* and *Oryza sativa* were combined

each branch. This indicates that the expansion of the RIP gene family in maize and sorghum was differentially affected by gene duplication.

5.2.3 Pooideae

Hitherto *B. distachyon* is the only Pooideae species for which a complete family of RIP genes can be retrieved.[2] The RIP gene family consists of four different type 1 and one type AD RIP gene and hence is far less complex than in rice, maize, and sorghum.

Though no genomic data are available for any other Pooideae species the vast amount of transcriptome data leave no doubt that, e.g., wheat and barley possess extended RIP families. The same applies most probably to *Secale cereale, Leymus cinereus, Aegilops speltoides,* and *Pseudoroegneria spicata.*

To corroborate the evolution of the RIP gene families within the Poaceae family the overall phylogeny was analyzed using three sets of sequences from rice, maize, and wheat, respectively. To reduce the complexity highly similar orthologs/paralogs (clustering in a single branch or side branch) were omitted (except one or two). As shown in Fig. 6, the dendrogram of the combined Poaceae sequences closely resembles that of the rice RIP gene family except that a branch with a type 2 RIP is introduced. This confirms the conclusion by Jiang et al. (2008) that the RIP gene family has largely evolved in parallel to species evolution within the family Poaceae. However, there seem to be important differences in what concerns the contribution of tandem duplications in different representatives. Moreover some species possess genes that are absent in others. For example, the type AB and AC genes present in and expressed by the maize genome are definitely absent from the rice genome. This finding indicates that the (still ongoing) evolution of the RIP gene family within the family Poaceae is to a certain extent determined by gene loss.

5.2.4 Relationships between the RIPs from Poaceae and Other Seed Plants

Though the Poaceae type 1 RIPs are usually regarded as a separate group, one can reasonably assume that they are somehow related to homologs from both other monocots and dicots. Therefore, a phylogenetic analysis was made of the combined sequences of type 1 RIPs from both dicots and all monocots (and supplemented with the A domain of type 2 RIPs). According to the results shown in Fig. 7, the issue of overall evolution of type 1 and type 2 RIP genes needs a thorough update. All sequences, apart from an orphan sequence found in wheat, cluster in two clades. One of these clades comprises all known dicot type 1 RIPs except that from poplar and all type 2 RIP sequences, whereas the second clade groups all monocot type 1

[2]These sequence data were produced by the US Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/.

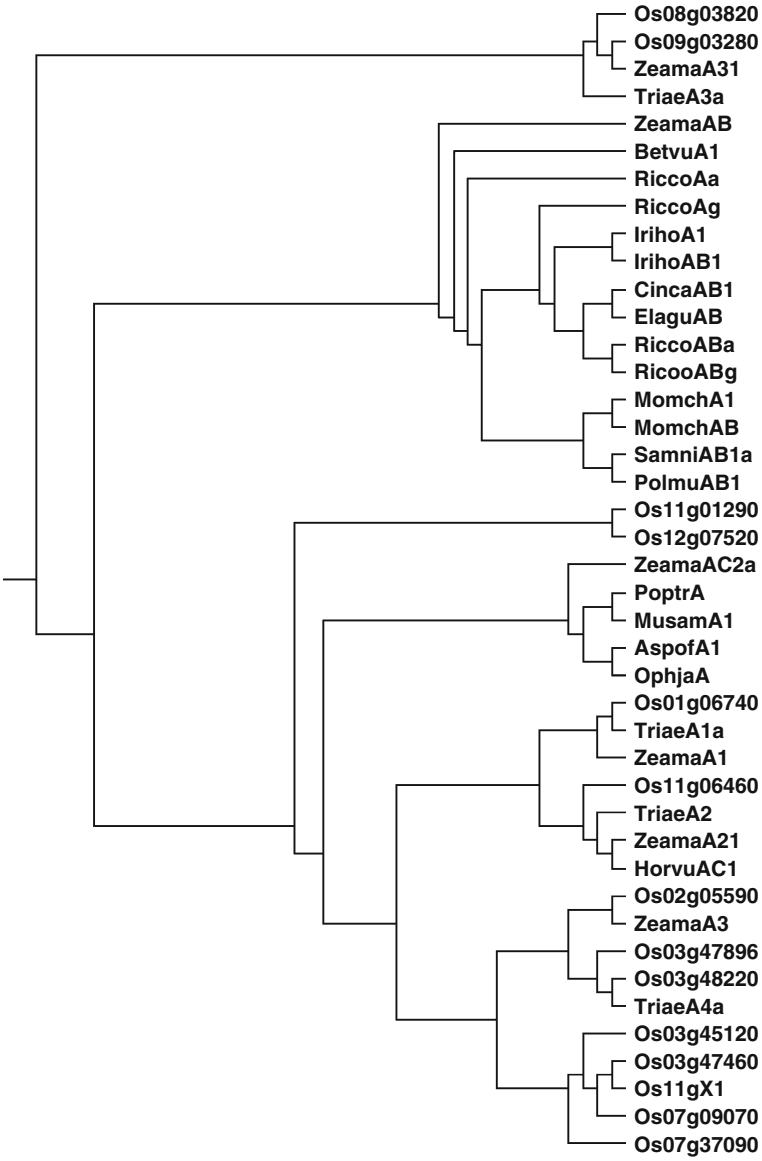

Fig. 7 Phylogenetic analysis of all plant RIPs

RIPs and the poplar sequence. This overall pattern clearly indicates a double phylogenetic origin. The second clade most probably represents a lineage that starts with an ancestral type A gene and leads to the monocot and poplar genes (and hence can be considered "primary type 1 RIPs" whereas the first clade represents a line of "secondary" type 1 RIPs that are derived from the type 2 RIP lineage through multiple B domain loss events.

6 What is the Relationship between Plant and Bacterial RIPs?

Several bacterial species (and bacteriophages) possess genes encoding one or more proteins with an RIP domain. Though it is generally accepted that the bacterial and plant sequences have a common origin, the exact relationships remain to be elucidated. Therefore, it seemed worthwhile to include the bacterial proteins in the same dendrogram as the plant proteins (Fig. 8). Only a limited number of bacterial proteins with an RIP domain have been identified. The best known are the Shiga toxin A subunit and (closely) related proteins found in *Escherichia coli*, *Shigella* sp., and some other Enterobacteria. All these proteins can be considered homologs of plant type 1 RIPs. In addition, a larger chimeric gene with an N-terminal RIP domain linked to an unrelated domain could be identified in *Micromonospora* sp. When added to the set of plant sequences the bacterial proteins do not cluster in a single prokaryotic clade but are placed at different positions. For example, the A domain of the *Micromonospora* protein forms a small side branch together with one of the type 1 RIP from wheat whereas the other bacterial proteins represent two small side branches of the type 2 RIP clade. Additional evidence against a monophyletic origin of the bacterial sequences comes from the observation that the *Micromonospora* protein is not retrieved by BLAST searches using, e.g., the Shiga toxin as a query, but is readily detected with the *Muscaria* type 1 RIP sequence. This, taken together with the narrow and patchly taxonomic distribution, makes it difficult to reconcile with a prokaryotic origin and therefore strengthens the idea that a few bacteria acquired the RIP domain by lateral transfer from a plant.

7 Chimeric RIPs Other Than Type 2 RIPs

Besides type 1 and type 2 RIPs two additional types of chimeric RIP proteins have been identified in Poaceae species.

7.1 JIP60 and Other Type AC Chimeric RIPs

A chimeric RIP protein was isolated from jasmonate treated barley leaves (Chaudhry et al. 1994). The so-called 60 kDa jasmonate-induced protein (JIP60) consists of an N-terminal A domain fused to an unrelated domain with no known function (which will further be referred to as the C domain) and was classified as a type 3 RIP (or AC-type). Hitherto, no homologs have been isolated but genome and transcriptome data revealed the occurrence of similar proteins in *Z. mays* and *Avena barbata*. Since there is no evidence for the presence outside the family Poaceae, the type AC RIP originated most likely within this family. To trace the origin of the RIP domain of these AC proteins the sequences of their respective A domains were

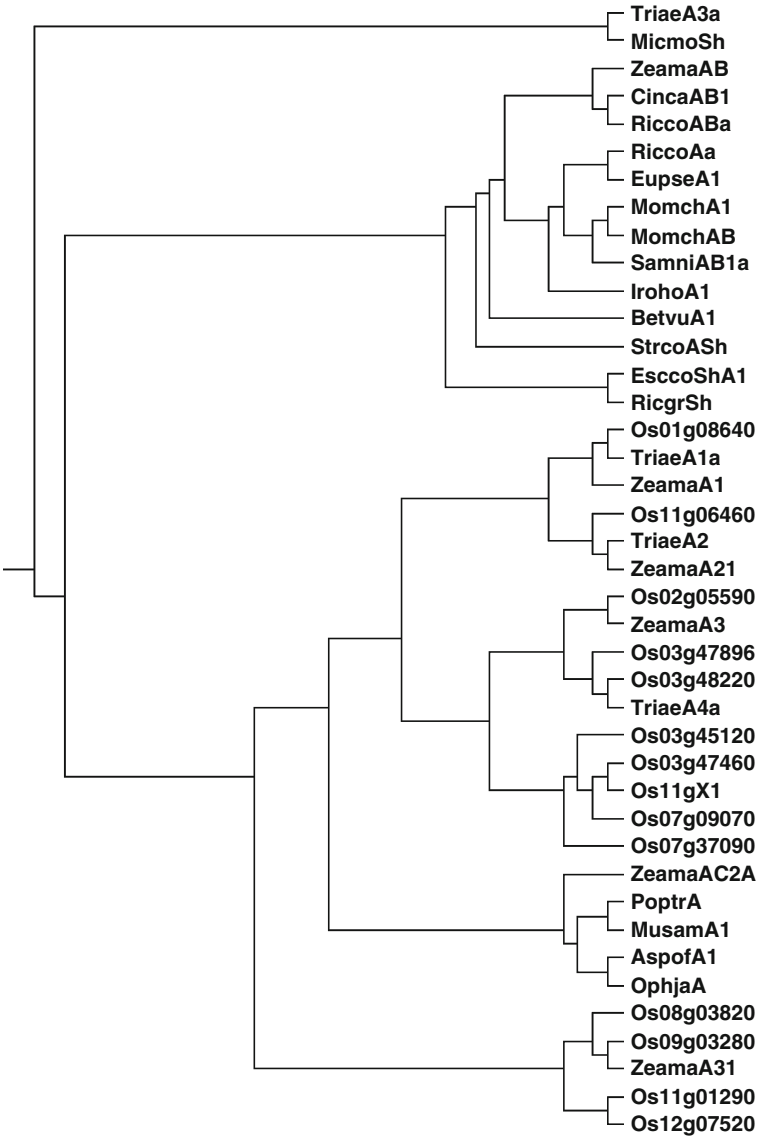

Fig. 8 Phylogenetic analysis of combined plant and bacterial RIP sequences

incorporated in a representative set of Poaceae type 1 RIPs for a phylogenetic analysis. As shown in Fig. 9 the RIP domains of AC proteins do not form a separate cluster but the A domain of HorvuAC (JIP60) and these from *Z. mays*/*A. barbata* are divided over two major branches. Moreover, one of the maize proteins is apparently more closely related to the oat homolog than to its conspecific paralog.

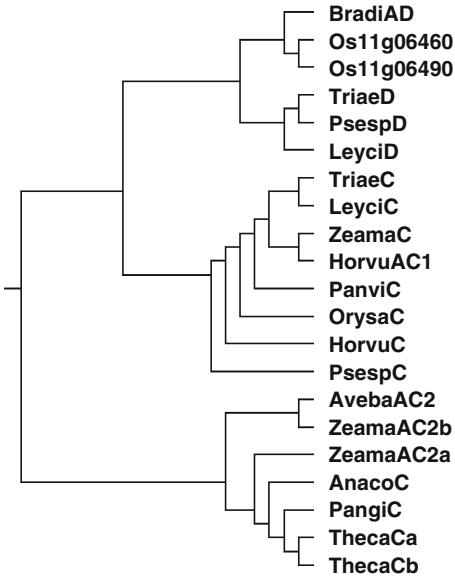

Fig. 9 Phylogenetic analysis of the C domain of the type AC RIPs, the D domain of type AD RIPs, and C and D proteins

The overall shape of the dendrogram and the obvious phylogenetic anomaly (one would expect that oat and barley cluster together) are indicative of two different gene fusion events: one that led to the AC1 type (as in barley) and another that yielded the oat and maize AC2 type. To check the presumed double origin a parallel phylogenetic analysis was made of the C domains found in the AC chimers and their parent genes. BLAST searches revealed that most but certainly not all Poaceae species possess (expressed) genes encoding a protein equivalent to the C domain of the type AC RIPs. For example, the C protein is found in wheat, barley, rice, maize, and *Panicum* but is absent in the *Sorghum* genome. Homologs are also expressed in other monocots (e.g., pineapple) and in some dicots (e.g., *P. ginseng* and *Theobroma cacao*). A phylogenetic analysis of all these sequences confirms that the barley type AC1 protein on the one hand and the maize and oat homologs on the other hand result most probably from two independent domain fusion events. The high sequence similarity between the barley AC1 protein and the C proteins found in other Poaceae indicates that the JIP60 chimer arose in an evolutionary recent past through a fusion event between a type 1 RIP gene of the TriaeA2 clade and a C domain gene. Since there is no information about the presence or absence of genuine JIP60 orthologs in other Poaceae, it is difficult to date the time point of the fusion event, but it might have taken place (relatively) recently in an ancestral Triticeae species. According to the dendrogram of the C domains, the origin of the AC2 type goes much further back in the time. Moreover, the position of the AC2 proteins in the dendrogram shown in Fig. 9 suggests that a different type 1 RIP gene was involved as in the fusion leading to the AC1 type chimer.

7.2 *Chimeric RIP with a C-terminal D Domain*

BLAST searches in the *B. distachyon* database (http://www.brachybase.org) yielded a (genomic) sequence sharing a high sequence similarity with the rice proteins Os11g06460 and Os11g06490. A perfectly matching (but short) EST sequence was retrieved indicating that the protein is expressed in *Brachypodium*. A closer examination revealed that the *Brachypodium* protein contains, besides an N-terminal RIP domain, a long terminal extension that shares a reasonably high sequence similarity with an unidentified protein (further referred to as the D protein) expressed in wheat, *P. spicata,* and *L. cinereus*. Using the *Leymus* D sequence as a model the *B. distachyon* genomic sequence could be correctly spliced. Once the exon–intron structure of the *Brachypodum* AD protein was determined the genomic rice sequences encoding Os11g06460 and Os11g06490 were reanalyzed and spliced correctly. Both rice proteins share a high sequence similarity with the *Brachypodium* AD chimer and comprise a C-terminal domain equivalent to the D protein from *Leymus*. The latter protein is still unidentified but shares sequence identity with the C proteins. For example, the C and D proteins share 24% identity concentrated in several well conserved regions. Therefore, the sequences of the AD chimers and the D proteins were included in the same phylogenetic analysis as the AC chimers and sole C proteins (Fig. 9). According to the dendrogram shown in Fig. 9 the A domain involved in the gene fusion leading to the AD chimers belonged to the same clade as the one recruited for the formation of the barley JIP60 AC1-type RIP. Given the occurrence of genuine orthologs in *Oryza* and *Brachypodium* the origin of the AD type RIP predates the division of the BEP clade into the Eahrhartoideae and Pooideae lineages.

8 Conclusions

The availability of novel genome and transcriptome data allowed updating the molecular evolution of the RIP family in plants (Fig. 10). All evidence suggests that the RIP domain itself was developed in plants before the Gnetophyta and Magnoliophyta lineages diverged from a common ancestor. Then the ancestral RIP domain followed two separate routes. A first route led directly to a subset of modern type 1 RIPs (primary type 1 RIPs). The second route started with a fusion (also before the Gnetophyta and Magnoliophyta lineages separated) of the RIP domain to a sugar-binding domain, which might be acquired by lateral transfer from a bacterium, and subsequently gave rise to (1) modern type 2 RIPs, (2) multiple lines of (secondary) type 1 RIPs through several domain B deletion events and (3) several lines of type B lectins through domain A deletion events. In many species, the RIP genes were purged from the genome whereas other species developed a whole set of RIP genes by gene and genome wide amplifications. Within the family Poaceae the original type 1 RIPs gave rise to (1) a complex set of type 1 RIPs

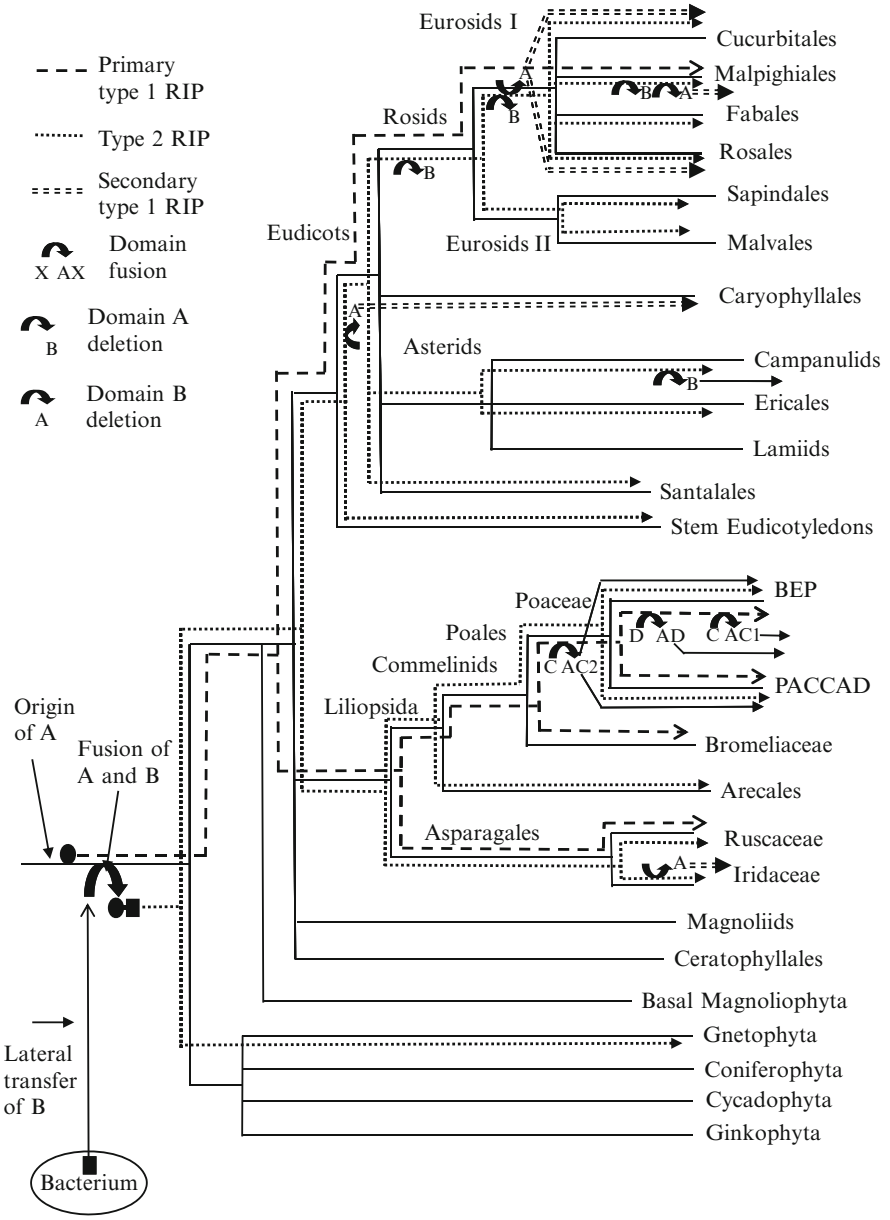

Fig. 10 Overall scheme of evolution of the RIP family in plants based on documented occurrence and phylogeny of type 1, type 2, and type AC and AD RIPs

through multiple gene and genome amplifications and (2) at least two different chimeric forms by two independent fusion events of a type 1 RIP to a C- and D-type protein, respectively. All evidence suggests that in the family Poaceae the RIP gene

family is still evolving by different mechanisms. A final remark concerns the origin of the bacterial RIP domain. Bacteria most probably did not develop their own RIP domain but acquired it through (multiple) lateral gene transfers from a plant.

Acknowledgements This research was supported by the Research Council of Ghent University and the Fund for Scientific Research (FWO-Vlaanderen, Brussels, Belgium).

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RNA *N*-Glycosidase Activity of Ribosome-Inactivating Proteins

Kazuyuki Takai, Tatsuya Sawasaki, and Yaeta Endo

Abstract Mammalian and bacterial ribosomes have ribosomal RNAs comprising 7,000 and 5,000 nucleotides, respectively. The RNA *N*-glycosidase activity of ricin and other ribosome-inactivating proteins (RIPs) specifically catalyzes removal of single adenine in the sarcin/ricin loop of the largest (28S or 23S) rRNA. Breakage of this single *N*-glycosidic bond is entirely responsible for the cytotoxicity. Ricin recognizes a highly ordered three-dimensional structure of the sarcin/ricin domain, which directly interacts with elongation factors to help switching through different states of the ribosome during the translation elongation cycle. Plants have an enzyme that specifically cleaves the phophodiester bond at the depurinated ricin site of 28S rRNA, named ribosomal RNA apurinic site-specific lyase (RALyase). The set of RIP and RALyase and the depurination and cleavage of the 28S rRNA are likely to have a role in senescence in plants.

1 Introduction

The ribosome is the central catalyst that supports synthesis of proteins within cells, not only translating nucleotide sequences into amino acid sequences, but also regulating gene expression. To understand the mechanisms by which genetic information in the genome is expressed as protein, it is essential to understand the mechanisms operating within the ribosome. Many three-dimensional structures representing different states of bacterial ribosomes during the course of sequential actions in the catalysis have been elucidated. While there is still much to be elucidated, the mechanisms in the ribosomes are gradually emerging. However, in the early 1980s, the ribosomes were very difficult subjects to study because of

K. Takai, T. Sawasaki, and Y. Endo (✉)
Cell-Free Science and Technology Research Center and Venture Business Laboratory, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan
e-mail: yendo@eng.ehime-u.ac.jp

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_2,

their complexity and large molecular sizes. Ribosomes vary in size from 2.5 to 4.5 MDa and consist of dozens of different proteins and several different rRNAs. Mammalian and *Escherichia coli* rRNAs comprise about 7,000 and 5,000 nucleotide residues, respectively.

Both toxins and antibiotics that act on the ribosomes have been used to investigate the mechanism of protein synthesis by the ribosomes. α-Sarcin is a proteinous toxin produced by the mold *Aspergillus giganteus*, first identified as an antitumor agent (Olson and Goerner 1965; Olson et al. 1965). It is a unique ribonuclease which cleaves the largest (25S) ribosomal RNA of yeast to generate a 320 nucleotide fragment (the α fragment) (Schindler and Davies 1977; Endo et al. 1983). The site of the phophodiester breakage was only at the 3′ side of G4325 of the 28S rRNA in rat liver ribosomes (Endo and Wool 1982; Chan et al. 1983). The sequence around this position is highly conserved among rat, yeast, and *E. coli* and forms a loop (the 17 nucleotides ranging from U4316 to C4332 in rat 28S rRNA) in the secondary structure of the rRNA (Fig. 1). Since this loop was later found to be modified also by ricin and other ribosome-inactivating proteins (RIPs) as below, it is now well known as the "sarcin–ricin loop (SRL)."

RIPs are RNA *N*-glycosidases that can catalyze depurination of a single adenylate residue within SRL. They occur widely in plants and other organisms, and are categorized into two types (Stirpe 1982, 2004). Type II RIPs comprise A- and B-chains connected by a covalent bond. The A-chain contains the catalytic domain, and the B-chain is responsible for entry of the A-chain into animal cells. The presence of the B-chain generally makes the type II RIPs highly toxic to animals. Ricin from *Ricinus communis* seeds and verotoxin (Shiga-like toxin) from *E. coli* are the best-characterized type II RIPs. Type I RIPs, which occur almost ubiquitously in the plant kingdom, contain only a single polypeptide with catalytic activity. Well-known type I RIPs include pokeweed antiviral protein from *Phytolacca Americana* and tritin from wheat seeds. They are frequently located in the intercellular space of whole plant tissues, and are thought to function by entering damaged cells to inhibit protein synthesis and to induce apoptosis. The detailed mechanisms are still unclear.

In this chapter, we review how ricin was characterized as an RNA *N*-glycosidase that acts on the SRL and the results of the subsequent research including the discovery of RALyase that catalyzes phophodiester bond breakage at the depurinated residue in the SRL.

2 Ricin as an RNA *N*-Glycosidase

2.1 28S rRNA as the Target of Modification by Ricin and Other RIPs

It was well known that ricin A-chain inactivates the 60S ribosomal subunits, but does not change the size of 28S rRNAs. Thus, the mechanism in the inactivation of

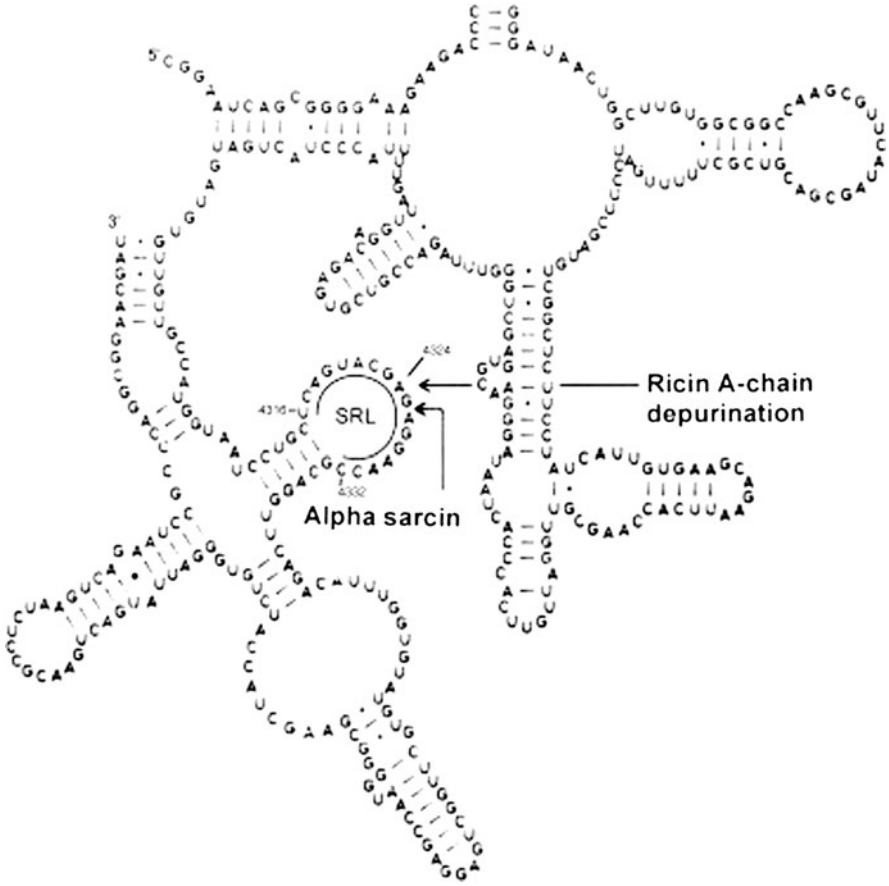

Fig. 1 Rat 28S rRNA and the sites of action of α-sarcin and ricin A-chain. The positions of the sarcin and ricin sites are shown in the secondary structure of rat 28S rRNA

the 60S subunit by ricin should be different from that by α-sarcin. However, as the effects of the treatments of the ribosomes with ricin and α-sarcin are common in that they affect the ribosomal functions related to eEF1 and eEF2 and in that they need no energy, we tested the possibility that ricin also acts on rRNA in the 60S ribosomal subunit as α-sarcin does. As a result (Endo et al. 1987), we found through a gel electrophoresis analysis that 28S rRNA from rat liver ribosomes treated with ricin A-chain migrates slightly slower than the sample from untreated ribosomes (Fig. 2a). The mobility of a 550-nucleotide fragment that is generated during rRNA preparation was also affected by the treatment with RIPs (ricin, abrin, or modeccin) (Fig. 2b). The other rRNAs, 5S, 5.8S, and 18S rRNA (or 16S in prokaryotes) were not affected. Therefore, RIPs modify the 28S rRNA in a different manner from main chain breakage.

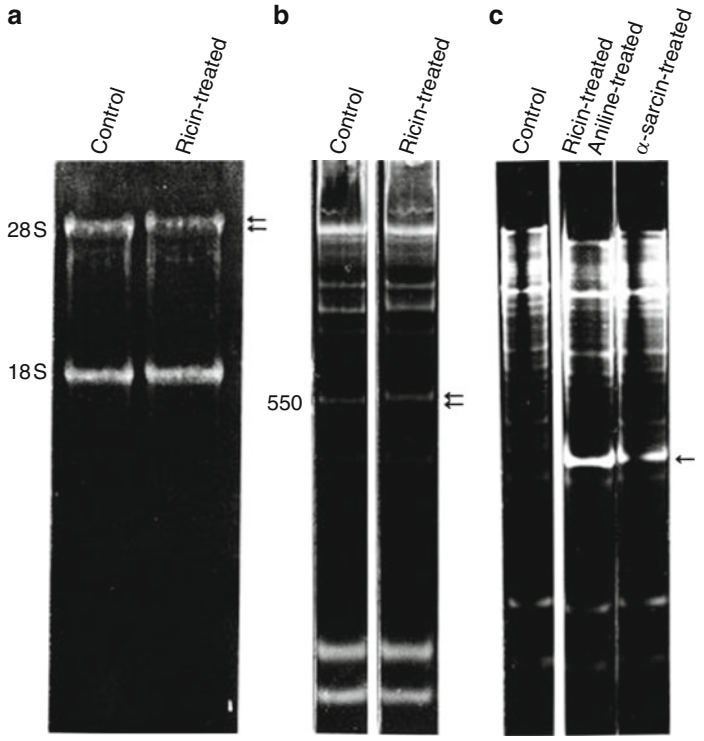

Fig. 2 Electrophoretic patterns of rat ribosomal RNAs treated with toxins. (**a**). A gel detecting the slight difference in the mobilities of unmodified and ricin-treated 28S rRNA samples. (**b**) A gel showing the upward shift of the band of the 550-nt fragment that is generated during purification of rRNA. (**c**) A gel showing that aniline treatment of the ricin-treated 28S rRNA gives a band with similar mobility to that produced by α-sarcin

2.2 RNA N*-Glycosidase Activity of Ricin A-Chain*

The RNA sequencing gels for the 550-nucleotide fragment from the ricin-treated ribosomes showed that the bands for G4323 and A4324 were absent (Fig. 3a). In addition, alkaline hydrolysis generated strong bands corresponding to the fragments cleaved at both G4323 and A4324 (Fig. 3a) (Endo et al. 1987). This strongly suggested that A4324 is modified and the phophodiester linkages at both sides of A4324 are sensitive to alkaline hydrolysis. By analyzing the bases released from the ribosomes by the action of ricin A-chain, adenine was revealed to be released stoichiometrically from the ribosome (Fig. 3b). All these and other pieces of evidence demonstrated that the adenine base of the A4324 residue is cut out from the 28S rRNA specifically by the action of ricin, which is an RNA *N*-glycosidase (Endo and Tsurugi 1987). As the depurinated site is specifically cleaved by a treatment with aniline at an acidic pH (Endo et al. 1987), this treatment has become a very specific and sensitive method for detection of RNA *N*-glycosidase activity.

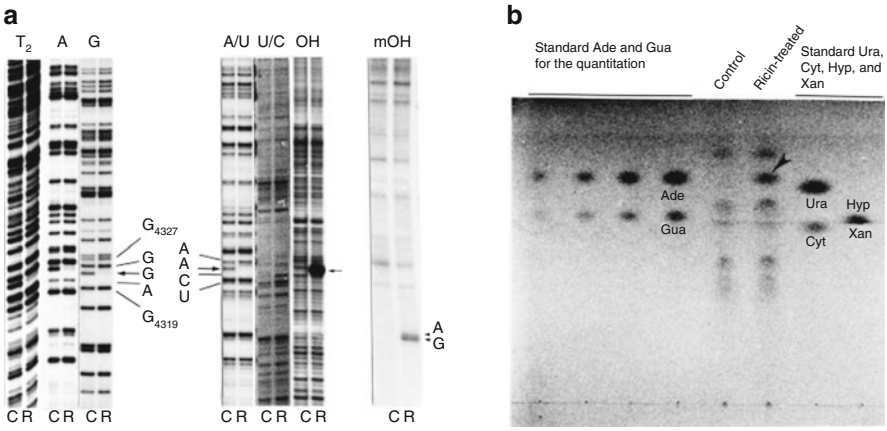

Fig. 3 Ricin as *N*-glycosidase specific for A4324 of 28S rRNA. (**a**) Patterns of the ricin-treated and -untreated rRNA 550-nucleotide fragment in RNA sequencing gels. "*C*" and "*R*" at the *bottom* indicate the untreated control sample and the ricin-treated sample, respectively. "*A*", "*G*", "*A*/*U*", and "*U*/*C*" indicate that the samples were partially digested with the specific RNases that cleave RNAs at the indicated nucleoside(s). "T_2" indicates the samples digested by RNase T_2. "*OH*" means the sample hydrolyzed by incubating it with an alkali, sodium carbonate. "*mOH*" means mild alkaline treatment. (**b**) A thin layer plate separating the bases from the ricin-treated ribosomes. An *arrowhead* indicates the spot of adenine showing an almost stoichiometric release from the rRNA. *Hyp*, hypoxanthine; *Xan*, xanthine

2.3 Other RIPs

It is very likely that the other plant RIPs including abrin and modeccin are also RNA *N*-glycosidases. Vero toxin (VT2, or Shiga-like toxin II) from *E. coli* O157: H7 and Shiga toxin from *Shigella dysenteriae* 1 were also shown to cause the band shift of the 28S rRNA, the alkaline-sensitive chain breakage, and the stoichiometric release of adenine (Endo et al. 1988a). Therefore, these bacterial cytotoxins are also rRNA *N*-glycosidases. Many other type II and type I RIPs have been shown to exhibit the same characteristics (Endo et al. 1988b, c, d, 1989).

2.4 Major Role of RNA in Protein Synthesis

It was surprising that the ribosome lost its major function in translation elongation by the breakage of a single bond, in particular, a single bond in its RNA but not in its protein. The identification of the SRL as the site of action of both α-sarcin and ricin clearly demonstrated for the first time the major role of RNA in the catalysis of protein synthesis. It was later shown that the ribosome is a ribozyme: what directly catalyzes the peptidyltransfer reaction is the ribosomal RNA, not any ribosomal protein (Noller et al. 1992; Yusupov et al. 2001).

3 Ribosomal Mechanisms Involving the Sarcin–Ricin Domain

3.1 *Eukaryotic Translation Can Be Inhibited Strongly by Dysfunction of a Small Fraction of the Ribosome Population*

Each cell contains a large number of ribosomes. It may seem odd that only a small number of the ricin A-chain molecule can show high toxicity. Although ricin and other RIPs cause pleiotropic effects that lead the cell to death, the severe inhibition of translation can be explained solely by their *N*-glycosidase activity: RIPs catalytically inactivate *translating* ribosomes and can inhibit translation by inactivating only a small fraction of the total ribosome population. To help the understanding of the high toxicity of RIPs with RNA *N*-glycosidase activity, it may be pertinent to see what would happen when the ribosomes lose their elongation activity in cells. Cellular translation occurs in the polysomes that are each made of 30–40 ribosomes that are translating a single mRNA molecule, particularly in the case of actively translated mRNA molecules. The depurination constitutes considerable damage to protein synthesis because inactivation of any one ribosome among the actively translating ribosomes on the same mRNA molecule would result in the blockage of the respective polysome and the cessation of translation of the mRNA molecule. This would not only stop translation of the mRNA molecule, but also inhibit translation of the other mRNA molecules by trapping the unaffected ribosomes in the stopped polysome. Therefore, depurination of 2–3% of the total ribosome population in a cell should result in almost total shut-off of the cellular translation. The turnover number of ricin A-chain on the depurination of rat liver ribosomes has been determined as 1,777 min^{-1} (Endo and Tsurugi 1988), which is much higher than that of α-sarcin (Endo et al. 1983). It may be reasonable to consider that this high catalytic activity can explain the ricin toxicity in cells.

3.2 *Difference in the Modes of Action between α-Sarcin and Ricin*

It had been noticed that the effects of α-sarcin and ricin are different from each other, while both inactivate the ribosomes through affecting the interactions with the elongation factors. By directly comparing the effects on the ribosomal activities, it was revealed that the effects of these toxins are different in the EF1-dependent aminoacyl-tRNA binding, in the EF2 binding to the ribosomes, and in the EF2-dependent GTPase activities. While α-sarcin blocks binding of the EF1–GTP–aminoacyl-tRNA ternary complex to the ribosomes, ricin only affects the turnover rate of the ternary complex binding. Inhibition of the EF2 binding to the ribosomes and stimulation of the EF2-dependent GTPase activity are stronger by ricin than by α-sarcin (Furutani et al. 1992).

3.3 Substrate Specificity

Ricin can act on naked rRNA extracted and deproteinized with SDS and phenol as above. By testing different rRNAs from rat and *E. coli*, it was revealed that a stem–loop structure with a GAGA sequence in the loop can be depurinated by ricin (Endo and Tsurugi 1988). This stem–loop domain is termed sarcin–ricin domain (SRD) hereafter.

It was also found that an oligonucleotide mimicking SRD can be a substrate of α-sarcin and ricin and is modified at each specific position by either enzyme (Endo et al. 1988e). This facilitated the analysis of substrate specificities of these enzymes (Endo et al. 1990, 1991; Glück et al. 1992, 1994). Both toxins need the stem and the GAGA sequence in the loop, and the correct position of the GAGA tetranucleotide is essential for the recognition (Endo et al. 1990, 1991). Thus, it was suggested that the loop is not a simple loop, but has a complex structure as revealed later. On the other hand, the two enzymes are different in the recognition of the GAGA sequence: while ricin needs the correct sequence surrounding the GAGA tetranucleotide, α-sarcin recognizes some loop mutants (Glück et al. 1992). Thus, it was suggested that SRL can adopt at least two conformations, one which is an "open" conformation that can be recognized only by α-sarcin but not by ricin, and the other which is a "closed" conformation that is recognized by ricin (Wool et al. 1992).

3.4 Structure of the SRL

The NMR solution structure of a 29mer oligonucleotide mimicking SRD was determined (Szewczak et al. 1993; Szewczak and Moore 1995). In the structure, all the bases except a guanine stack together to form a single bulged helix turning at between G4323 and A4324 with A4324 at the top of the helix stacking to the 3′ side and G4323 stacking to the 5′ side forming a noncanonical base pair with A4326. This helix contains several other noncanonical base pairs and a cross-strand stack that stabilizes the helix. There is a flexible region in the middle of the helix, in which noncanonical U–C and a C–C pairs without hydrogen bonds connect the base stacks between the stem and the loop regions. The 5′ C and 3′ G bases flank the GAGA sequence pair with each other to form a Watson–Crick pair. Thus, SRL can be viewed as a hairpin with a GAGA tetraloop. This structure can explain the narrow specificity of ricin A-chain that attacks the A base at the top of the structure only when the loop has this closed structure. Actually, ricin A-chain requires a Watson–Crick pair between these closing nucleotides. α-Sarcin has to melt the loop at least in part to access the phosphodiester bond, and does not require a base pair between the bases flanking the GAGA tetranucleotide (Glück et al. 1994).

Therefore, it seemed possible that the SRL temporarily adopt the open conformation during the course of translation elongation, and this conformation is the target of α-sarcin. It was thus speculated that the transition between the two

conformations is associated with the transitions from the pre- to the posttranslocation states.

On the other hand, a crystal structure of the same 29mer was reported later (Correll et al. 1998) (Fig. 4). This structure was a bit different from the NMR structure in the stacking pattern at the top of the loop and in the structure of the flexible region. As the 3′ phosphate of G4325 is exposed in the X-ray structure, α-sarcin may be able to access the phosphate without melting the loop.

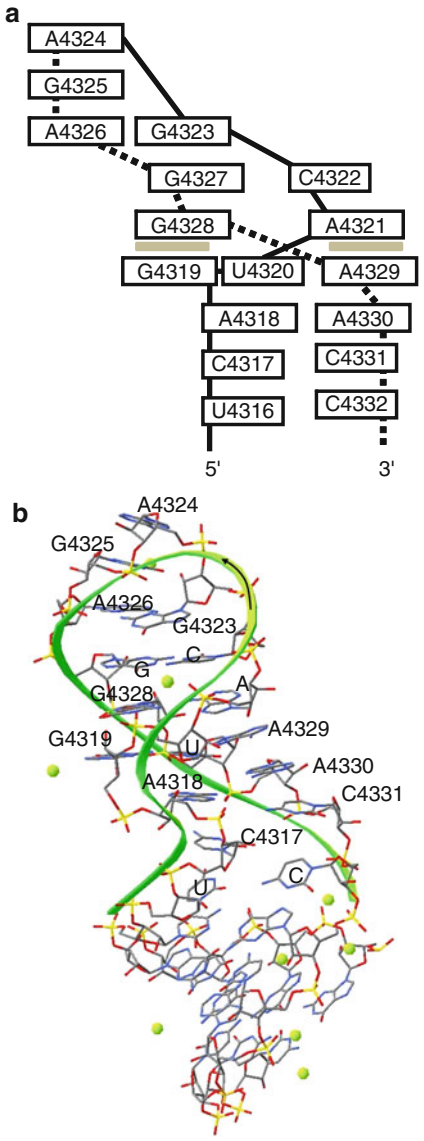

Fig. 4 The crystal structure of a 29mer RNA mimicing the SRD of rat 28S rRNA (Correll et al. 1998). (**a**) A stacking diagram deduced from the crystal structure of the SRD oligonucleotide in (**b**). *Thick gray lines* between A4321 and A4329 and between G4328 and G4319 show the cross-strand stacking interactions that stabilize the structure. *Thick solid lines* represent the sugar-phosphate backbone from the 5′ terminus to A4324, and the *dotted lines* from A4324 to the 3′-terminus. (**b**) The crystal structure of the SRD oligonucleotide from Protein Data Bank (PDB ID: 430D) drawn with Swiss-PdbViewer. *Light green spheres* represent magnesium atoms. The *green ribbon* traces the sugar-phosphate backbone within SRL. The *arrow* in the ribbon shows the direction of the backbone from 5′ to 3′. The A4324 ricin site is at the *top* of the figure

The difference in the structure of the flexible region makes the relative orientations of the stem to the SRL quite different. This may mean that two conformations are possible, one of which is similar to the NMR structure, and the other to the X-ray structure. Therefore, the hypothetical conformational transition is more likely the one between these two conformations if any, rather than the one between the "open" and "closed" conformations. The crystal structure of an *E. coli* SRD oligoribonucleotide (Correll et al. 1999) was essentially the same as that of rat except that the flexible region of the *E. coli* SRD is slightly shorter than that of the rat because the number of the base pairs in the flexible region is different. It has been observed that the orientation of the SRL in the cryo-electron microscopy structure of the *E. coli* 70S ribosome with bound formylmethionyl-tRNA is different from that of the crystal structure of the *Haloarcula marismortui* 50S ribosome (Gabashvili et al. 2000). It is not clear whether this conformational change is associated with the coordinated structural changes of the ribosome during the elongation cycle.

Chemical modification studies showed that the EF-Tu and EF-G protect the tip of SRL (Moazed et al. 1988), and that the other side of SRL is buried in the ribosome (Uchiumi et al. 1999). Three-dimensional structures of the ribosome complexed with elongation factors (Stark et al. 2002; Connell et al. 2007) clearly showed that the elongation factors directly bind to the tip of SRL, where the target of RIPs is present. The present model of translocation involves relative rearrangements of domains in EF2 (EF-G or eEF2) with a ratchet-like subunit rearrangement in the ribosome (Spahn et al. 2004). The "switch" regions of EF-G are thought to switch the domain arrangements during the changes in the guanine nucleotide phosphate states. SRL directly interacts with "switch II" of EF-G and with "P-loop" that directly bind the phosphate of the guanine nucleotide (Connell et al. 2007). Therefore, it is likely that SRL helps regulation of the domain rearrangement of EF-G (EF2) according to the guanine nucleotide phosphate states which accompanies the ratchet-like subunit rearrangement of the ribosome during translocation.

4 Ribosomal RNA Apurinic Site-Specific Lyase: Intrinsic Stability of the Ribosome

Wheat has a type I RIP named tritin (Roberts and Stewart 1979). It is distributed in seeds and other tissues including leaf and stem. Tritin strongly inhibits mammalian cell-free translation by inactivating the ribosomes, while it was suggested that it does not act on the wheat-germ ribosomes. On the other hand, wheat-germ cell-free translation systems had been unstable, producing much smaller amounts of proteins than the cell-free translation systems from *E. coli*. It had been generally believed that the eukaryotic cell-free protein synthesis should inevitably be unstable on extraction from the cells. However, we were trying to develop a stable cell-free protein production system from wheat embryos, speculating that the wheat cell-free system was unstable primarily because catalytic inhibitors, such as tritin, inhibited

the cell-free translation reaction. We finally found that the cell extract from embryo particles intensively washed to remove endosperm provides a highly efficient and stable cell-free translation system (Madin et al. 2000). The embryo cells do not have tritin and the ribosomes are intrinsically quite stable.

At the same time, we found that the 28S rRNA is cleaved near the site of SRL during cell-free translation with the extract from unwashed embryos. As tritin only depurinates SRL but does not cleave the main chain of the RNA, there should be another enzyme that cleaves the backbone. The fragment of wheat 28S rRNA generated by the enzyme has almost the same size as that generated by α-sarcin and by an RIP followed by the aniline treatment. By a reduction/oxidation experiment and the analysis of the 5′-terminus of the generated fragment, the enzyme was considered to be a lyase that generates α-hydroxy-α,β-unsaturated aldehyde at the 3′ terminus and a phosphate at the 5′ terminus (Fig. 5). It was purified, and its cDNA was cloned, sequenced, and expressed in a wheat cell-free translation system. Only the depurinated site generated by RIPs on the ribosome can be cleaved by the enzyme. Thus, the enzyme was named RALyase, after "ribosomal RNA apurinic site-specific lyase" (Ogasawara et al. 1999). A similar enzyme was also found in rice (Ito et al. 2002).

The presence of tritin only in endosperm and not in embryo cells and that of RALyase in the embryo cells suggest that tritin has a biological function when the embryo cells somehow incorporate the endosperm materials, such as when the embryo cells are injured. Thus, it is likely that tritin and RALyase constitute a part of a molecular system that leads the cell to programmed death. If so, why is it needed to inactivate the ribosome by the two steps, instead of cleaving the SRL main chain by one step as α-sarcin does? It was found that the RIP-treated wheat ribosome retains weak poly(Phe) synthesis activity and shows an elevated activation of eEF2 GTPase. The ribosomes are completely inactivated by the action of RALyase (Ozawa et al. 2003). It is possible that the residual activity of the ribosomes is also a part of the pathway to programmed cell death. It is well

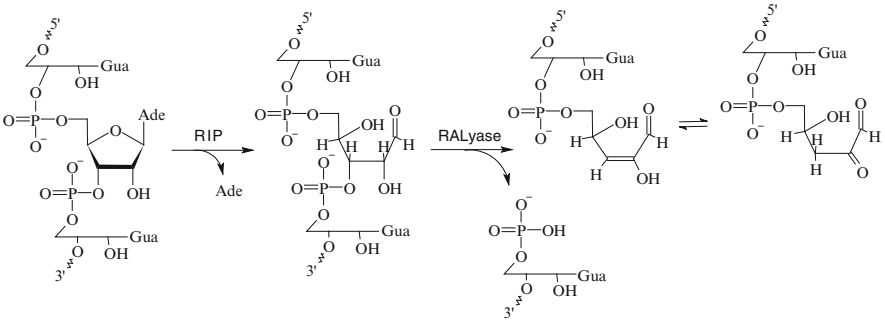

Fig. 5 Reactions catalyzed by RIP and RALyase. RALyase catalyzes a β-elimination reaction producing a 5′-phosphate end of the 3′ fragment (α-fragment) of the 28S rRNA and an α-hydroxy-α, β-unsaturated aldehyde end of the 5′-fragment. The aldehyde end should be in the keto–enol equilibrium

known now that RIPs and α-sarcin not only inactivate the ribosomes but have pleiotropic effects related to apoptosis as reviewed in this volume.

It was recently found that expression of tritin in wheat coleoptile cells is related to senescence (programmed cell death) in plants. RALyase is constitutively expressed, and tritin is expressed during the late stage of senescence, when the cytoplasmic ribosomes are depurinated at SRL and are immediately cleaved at the depurinated site (Sawasaki et al. 2008). Therefore, RIP and RALyase are integrated in a unique plant system that mediates programmed cell death.

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Enzymatic Activities of Ribosome-Inactivating Proteins

Martin R. Hartley

Abstract Ribosome-inactivating proteins (RIPs) constitute a diverse group of proteins that share an RNA *N*-glycosidase activity that acts very specifically on the ribosomal RNA of the 50S/60S ribosomal subunit to inhibit protein synthesis. Additionally, the majority of RIPs act on non-ribosomal RNA and DNA in a sequence context-independent fashion, releasing multiple adenines and sometimes guanines. One such activity depends on the presence of a 5′ cap structure, and may be responsible for the anti-viral properties of some RIPs. In addition to their *N*-glycosidase activity on nucleic acids, some ribosome-inactivating enzymes have been reported to be bifunctional with another, unrelated activity. No active sites for these unrelated activities have been found, and their presence in preparations of RIPs may be due to contamination.

1 Introduction

The discovery of the highly specific RNA *N*-glycosidase activity of RTA (ricin A-chain) (Endo and Tsurugi 1987) and other ribosome-inactivating proteins (RIPs) towards ribosomes was widely regarded as being entirely responsible for their cytotoxic action. However, in recent years this straightforward explanation has been questioned by the finding that many RIPs can act in a much less specific manner on a variety of RNA and DNA substrates, and also possess a number of apparently unrelated activities such as superoxide dismutase, phospholipase, and pectin methylesterase. Clearly, this challenges the notion of the "unity of biochemistry" especially when taking into account the fact that all of the RIPs from diverse sources for which crystal structures exist are essentially similar in structure, with

M.R. Hartley
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
e-mail: mhartley@bio.warwick.ac.uk

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_3,

rms deviations of <2.8 Å from RTA (reviewed by Robertus and Monzingo 2004). In addition to their cytotoxic effects, many RIPs have additional biological effects on cells and/or organisms which may or may not require the functional RNA *N*-glycosidase active site that acts on ribosomes. These include anti-viral, anti-fungal, and apoptosis-inducing activities (Lodge et al. 1993; Zoubenko et al. 1997; Sikriwal et al. 2008). To complicate matters further, the specific depurination of rRNA in ribosomes and the consequent inhibition of protein synthesis have been reported to be insufficient for cytotoxicity (Hudak et al. 2004), a contention which would seem to call into question a basic premise of Molecular Biology. In this review, I attempt to make a critical analysis of some of the recent and contentious issues surrounding the supposed activities of RIPs. It should be borne in mind that RIPs as a group exhibit considerable diversity in their substrates and in their biological effects for which a molecular explanation is not yet available, despite the fact that they share a similar tertiary structure.

2 Action of RIPs on Ribosomes and rRNA

2.1 Site of Modification by RIPs

Early studies on the effects of the A-chains of the type 2 RIPs ricin, abrin, and modeccin on the inhibition of protein synthesis in cell-free systems showed that the 60S ribosomal subunit alone was affected and that one A-chain molecule could inactivate ca. 1,500 ribosomes per minute (Olsnes and Pihl 1982). The nature of the enzymatic modification responsible was shown by Endo's group to be the removal of an adenine (A4324) and/or guanine (G4323) residue) from 28S rRNA in the universally conserved sequence AGUACGA*GAGGAA (in which A* is removed) present in domain VII, some 400 nucleotides from the 3′ end (Endo et al. 1987; Endo and Tsurugi 1987). When this discovery was made, this conserved sequence was depicted as being part of a single-stranded loop closed off by a double helical stem, the so-called sarcin/ricin domain (SRD – see below). From Endo's initial studies, in which the RIP depurination site was revealed following cleavage of the rRNA backbone with acidic aniline, it is clear that A4324 is the major depurination site, although additional, minor sites could not be excluded. However, other work in which the large and small aniline fragments from 28S rRNA were quantified revealed a near 1:1 stoichiometry, indicative of a single depurination site (Osborn and Hartley 1990). This pattern of specificity is maintained over a wide range of (enzyme):(substrate) ratios, and indicates that the interaction between the RIP and its target site is highly specific. Several of the "ribocentric" researchers who have used RIPs to perturb the structure of the ribosome believe that the cytotoxic effect of type 2 RIPs can be explained entirely by the inhibition of protein synthesis resulting from the above modification to rRNA (Wool et al. 2000), a view supported by the fact that many mutations in the SRD show a dominant-lethal phenotype

(Marchant and Hartley 1994). However, in recent years the importance of SRD depurination in cytotoxicity has been greatly diminished by some workers (Park et al. 2004; Hudak et al. 2004). In the Hudak et al. (2004) work, mutations were introduced into the cDNA for the type 1 RIP pokeweed anti-viral protein (PAP). This encodes a cleavable 22 amino acid signal peptide, 262 amino acids of the mature polypeptide, and a cleavable C-terminal extension of 29 amino acids. The mutants were inserted into a yeast expression vector under control of the GAL1 promoter, and induced transformants assayed for cytotoxicity by their ability to grow on galactose plates and doubling time in liquid media, protein synthesis by ^{35}S-methionine incorporation, and ribosome depurination by primer extension on rRNA. One of the mutants, Y123I in the active site, was non-cytotoxic, had a doubling time of 87% of that of an empty vector control, and its level of protein synthesis was 56.5% of the control; yet its ribosomes were depurinated to 81% of the wild-type PAP control level. From these data, it was concluded that ribosome depurination is not sufficient for cytotoxicity. However, Takai et al. in this volume state that "depurination of 2–3% of the total ribosome population of a cell should result in almost total shut off of cellular translation". How might these conflicting views be reconciled? In subsequent work on PAP expression in yeast, Parikh et al. (2005) showed that the PAP signal sequence is functional in yeast, as PAP was found to be localised in the ER. Cytotoxicity was attributed to retrotranslocation of a fraction of PAP in the ER into the cytosol, using the ER-associated degradation (ERAD) pathway in a similar manner for that proposed for RTA, where it refolds and depurinates ribosomes (see chapter, "How Ricin Reaches its Target in the Cytosol of Mammalian Cells" by Spooner et al. in this volume). In these studies, ribosomes were prepared from yeast transformants by differential centrifugation following cell breakage in aqueous buffers, and during this procedure vesicles from the endomembrane system containing PAP could rupture and their contents come into contact with ribosomes and depurinate them, thus giving rise to a higher level of depurination than that in situ before cell breakage. This situation has been shown to occur in the preparation of ribosomes from several plant species producing type 1 RIPs, including pokeweed (Taylor and Irvin 1990; Prestel et al. 1992; Massiah and Hartley 1995). It is also possible that some of the mutations introduced into PAP could have affected their ability to act as ERAD substrates and/or their sensitivity to degradation by proteases, raising the possibility that they could affect the level of ribosome depurination as the result of differences in their concentration in the cytosol, rather than their intrinsic enzymatic activities on ribosomes.

2.2 Structural Requirements in Ribosomal RNA for RIP Action

The first indications of the nature of the RNA structural requirements for a RIP substrate came from a study on the action of ricin A-chain on naked (deproteinised) rRNA (Endo and Tsurugi 1988). Ricin A-chain depurinated naked 28S rRNA with an identical specificity to that of 28S rRNA in native ribosomes. Informatively,

ricin A-chain was also active on naked rRNA from *Escherichia coli* (cf. native *E. coli* ribosomes which are completely refractory): here two depurination sites were identified – one in 23S rRNA at A2660 corresponding to A4324 in rat 28S rRNA and a second in 16S rRNA at A1014. Inspection of the structures around these depurination sites reveals that both of them contain the motif GAGA in a tetraloop structure closed off by Watson–Crick base pairs. However, *E. coli* rRNA contains further eight such GAGA-containing structures that are not substrates for ricin A-chain in the context of intact rRNAs (Endo and Tsurugi 1988). An analysis of the kinetic parameters for the depurination of rat liver ribosomes and naked 28S rRNA by ricin A-chain revealed that the K_m values were similar (2.6 and 5.8 μM respectively, whilst the turnover number (K_{cat}) differed by a factor of ca. 10^5 (1,777 min^{-1} and 0.02 min^{-1} respectively) (Endo and Tsurugi 1987). This suggests that ribosomal proteins in the native ribosome are important for efficient catalysis. The role played by ribosomal proteins in the interaction of RIPs with ribosomes has been the subject of considerable work in recent years, and although incomplete, a picture corroborated by several laboratories is starting to emerge. The acidic ribosomal proteins P0, P1 and P2 which form the pentameric complex (P0, $(P1)_2$, $(P2)_2$) of the central protuberance (stalk) of the 60S subunit have been implicated in binding of some, but not all, RIPs to the ribosome. These three proteins share a conserved, flexible C-terminal tail ($SD^D/_EDMGFGLFD$) involved in the binding of protein synthesis initiation, elongation and termination factors (Helgstrand et al. 2007). Trichosanthin, a type 1 RIP with anti-tumour and anti-HIV properties had been shown to bind to P2 through electrostatic interactions between three basic residues (K173, R174 and K177) and the DDD motif of P2 (Chan et al. 1997). A triple alanine variant (K173A, R174A and K177A) of trichosanthin failed to bind to P2 and had 18-fold lower activity in inhibition of protein synthesis than the wild-type RIP. There does seem to be a general consensus that electrostatic interactions between RIPs and the ribosome are important in promoting the unusually fast second order rate constants (K_{cat}/K_m) in the order of 10^9–10^{10} M^{-1} S^{-1} for the depurination reaction (Korennykh et al. 2007). In subsequent work, a crystal structure was solved for the trichosanthin/conserved peptide structure in which the N-terminal region of the peptide interacts with K173, R174 and K177 in trichosanthin, and its C-terminal region is inserted into a hydrophobic pocket. Interestingly, this P protein peptide can similarly dock to other, diverse RIPs, including Shiga-like toxin 1A, ricin A-chain and saporin (SO6), but not to others, including PAP and Shiga-like toxin A (SLT A) (Too et al. 2009). Although this observed interaction does not provide direct evidence that it is of significance for the action of RIPs on intact ribosomes, the finding that a single-chain antibody fragment (scFvC5) against the C-terminal end of *Trypanosoma cruzi* ribosomal P proteins protected *T. cruzi* ribosomes from depurination by trichosanthin, but not PAP, suggests it is of physiological significance (Ayub et al. 2008). This is also supported by the demonstration, using surface plasmon resonance, that RTA binds to P1 and P2 proteins in yeast ribosomes, and that mutants in P1 and P2 confer partial resistance against depurination of the ribosome by RTA *in vitro* and are more resistant to RTA expression *in vivo* (Chiou et al. 2008).

The suggestion from modelling studies that PAP does not interact efficiently with P proteins is in keeping with earlier work on a yeast ribosomal protein mutant (the *mak8-1* allele of L3 which lies close to the SRD) conferring resistance to PAP expression *in vivo* (Hudak et al. 1999). Although PAP interacts with both free wild-type L3 and the mak8-1 protein, it does not interact with the mutant protein in ribosomes *in vitro*, suggesting that the mutant protein alters its conformation in the ribosome. Interestingly, this mutation does not confer resistance against RTA (Tumer, personal communication). The interaction between L3 and PAP is apparently mediated through the active site cleft of PAP, as deduced from the binding characteristics of active site cleft mutants in residues not directly involved in catalysis (Rajamohan et al. 2001). However, Ayub et al. (2007) have questioned this work, claiming that the mutants with impaired binding to L3 also have reduced ability to depurinate naked rRNA where L3 is absent.

In conclusion, it appears that two different sets of ribosomal proteins can account for a rate enhancement in RIP action on ribosomes compared to naked rRNA. Ribosomal P proteins are involved in binding a subset of RIPs, including ricin A-chain, trichosanthin, and SLT 1A and saporin SO6, whereas L3 is involved in binding PAP.

The identity of the structural elements in RNA required for RTA recognition and catalysis was determined by monitoring its action on a 35-residue oligoribonucleotide that mimics the SRD (Endo et al. 1991). The rationale behind this is that the oligoribonucleotide based on sequence of the SRD in rat rRNA acts as a substrate for RTA, with depurination at the same site as in native ribosomes. However, certain variant oligoribonucleotide were not substrates for ricin A-chain, and from this it was concluded that the minimum structure required for RTA action is a GAGA motif flanked on either side by two bases capable of forming Watson–Crick base pairs. Following this work, both crystal and solution NMR structures have been solved for the SRD and revealed a complexity of structure not apparent from the proposed secondary (see chapter "RNA *N*-Glycosidase Activity of Ribosome-Inactivating Proteins" by Takai et al. in this volume).

The vast majority of the work on RIP substrates requirements has been done for RTA, and because nearly all other RIPs for which information exists act on eukaryotic ribosomes in a similar manner, it has been tacitly assumed that the "identity" elements in RNA required for RIP recognition and catalysis are essentially the same for all RIPs. An additional consideration is whether the identity element rules established from the action of RIPs on oligoribonucleotides are also applicable to rRNA in the context of the native ribosome. The finding that PAP and several other type 1 RIPs are active on *E. coli* ribosomes (unlike ricin A-chain, which is active only on naked *E. coli* rRNA) made it feasible to introduce mutations into the SRD of 23S rRNA, and assay the mutant ribosomes and naked rRNA for susceptibility to PAP, and the latter for susceptibility to RTA (Marchant and Hartley 1995). It was found that for RTA, the identity element rules established with oligoribonucleotides were also applicable for intact rRNA (i.e. the requirement for the sequence GAGA flanked by bases capable of forming Watson–Crick pairs). However, PAP was active both on ribosomes and naked rRNA in which the bases

flanking the GAGA motif could not form canonical base pairs, implying that the tetraloop structure was not required, and also on mutants in which G* in the sequence GAG*A had been changed to C. Thus, the recognition elements for PAP are considerably less stringent than those for RTA even though, counter intuitively, their target sites in ribosomes and rRNAs are identical.

3 Polynucleotide:Adenosine Glycosidase Activity

3.1 5′ Cap-Independent Activity

The general applicability of the mantra of very stringent substrate requirements for recognition and catalysis by RIPs on ribosomes and naked rRNA established largely through the work of the groups of Endo and Wool has been challenged by observations originating from Stirpe's group. They found by chemical analysis that some saporin isoforms released more than one molecule of adenine per ribosome (Barbieri et al. 1992; see also the chapter by Lombardi et al. in this volume), and subsequently extended these studies to many other RIPs and various nucleic acid substrates (Barbieri et al. 1997). In an extreme case, it can be calculated from the data presented that saporin-L2 released ~1,300 adenines from the rRNA equivalent of one *E. coli* ribosome, meaning that practically all the adenosyl residues in the rRNA were depurinated. At the other extreme, RTA released only one adenine per RNA molecule. Of the 27 type 1 RIPs and five type 2 RIPs tested, all released adenines from herring sperm DNA, the majority were active on rRNA and a minority on poly(A). This non-specific deadenylation activity variously termed polynucleotide:adenosine glycosidase (PAGase) and adenine polynucleotide glycosylase (Girbés et al. 2004) has been proposed to replace the term RIP to describe this class of proteins, although this has failed to gain general usage. The proponents of PAGase argue that this activity, rather than specific ribosome depurination, could be responsible for many of the biological effects of RIPs, including anti-viral activity, senescence promotion, and apoptosis by DNA modification (Girbés et al. 2004). There are a number of reports which support this contention. Park et al. (2004) investigated the effects of ME1 (a type 1 RIP from *Mirabilis expansa*) on a variety of different RNA substrates in native and partially heat-denatured states, and concluded that adenines, and to a lesser extent guanines, were randomly removed from single-stranded regions depending on their accessibility by the RIP. The same enzyme preparation generated a diagnostic "aniline" fragment when assayed on yeast and *M. expansa* ribosomes, showing it to possess Endo's site-specific activity in addition to PAGase activity. In an attempt to determine whether *M. expansa* ribosomes, or some other non-ribosomal RNA is the likely target site for ME1, the activity on ribosomes was assayed in the presence of increasing concentrations of a synthetic 23mer oligoribonucleotide containing a single A residue at its centre flanked on either side by G and C residues capable of

forming a perfect intramolecular A-form double helix. The oligoribonucleotide protected the ribosomes from ME1 action, a finding the authors' interpreted to show that ribosomes are unlikely to be the primary target for RIP activity. However, they did not present evidence to show whether the oligoribonucleotide was a substrate for ME1. There is evidence of a positive correlation between PAGase activity and anti-viral efficacy (Park et al. 2004). RIPs such as RTA, which lack PAGase activity lack anti-viral activity, whereas the converse holds for RIPs with high activity such as PAP. However, this is contentious because in the case of PAP its anti-viral action *in planta* is dependent on an intact C-terminus, whereas its *N*-glycosidase activity is not (Tumer et al. 1997).

Opinion as to the possible physiological relevance of the PAGase activity of RIPs is sharply divided, and the fact that it can be demonstrated to occur *in vitro* does not necessarily mean it has a significant role *in vivo*. For example, it is clear that most RIPs possess PAGase activity on DNA, but as there is no evidence of their entering the nucleus, they may never have the opportunity to exercise this activity *in vivo*. The physiological relevance of PAGase has also been questioned on the grounds of its catalytic inefficiency in relation to the "classic" activity on ribosomes (Robertus and Monzingo 2004). For example, using data published from Rajamohan et al. (1999a, b) for the release of adenine from HIV-1 RNA by PAP (250 pmol of PAP released only 168 pmol of adenine per hour), they calculated the activity to be 30,000–100,000 lower than for the release of adenine from ribosomes. Also, there is no evidence that non-cap-independent PAGase of RIPs occurs *in vivo*, yet extensive evidence exists that ribosome depurination is an early event following intoxication of cells by type 2 RIPs.

3.2 5′ Cap-Dependent Activity

In addition to PAGase activity, PAP also possesses a deguanylation activity, releasing approximately equimolar amounts of guanine and adenine from HIV-1 RNA and other RNA substrates (Rajamohan et al. 1999a, b). Modelling studies showed that guanine is able to fit into that active site of PAP very much like adenine. This may also be pertinent to the finding that PAP depurinates certain capped, but not uncapped, RNAs *in vitro* (Hudak et al. 2000). PAP binds to the m^7Gppp structure of luciferase mRNA, and although it does not depurinate the cap structure, it removes A and G residues throughout the mRNA, as revealed by the positions of numerous primer extension products, suggesting that it acts in *cis* after binding to the cap. The sequence context of the depurination sites does not reveal any conserved features. This work was extended to the translation *in vitro* of the RNAs of the plant virus brome mosaic virus (BMV) and potato virus X (PVX) (Hudak et al. 2000). Translation of capped, but not uncapped RNAs was inhibited by wild-type PAP and certain PAP mutants that were unable to depurinate reticulocyte ribosomes. The inhibition was overcome by the presence of the cap analogue m^7GpppG but not GpppG or GTP suggesting, as above, that PAP recognises the cap structure. The authors suggest that

this activity could be responsible for the anti-viral activity of PAP. However, Peumans et al. (2001) raised a number of concerns about this work which are worthy of repetition. First, the mutants that were used were PAPx, an active site mutant (E176V), PAPn, a G75D substitution near the N-terminus and PAPc, in which the C-terminal 25 residues were deleted. All three mutants were reported to be inactive on tobacco and reticulocyte ribosomes, whereas in previous work (Tumer et al. 1997), PAPc was reported to inhibit translation by reticulocyte ribosomes. Second, the authors state that wild-type PAP, PAPc and PAPn (the latter two reportedly inactive on ribosomes) have a direct effect on capped RNAs that significantly reduces their translation. In the case of PAP, it is clearly shown that capped RNAs are depurinated, but no data are shown for PAPc and PAPn, so it is unclear whether they can also depurinate capped RNAs. This omission is important because the authors state that inability of these mutants to depurinate ribosomes is because of an altered association, and not a general impairment of activity.

Another effect of PAP expression in yeast is to destabilise its own mRNA by a mechanism that requires depurination, as evidenced by the need for a functional active site, but which can be separated from the depurination of ribosomes, as evidenced by the finding that a mutation in the N-terminal region (L71R) was active in ribosome depurination, but not in mRNA destabilisation (Parikh et al. 2002). Expression of PAP did not cause a general reduction in mRNA levels because the levels of four constitutively expressed yeast transcripts were unaffected by PAP. It is difficult to envisage what the physiological role of PAP mRNA destabilisation might be, and how the apparent specificity for targeting PAP mRNA, and the viral RNAs described above, might be achieved, especially taking into account that it is also observed with capped luciferase mRNA. However, the authors do consider that capped PAP mRNA could be a physiological substrate for PAP, based on their finding that the affinity of PAP for capped message is only fourfold lower than for naked rRNA, as determined by equilibrium binding (Hudak et al. 2002). But the relevant comparison here is with ribosomes, and not naked RNA, because ribosomes are the physiological substrate, and for which the catalytic efficiency of depurination by RIPs is several orders of magnitude higher than naked RNA.

The proposal that the anti-viral activity of RIPs could operate through a cap-dependent adenosine and/or guanosine glycosylase is an attractive one, and gains support from its demonstration *in vivo*. However, as with non-cap-dependent PAGase, its activity is several orders of magnitude lower than for the "classic" depurination of ribosomes. Proponents of an anti-viral role may argue that the finding that the anti-viral and ribosome-inactivating activities can be separated from each other in certain RIP mutants is strong evidence for their stance, but such mutants have not been found to occur in nature. An anti-viral mechanism based on RIPs which did not damage ribosomes could be highly advantageous to plants, yet the fact that mutants inactive on ribosomes can be generated by single amino acid substitutions in the laboratory, but are not found in nature, is evidence of a strong selective pressure to maintain ribosome-inactivating activity during evolution. Of course, the biotechnological exploitation of RIPs based on such mutants may offer considerable promise.

4 DNA Lyase

Structural and biochemical studies on *Mirabilis* anti-viral protein 30 (MAP30) from the bitter melon, whose extracts have been used as therapeutic agents for centuries, have shown that this type 1 RIP acts as both a DNA glycosidase and an apurinic site (AP) lyase (Wang et al. 1999). MAP30 exhibits potent anti-tumour activity against human cancer cell lines, inhibits HIV-1 infection of lymphocytes and monocytes and inhibits viral replication in infected cells. From structural studies, it has been proposed that AP sites in DNA, generated by MAP30's *N*-glycosidase activity bind to a conserved tryptophan residue (Trp190) located on the protein surface in the vicinity of the *N*-glycosidase active site cleft, which brings the AP site in close contact with a conserved lysine side chain (Lys195). DNA backbone cleavage is postulated to occur through the nucleophilic attack by the lysine amino group on the C1′ deoxyribose of the AP site. Gelonin, a type 1 RIP from *Gelonium mutiflorum* seeds, and PAP have been found to degrade single-stranded DNA in the presence of Zn^{2+}, first by removing an RIP-specific set adenines, followed by the formation of an enzyme-imino intermediate characteristic of DNA glycosides/AP lyases, and finally strand cleavage at the 3′ of the abasic sites through a β-elimination reaction (Nicolas et al. 1998). It has been proposed that the glycosylase/AP lyase activity explains MAP30's inhibition of the HIV-1 integrase, its ability to irreversibly relax supercoiled DNA, and may contribute to the non-cytotoxic pathway leading to its anti-tumour and anti-HIV-1 activities (Wang et al. 1999). However, it has been pointed out by Peumans et al. (2001) these conclusions are based on circumstantial evidence because the purity of the enzyme preparations has not been properly assessed. It is noted also that PD-L4, a single-chain RIP from *Phytolacca dioica* which contains a lysyl residue corresponding to K195 of MAP30 does not show DNA nicking activity (chapter, "Type 1 ribosome-inactivating proteins from the ombú tree (Phytalacca dioica L.)" by Parente et al. in this volume). A broader review of the relevant literature on the action of RIPs on DNA is also given in this chapter of this volume and the reader is directed there.

5 Bifunctional Enzymes with RIP Activity in Which the Non-RIP Activity Acts on Non-Nucleic Acid Substrates

5.1 Lipase

An initial observation by Moulin et al. (1994) that ricin and a lipase from another member of the Euphorbiaceae (the same family of *Ricinus communis*) share significant sequence homology prompted an investigation into possible lipase activity of ricin. Lipase activity, with specificity towards neutral lipids, was discovered raising the possibility that this could facilitate access to the cytosol by

providing local destabilisation of the membrane (Lombard et al. 2001). Further characterisation of this activity showed it to involve both the subunits (RTA and RTB) of ricin holotoxin, but not RTA and RTB separately, and that from theoretical considerations, activity resided in the subunit interface, comprising the lipase catalytic triad residues Ser211 and His40 from RTA and Asp94 from RTB on the basis of the position of these residues in relation to those in a reference lipase active site (Molon-Guyot et al. 2003). These residues are conserved in the toxic type 2 RIP abrin, but not in the barely toxic type 2 RIPs ebulin 1 and mistletoe lectin 1. Mutation of Ser211 to Ala resulted in the loss of lipase activity of the reconstituted holotoxin, but did not affect intracellular routing by mouse lymphocytes or RNA *N*-glycosidase activity. It was calculated that the mutant RTA translocated into the cytosol at a rate of 64% of wild-type RTA from the assumption that translocation is the rate-limiting step in cytotoxicity, and thus implying a role for lipase activity in translocation. However, the mutation could affect the cytotoxicity of RTA for other reasons, for example its ability to refold following translocation or its protease sensitivity in the cytosol. Another imponderable is that the specificity of the lipase activity is towards triglycerides which are only minor components of membranes, although the compartments from which RTA translocation is thought to occur are enriched in triglycerides compared with the plasma membrane (Molon-Guyot et al. 2003). Because of these uncertainties, and the fact that the lipase activity of ricin makes only a small contribution to its cytotoxiciy, its role remains an open question.

5.2 Chitinase

In plants, chitinases form part of a defence mechanism against fungal pathogenesis as one of a group of pathogenesis-related proteins, and they have been reported to act synergistically with RIPs to provide protection against the pathogenic fungi *Trichoderma reesei* and *Fusarium sporotrichioidies* (Leah et al. 1991). Shih et al. (1997) reported that three isoforms of the type 1 RIP trichosanthin isolated from cell suspension cultures of *Trichosanthes kirilowii* exhibited chitinase activity in addition to *N*-glycosidase activity. Two of the isoforms were of a mass typical of type 1 RIPs (ca. 28 kDa), whereas the third was much smaller (ca. 15 kDa). It was claimed that the RIP preparations were highly pure, as judged by silver-stained gels, but as more rigorous methods of ascertaining purity were not applied, this claim must remain questionable.

5.3 Superoxide Dismutase

It was thought for many years that tobacco (*Nicotiana tabacum*) did not produce an RIP. However, leaves were shown to contain small amounts (ca. 0.01% of total soluble protein) of a 26 kDa protein which clearly exhibited classical *N*-glycosidase

activity on yeast ribosomes (Sharma et al. 2004). The RIP (termed TRIP) was subjected to sequencing, and a partial sequence of 15 residues obtained from a tryptic digest fragment did not share homology with any known RIP, but was identical to a sequence found in Fe-superoxide dismutase (Fe-SOD) of *Nicotiana plumbaginifolia*, *Arabidopsis* and potato, whose main function in plants is to dismutate the chemically aggressive superoxide radicals formed in photosynthesis when electrons are transferred from PS1 to oxygen, forming hydrogen peroxide and reducing a metal ion such as Fe^{3+} (Heldt 1997). The full length sequences of these SODs in the database show no homology to RIPs, but TRIP did indeed show SOD activity, suggesting that its RIP activity could be a contaminant. Because TRIP showed an exact sequence homology with Fe-SOD, the authors tested a commercially available Fe-SOD from *E. coli* and bizarrely this had classical *N*-glycosidase activity against yeast ribosomes. It seems very unlikely that the *E. coli* strain used was enterohemorrhagic, producing Shiga-like toxin which contaminated the Fe-SOD. So we are left with two possibilities: either one or more of the common reagents used for RIP assays in this study was contaminated with an RIP from some other source, or TRIP and Fe-SOD are indeed truly bifunctional enzymes, and for which the RIP activity resides in a unique active site.

6 Conclusions

The nature of the enzymatic modification to ribosomes by the action of RIPs is well understood and defines this class of enzymes. The structure of the site of action of RIPs – the SRD – is known at the atomic level and allows a non-base-stacked adenosyl residue to interact with the active site of RIPs. Although a few reports have cast doubt on the inhibitory effect that ribosome depurination is generally recognised to have on protein synthesis, these are most probably in error. Recent progress has shown that two distinct sets of ribosomal proteins are responsible for binding RIPs and accounting for the enhancement in the rate of RIP modification compared to naked rRNA. The P proteins of the central stalk of the 60S subunit are responsible for binding one subset of RIPs, including RTA, whilst L3 near the peptidyl transferase binds another subset, including PAP. In addition to their specific action on ribosomes, all RIPs show a sequence context-independent depurination activity on DNA and RNA, although the level of promiscuity shown by individual RIPs on these substrates varies widely. Some of the biological effects of RIPs, notably their anti-viral activities, correlate with the RIP's ability to depurinate capped viral RNAs and although this activity requires a functional *N*-glycosidase active site, it can be separated from ribosome inactivation by mutations elsewhere in the RIP. How such an activity could specifically target viral and certain other capped mRNAs, including that for PAP, is unknown. The history of research on RIPs has been bedevilled by reports of activities in addition to the *N*-glycosidase in supposedly pure preparations of RIPs. Many of these do not stand up to close scrutiny and should be viewed with caution.

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Type I Ribosome-Inactivating Proteins from *Saponaria officinalis*

Alessio Lombardi, Richard S. Marshall, Carmelinda Savino, Maria Serena Fabbrini, and Aldo Ceriotti

Abstract Saporins are ribosome-inactivating proteins (RIPs) extracted from different tissues of the soapwort plant (*Saponaria officinalis* L.). While the biosynthesis of these proteins and their roles in planta have received little attention, saporins have been extensively used for the production of targeted toxins for therapeutical and research applications. The biochemical features of one group of closely related saporin isoforms, collectively named SO6, have been characterized in considerable detail. In this chapter, we summarize available information on the saporin family of proteins, including their catalytic activity, 3D-structure, and biosynthetic and intoxication pathway(s), emphasizing the differences between the different family members and the characteristics that distinguish saporin from the catalytic subunit of the prototype Type II RIP ricin. The use of heterologous systems for the production of saporin and saporin-based chimeric toxins is also described.

1 Introduction

Ribosome-inactivating proteins (RIPs) are potent inhibitors of protein synthesis that act by catalytically depurinating an adenine residue (A4324 in rat) present in a conserved stem-loop region of 23/26/28S ribosomal RNA (rRNA), causing an irreversible arrest in protein synthesis (Endo and Tsurugi 1987, 1988; Endo et al. 1988; Hartley et al. 1991). The prototype RIP is the ricin AB dimer, whose

A. Lombardi, R.S. Marshall, M.S. Fabbrini, and A. Ceriotti (✉)
Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Via Bassini 15, 20133 Milano, Italy
e-mail: a.lombardi@ibba.cnr.it; marshall@ibba.cnr.it; fabbrini@ibba.cnr.it; ceriotti@ibba.cnr.it
C. Savino
Istituto di Biologia e Patologia Molecolari, Consiglio Nazionale delle Ricerche, c/o Università "Sapienza" di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy
e-mail: linda.savino@uniroma1.it

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_4,

biochemical features, catalytic activity, biosynthetic pathway, and intracellular transport have been studied in great detail. Ricin is synthesized as an inactive single-sized precursor that is transported to the protein storage vacuoles of castor bean endosperm cells and processed into disulphide-linked A (RTA) and B (RTB) chains (Butterworth and Lord 1983; Hiraiwa et al. 1997). The mechanism by which the ricin dimer intoxicates mammalian cells has also been thoroughly characterized (see chapter, "How Ricin Reaches its Target in the Cytosol of Mammalian Cells" by Spooner et al. in this volume) (Sandvig and van Deurs 2000; Lord et al. 2003). In contrast, little is known about the biosynthesis and trafficking of Type I (single chain) RIPs, their physiological function(s) in planta, and the mechanism(s) by which they reach the cytosol after uptake by mammalian cells.

The name saporin collectively identifies a family of RIPs that accumulate in different soapwort (*Saponaria officinalis* L.) tissues. Several cDNA and genomic clones coding for different members of the saporin family of proteins have been isolated, and individual isoforms (or mixtures of closely related isoforms) have been purified. The three-dimensional structure of one isoform has been solved, and the enzymatic activity of individual family members has been studied in some detail. While some characteristics of the saporin proteins, such as key catalytic residues and overall three-dimensional fold, are shared with RTA, certain biochemical and functional properties clearly diverge. In this chapter, we will describe the principal characteristics of the saporin protein family, highlighting both the differences between different family members and the specific features that distinguish these proteins from RTA and from other Type I RIPs.

2 Saporin Multigene Family and Saporin Isoforms

Saporins are encoded by a small multigene family (Fordham-Skelton et al. 1990; Barthelemy et al. 1993). While saporins are often designated on the basis of the tissue of origin and the number of the chromatographic peak in ion-exchange chromatography, it should be stressed that these chromatographic peaks can contain two or more closely related isoforms, and that the use of different purification procedures implies that peaks having the same or similar names cannot be assumed to contain an identical set of proteins. In addition, some recombinant proteins have in some instances been given a name similar to the one used for chromatographic peaks. Thus, saporin nomenclature is somewhat confusing, and attention must be paid when comparing data from different sources.

The presence of multiple RIP isoform has been reported for different members of the Caryophyllaceae family of plants, such as *Dianthus caryophyllus* (Stirpe et al. 1981) (a plant belonging to the same subfamily as *S. officinalis)*, *Lychnis chalcedonica* (Bolognesi et al. 1990), and *Petrocoptis glaucifolia* (Arias et al. 1994). The tissue distribution of dianthin 30 and 32 in *D. caryophyllus* was investigated by the use of anti-dianthin antibodies; while dianthin 30 was found throughout the plant, seeds included, dianthin 32 was detected only in leaves and

growing shoots (Reisbig and Bruland 1983). Both isoforms accumulate in old tissues, where they represent between 1 and 3% of the total extractable protein.

The tissue distribution of saporin, like that of dianthin, contrasts with that of the RIPs present in the Gramineae family (Coleman and Roberts 1982), or in *Ricinus communis* (Tregear and Roberts 1992), where the RIPs are apparently confined to the seeds. When translation inhibitory activity was monitored in different soapwort tissues, it was detected in all those that were examined (leaves, stems, roots, flowers, and fruits), except immature seeds (Ferreras et al. 1993). A high level of activity was found in roots and mature seeds, while old and young leaves contained similar activity. The expression of saporin has also been studied in callus, cell suspensions, and root cultures from soapwort explants (Di Cola et al. 1997). High specific activity was found in callus extracts, while lower levels were present in root extracts. In addition, culture senescence and abscisic acid were found to induce saporin production in cultures of soapwort roots (Di Cola et al. 1999). These results suggest that callus and cell cultures may be a suitable model system to study saporin biosynthesis and biological function.

After fractionation of soapwort plant extracts, most of the translation inhibitory activity was found to be associated with three chromatographic peaks in seeds, two in leaves, and three in roots (Stirpe et al. 1983; Ferreras et al. 1993). N-terminal sequencing suggested that saporins present in these chromatographic peaks could be divided into three groups, each group being specific to one of the three organs, with the exception of one root isoform (R2) that has an N-terminal sequence similar to the ones of the two leaf isoforms (L1 and L2) (Ferreras et al. 1993). Notwithstanding the identical N-terminal sequence, saporin L1 and R2 have distinct biochemical properties. Two out of three root saporins (R1 and R3) were reported to be glycosylated and to contain cysteine residues, an amino acid which is absent in all other saporin isoforms (Ferreras et al. 1993).

Two of the three (5, 6, and 9) major peaks of activity identified in seed extracts have been characterized in detail (Stirpe et al. 1983). SO6 saporin represents the major peak (peak 6) and constitutes about 7% of the total seed protein. Direct sequencing of the protein revealed heterogeneity at two positions, with either aspartic or glutamic acid in position 48, and either lysine or arginine present in position 91 (Maras et al. 1990; Barra et al. 1991). These data indicate that the SO6 peak contains a set of closely related saporin isoforms. Indeed, RP-HPLC analysis confirmed the presence of atleast three different isoforms in SO6 preparations (Fabbrini et al. 1997a).

The primary structure of SO9 saporin (peak 9) has also been determined (Di Maro et al. 2001). The protein contains four histidine residues, an amino acid which is absent in all the other known seed isoforms, and presents 22 amino acidic substitutions when compared to SO6. No heterogeneity was found in this case, indicating that the SO9 peak contains a single saporin isoform. A preliminary crystallographic characterization of this protein has also been reported (Kumar et al. 1999).

The first DNA sequence coding for a saporin isoform was isolated from a leaf cDNA library (Benatti et al. 1989). Comparison with the sequence of

seed-extracted SO6 suggests that the polypeptide encoded by this cDNA clone contains both a signal peptide and a C-terminal extension. The predicted mature protein contains 11 amino acid differences when compared to SO6.

Three genomic clones, termed Sap2, Sap3, and Sap4, were also successively isolated (Fordham-Skelton et al. 1990, 1991). Two of them (Sap3 and Sap4) were truncated, while Sap2 was found to encode a full-length saporin precursor. Comparison of the sequence encoded by the Sap2 clone with the one of SO6 reveals again the presence of a signal peptide for insertion in the endoplasmic reticulum (ER) and of the C-terminal propeptide that must be removed to generate the native SO6 C-terminus (Fig. 1b). After removal of the signal peptide and of a C-terminal propeptide, the Sap2-encoded protein would be identical to one of the four putative isoforms potentially present in the SO6 peak.

Subsequently, five further partial clones (numbered 1–5) were isolated by PCR amplification of soapwort genomic DNA (Barthelemy et al. 1993). Of the encoded proteins, two are similar to the one encoded by the leaf cDNA clone (Benatti et al. 1989) while the others encode SO6 or SO6-like polypeptides. Three of these isoforms showed identical translation inhibition activity when recombinantly expressed in *E. coli* (Fabbrini et al. 1997a). In contrast, the protein encoded by the leaf cDNA clone (Benatti et al. 1989), also termed saporin-C, was 10-fold less active in the same assays. Consistently, the sequence of clone 5 described by Barthelemy et al. (1993) codes for a protein which is closely related to saporin-C and which is less active than the product of clone 2, which codes for one of the components present in the SO6 peak (Bagga et al. 2003a).

A few further cDNA sequences encoding saporin polypeptides have been deposited in the DNA data banks.

3 Saporin Biochemical Features

3.1 Saporin Structure

SO6 polypeptides are composed of 253 residues, corresponding to a molecular weight of ~28,600 Da (Maras et al. 1990). The proteins contain a net positive charge with an isoelectric point (pI) above 9.5 (Lappi et al. 1985; Di Maro et al. 2001). Lysine residues, which represent ~9% of the total, are particularly abundant. The SO6 proteins are also characterized by high resistance to chemical denaturation and proteolytic degradation in vitro (Santanché et al. 1997). Deletion of the first 20 amino acids has been shown to drastically affect saporin folding (Bonini et al. 2006), while deletion of the last 19 amino acids has a detrimental effect on catalytic activity (Pittaluga et al. 2005).

Despite the structural similarity between saporin, RTA, and other Type I RIPs, sequence identity is low: only 62 residues (about 22%) are conserved between RTA and SO6, and 44 residues (about 15%) between the latter and trichosanthin (TC).

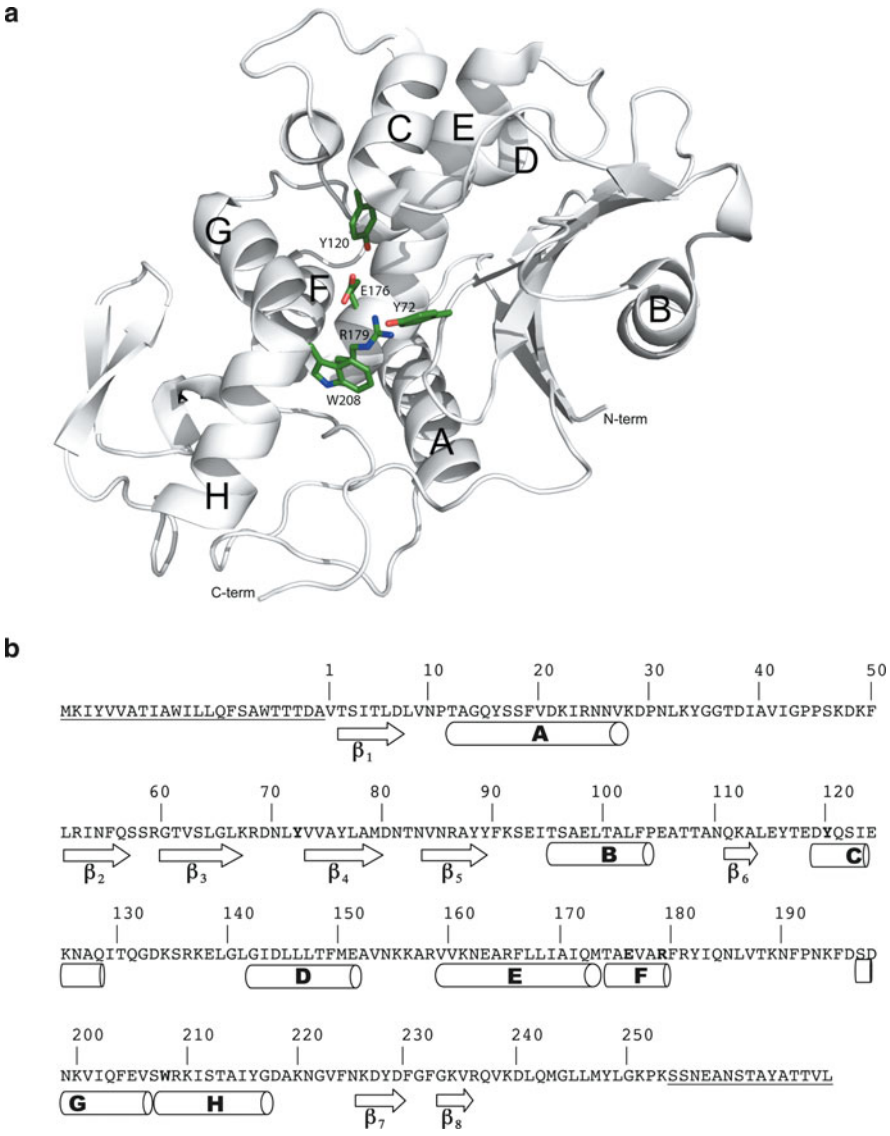

Fig. 1 Overall 3D-structure of saporin. (**a**) Ribbon representation of SO6 structure (PDB code: 1qi7) in which the secondary structure elements are shown. The five conserved residues in the active site (Tyr 72, Tyr 120, Glu 176, Arg 179, and Trp 208) are shown in *stick representation*. (**b**) Amino acid sequence of a putative SO6 saporin precursor, as deduced by the DNA sequence of the Sap2 clone (Fordham-Skelton et al. 1991). Helices are shown as *cylinders* and are named following the canonical RIP nomenclature. Strands are shown as *arrows*. The conserved active site residues are shown in *bold*. The N-terminal signal peptide and the C-terminal propeptide sequences are *underlined*. Numbering starts with the first amino acid of the mature protein

On the contrary, a high degree of sequence identity (around 80%) is found between saporin and dianthin, both of which are synthesized by plants belonging to the subfamily Silenoideae of the Caryophyllaceae family.

The three-dimensional structures of different Type I RIPs including momordin (Husain et al. 1994), pokeweed antiviral protein (PAP) (Monzingo et al. 1993), TC (Zhou et al. 1994), gelonin (Hosur et al. 1995) and, more recently, dianthin (Fermani et al. 2005) have been determined. SO6 has been crystallized (Savino et al. 1998) and the crystal structure resolved (Savino et al. 2000). Saporin shares with other Type I RIPs and with RTA a common "RIP fold" characterized by the presence of two major domains: an N-terminal domain which is predominantly β-stranded, and a C-terminal domain that is predominantly α-helical (Figs. 1 and 2). Insertions and deletions as compared to PAP, momordin, and RTA lie mainly in the random coil regions. Most of the secondary structural elements are comparable between saporin and other Type I RIPs. The deviations are seen mainly in some surface-located loop regions, particularly: (1) between strands β_4 and β_5 (residues 79–85), (2) helix B and the loop connecting this helix to strand β_6 (residues 95–109), and (3) between helices C and D (residues 128–134). Figure 2 shows, as an example, a superimposition of the SO6 and RTA structures. Interestingly, the loop connecting strands β_7 and β_8 located at the C-terminal domain, whose length is variable among RIPs, is very short in SO6 (only three residues), and in dianthin 30 (Fermani et al. 2005), but is longer in PAP and RTA. This region contains three lysine residues (220, 226 and 234), which seem to be involved in the molecular recognition of the ribosome. The reduced length of this loop could determine an increased accessibility to the substrate for both saporin and dianthin. The catalytic cleft is almost perfectly superimposable in all RIPs, including saporin, except for

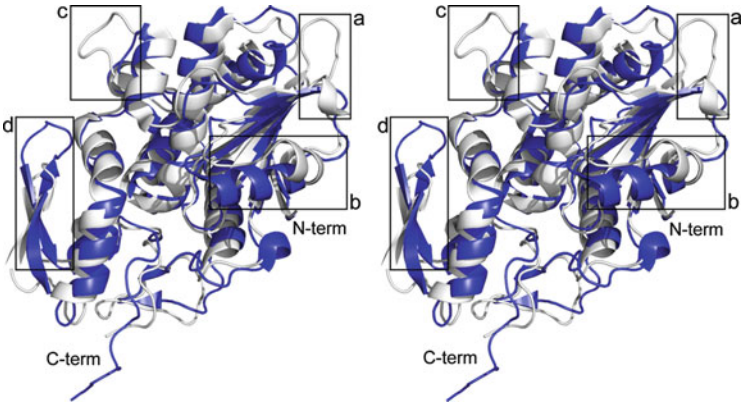

Fig. 2 Structural comparison based on superimposition of secondary structure elements of SO6 (PDB code: 1qi7, *white*) with the A chain of ricin (PDB code: 1j1m, *black*), stereoview. The regions with the largest deviation are included in *boxes* and *labeled*: (**a**) loop between strands β_4 and β_5 (residues 79–85), (**b**) helix B and the loop connecting this helix to β_6 (residues 95–109), (**c**) CD loop (residues 128–134) and (**d**) loop connecting strands β_7 and β_8 (residues 230–233)

the orientation of one of the two tyrosine residues (Tyr 72 in saporin) that are involved in the interaction with the target adenine (Fermani et al. 2009).

3.2 Saporin Catalytic Activity

Saporin is an RNA *N*-glycosidase that removes a specific adenine residue (A4324 in rat) located in the highly conserved GAGA-tetraloop, also termed the α-sarcin/ricin-loop, present in 23/26/28S rRNA (Endo et al. 1988). Like RTA (Endo and Tsurugi 1987), SO6 has been found to release a single adenine from 80S ribosomes (Sturm et al. 2009). However, removal of a second adenine residue has also been reported (Fermani et al. 2009).

Typically, saporin SO6 preparations and recombinant SO6 proteins are found to inhibit translation in a rabbit reticulocyte lysate with an IC_{50} in the low picomolar range (Ferreras et al. 1993; Fabbrini et al. 1997a; Bagga et al. 2003a, b; Pittaluga et al. 2005). While *E. coli* ribosomes are more resistant than mammalian ribosomes to the action of different saporins (Ferreras et al. 1993; Girbés et al. 1993), the difficulties in expressing various saporin isoforms in *E. coli* suggest that they are toxic to this host (Fabbrini et al. 1997a). Indeed dianthin 32, which is highly similar to SO6 saporin, was found to be both ~500 times more active on yeast than on *E. coli* ribosomes, and specifically to depurinate 23S rRNA at a site which is equivalent to A4324 in rat 28S rRNA (Hartley et al. 1991).

Different saporin preparations have been found to be active in inhibiting protein synthesis from plant (*Vicia sativa*, *Cucumis sativus*, wheat germ) ribosomes (Ferreras et al. 1993). In particular, a chromatographic fraction named saporin 5 was shown to depurinate *V. sativa* ribosomes at a site corresponding to A4324 in rat 28S RNA, although other depurination sites were evident as well (Iglesias et al. 1993). We have also found that saporin expression is highly toxic to tobacco protoplasts and that this toxicity depends on the presence of an intact active site (our unpublished observation).

Several saporin fractions have been shown to depurinate naked nucleic acids such as viral genomic RNA, herring sperm DNA, rRNA, and poly(A) RNA, and to release more that 1 mole of adenine per mole of ribosomes, thus possessing polynucleotide:adenosine glycosidase (PNAG) activity (Barbieri et al. 1992, 1994, 1997). This activity has been characterized in detail for the saporin fraction L1. The protein was found to inhibit translation in a reticulocyte lysate system with an IC_{50} of ~45 pM (Sturm et al. 2009) and to release ~6.5 adenines from rat liver ribosomes before 50% inhibition was observed in an in vitro assay with poly(U) transcript (Barbieri et al. 1996). Under appropriate conditions, saporin L1 was found to depurinate DNA extensively and released adenine from all adenine-containing polynucleotides tested. Characterization of the kinetic parameters indicated that poly(A) RNA depurination proceeds with a K_m of 639 ± 32 μM and a k_{cat} of 61 ± 1 min^{-1} at pH 7.8 and 25°C. The catalytic efficiency of L1 on this substrate thus appears to be considerably lower compared to the action of a typical

RIP, such as ricin A chain, on intact rat ribosomes, which has been reported to occur with a K_m of 2.6 μM and a k_{cat} of 1,777 min^{-1} (Endo and Tsurugi 1988). Determination of the kinetics of ribosome depurination at different sites in relation to the inhibition of protein synthesis will be required to understand the mechanism of 80S ribosome inactivation by saporin L1.

In addition to saporin L1, other members of the saporin family have been shown to be endowed with PNAG activity. All tested saporins were active on herring sperm DNA at pH 4.0, while activity on poly(A) RNA, rRNA, and viral RNA varied widely between different members of the family. Two leaf saporin fractions and one root fraction (all sharing similar N-terminal sequences) appeared to be generally more active than other isoforms on poly(A) RNA, rRNA, and viral RNA (Barbieri et al. 1997). The activity of seed-extracted SO6 was found to vary between different batches or experiments when poly(A) or viral RNAs were used as substrates (Barbieri et al. 1997; Fermani et al. 2009). Recombinant saporins were instead essentially inactive on poly(A) and viral RNAs, but active on rRNA at pH 4.0 (Barbieri et al. 1997).

The biological significance of the activity against rRNA at sites different from the one attacked by ricin, and on substrates other than the ribosome (DNA, viral RNA, poly(A) RNA), remains to be established. When rRNA was extracted from mammalian cells treated with an SO6 saporin fraction, rRNA was found to be depurinated at a single site most likely corresponding to the ricin target (Vago et al. 2005). Analysis of the in vivo activity of other saporin fractions (such as fraction L1) will be required to assess whether multiple depurination plays any major role in the intoxication process.

In addition to these depurinating activities, saporin has been proposed to have DNase-like activities (Roncuzzi and Gasperi-Campani 1996; Ghosh and Batra 2006). However, several lines of evidence indicate that the DNase activity associated with saporin (and with other RIPs) may be due to contamination (Day et al. 1998; Barbieri et al. 2000; Peumans et al. 2001; Lombardi et al. 2010).

3.3 Residues Important for the Catalytic Activity

The active site of the SO6 protein includes a number of residues that are conserved in the RIP family of proteins: Tyr 72, Tyr 120, Glu 176, Arg 179, and Trp 208 (Fig. 1). The role of these residues in the catalytic activity of a component of the SO6 peak (named saporin 6), has been systematically investigated by mutating them to Ala (Bagga et al. 2003b). Mutating Tyr 72 had a stronger impact on saporin 6 catalytic activity than mutating Tyr 120. This is similar to what has also been observed for the corresponding tyrosine residues in RTA where mutation of Tyr 80 and Tyr 123 reduced RTA catalytic activity 160- and 70-fold, respectively (Monzingo and Robertus 1992).

Both Glu 176 and Arg 179 are thought to be directly involved in saporin 6 catalysis. However, while the Glu 176 mutant was 20-fold less active than

wild-type saporin 6 in inhibiting translation in a reticulocyte lysate, the Arg 179 mutant was 200-fold less active. In RTA, the same mutation in Glu 177 facilitates the nearby Glu 208 to move into the active site, fulfiling the role of Glu 177 (Frankel et al. 1990; Kim et al. 1992). It has therefore been proposed that, in the corresponding saporin 6 mutant, Glu 205 occupies the position of the mutated Glu 176, but that the carboxylate of Glu 205 provides less stabilization to the oxycarbonium ion transition state than Glu 176 (Bagga et al. 2003b). Interestingly, a Glu 176 change to a lysine (a residue bearing an opposite charge) led to a more drastic increase in the IC_{50} in an in vitro assay (Pittaluga et al. 2005).

A complete loss of in vivo toxicity was obtained by mutating both Glu 176 and Arg 179 into lysine and glutamine, respectively. This double mutant (termed KQ) is devoid of the detrimental effects associated with RIP expression in several different hosts (Zarovni et al. 2007; Lombardi et al. 2010). Intriguingly, no correlation between the in vitro enzymatic activity and cytotoxicity was reported for a saporin mutant at Trp 208 (Bagga et al. 2003b) while this same residue was seen to be crucial for the structural integrity of PAP (Rajamohan et al. 2000).

Tyr 16 and Arg 24 are two other invariant residues lying outside the active site and present among various RIPs, but whereas mutation at Arg 24 did not have any effect on the enzymatic activity of saporin 6, mutation at Tyr 16 resulted in a complete loss of the RNA *N*-glycosidase activity (Bagga et al. 2003b). In contrast, deletion of residues 21–23 of RTA (including a tyrosine residue equivalent to Tyr 16 in saporin 6) did not affect the functional activity of the protein (Morris and Wool 1992), while mutation of Tyr 14 to Phe in TC resulted in a relatively small (5-fold) decrease in RIP activity (Shaw et al. 1997). The basis of these differential effects remains to be clarified.

A contribution from Asn 162 of saporin 6 has been highlighted by a study in which the catalytic activity of two saporin isoforms, encoded by two different genomic clones (corresponding in sequence to clone 2 and clone 5 described by Barthelemy et al. 1993) has been compared (Ghosh and Batra 2006). The product of clone 2 (saporin 6) is about 10-fold more active than the product of clone 5 (saporin 5) in inhibiting protein synthesis (Bagga et al. 2003a). Among the 12 amino acid differences between the two proteins, six are conservative in nature. Five other amino acid differences were found to be irrelevant, but when Asn 162 of saporin 6 was replaced with Asp (the amino acid found at that position in saporin 5), the IC_{50} of the protein in an in vitro translation system increased $\sim$10-fold. Asn 162 is proximal to a set of hydrophobic residues placed on a neighboring helix, and the introduction of a negative charge at this position has been proposed to affect the stability of the active site (Ghosh and Batra 2006).

3.4 Interaction with the Ribosome

Although RTA can depurinate naked rRNA, the k_{cat} value of such a reaction is 10^5-fold lower than that for rRNA associated with proteins in the ribosome

(Endo and Tsurugi 1988). This suggests that the interaction between the RIP and the ribosomal proteins is essential to achieve optimal enzymatic activity. RTA can be cross-linked to mammalian ribosomal proteins L9 and L10e (Vater et al. 1995), whereas PAP gains access to the ribosome by recognizing L3 (Hudak et al. 1999). In eukaryotic ribosomes, P0, P1, and P2 proteins form a pentameric P-complex (P0 $(P1)_2(P2)_2$) which constitutes the so-called ribosomal stalk (Tchorzewski 2002). The P-complex docks onto the ribosome through an interaction with 28S rRNA and forms the GTPase-associated center for binding elongation factors during protein synthesis (Uchiumi and Kominami 1992). The ribosomal stalk has been implicated in the binding of TC, RTA, and Shiga-like toxin 1 A_1 chain to the ribosome (Chan et al. 2007; McCluskey et al. 2008). In the case of TC, three key basic residues (Lys 173, Arg 174 and Lys 177) located in the C-terminal domain are involved in P2 binding.

Chemical cross-linking studies suggest that at least one 30 kDa ribosomal protein from the 60S yeast ribosomal subunit comes into contact with saporin (Ippoliti et al. 1992). Savino and coworkers further studied the molecular interaction(s) between SO6 and the yeast ribosome by differential chemical modifications and identified a contact surface within the C-terminal region of saporin which includes three lysine residues in positions 220, 226, and 234 (Savino et al. 2000).

A negative electrostatic potential, arising from both the negatively charged phosphodiester backbone and from conserved solvent-exposed acidic patches on the ribosomal proteins, covers much of the ribosomal surface (Baker et al. 2001). The net positive charge of saporin and its high content of basic residues are likely to be critical for the recognition of the ribosomal surface. In RTA, a set of arginine residues in the region of the active site are involved in electrostatic interaction with the phosphodiester backbone of the sarcin–ricin loop (Monzingo and Robertus 1992; Marsden et al. 2004). Both RTA- and saporin-catalyzed rRNA modification shows a net dependence on salt and ion concentrations, indicating that these toxins exploit multiple electrostatic interactions with the target ribosomes (Korennykh et al. 2007). These recent studies confirm that Coulomb interactions play a crucial role in helping saporin (and other RIPs) in finding their ribosomal target sites, and may explain how RIPs can operate on the ribosome with k_{cat} and K_m exceeding their basal encounter frequency by more than an order of magnitude.

3.5 Saporin Inhibitors

During the last few years there has been an increasing interest in identifying small molecules which may act as inhibitors of RIPs for diagnostics, as antidotes to poisoning, or to avoid side-effects following administration of immunotoxin therapies, such as the post-therapy vascular leak syndrome (Baluna and Vitetta 1997). Although RNA aptamers have been considered as potential inhibitors of RIPs (Hesselberth et al. 2000), previous studies identified small ring molecules, such

as formycin, that interfered with ricin enzymatic activity (Yan et al. 1998). The 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP) adenine analog was also shown to inhibit different RIPs to several different extents; whereas it was ineffective on ricin, it had some effect on Shiga toxin and SO6 (Brigotti et al. 2000), indicating that RIPs differ in their ability to fit adenine analogs within the active-site cleft, presumably due to local sequence variability. Interestingly, linear, cyclic, and stem–loop oligonucleotides mimicking the catalytic transition state showed potent inhibitory effect on the leaf saporin L1 isoform but not on seed-extracted saporin SO6 (Sturm et al. 2009).

4 Saporin Trafficking and Toxicity in Eukaryotic Cells

4.1 Subcellular Distribution of Saporin Isoforms in Soapwort Tissues

RIPs have been found to accumulate both intracellularly and in the apoplast. For instance, ricin accumulates in the matrix of vacuolar protein bodies (Tully and Beevers 1976; Youle and Huang 1976; Lord et al. 1994), while PAP is deposited extracellularly in leaves (Ready et al. 1986). A detailed study of the subcellular localization of saporin in soapwort seeds and leaf tissue has been performed using immunogold labeling techniques and anti-SO6 antibodies (Carzaniga et al. 1994). During seed development, saporin was found to accumulate in the perisperm, a maternal tissue derived from the nucellus that represents the major storage tissue in the Caryophyllaceae. In the perisperm, saporin was localized both intracellularly and extracellularly. In developing seeds, saporin was immunolocalized to the ER and cytoplasmic vesicles, and accumulated within the large central vacuole, either in small isolated deposits or in large protein aggregates. Outside perisperm cells, saporin was found in the intercellular spaces and the paramural region, between the plasmalemma and the primary cell wall, but was not detected within the cell wall matrix. Interestingly, saporin was also found within the residue of pollen-tube exudates. No accumulation of the toxin was observed within the embryo in either developing or mature seeds, indicating that expression of saporin genes is strictly tissue-specific. While these data indicate that saporin accumulates at several sites in the perisperm, it remains unclear whether this distribution is due to the expression of differentially targeted isoforms, to the presence of inefficient vacuolar targeting signals, or to both of these factors.

In leaves, immunolocalization using an anti-SO6 antibody showed saporin to be associated with the intercellular spaces within the chlorenchima, while no labeling was observed within the protoplasm (Carzaniga et al. 1994). However, the lack of an intracellular signal may be due to the presence of saporin isoforms that are not recognized by the anti-SO6 serum. More recently, a study based on mass spectrometry analyses and N-terminal sequencing of the apoplastic and intracellular leaf

isoforms identified saporin-L1 as the most abundant saporin vacuolar isoform, while the apoplastic forms were more related to seed-like isoforms (De Angelis et al. 2001).

4.2 Saporin Biosynthesis and Role in Planta

Signal peptide-mediated targeting of saporin precursors to the ER and segregation within the secretory pathway is likely to be essential to protect soapwort ribosomes from inactivation. Indeed, several saporin encoding cDNAs and genomic clones have been shown to encode a precursor form containing an N-terminal signal peptide (Benatti et al. 1989; Fordham-Skelton et al. 1991). In addition to an N-terminal signal peptide, different saporin isoforms may also contain a C-terminal propeptide. Conclusive evidence for the presence of both a signal peptide and a C-terminal extension has been obtained in the case of SO6. One of the clones (Sap2) described in Fordham-Skelton et al. (1991) encodes a protein whose sequence corresponds to one of the four possible components of the SO6 peak. Comparison of the predicted amino acid sequence of the Sap2 genomic clone with the one of the SO6 protein (Maras et al. 1990; Savino et al. 1998) reveals that SO6 is synthesized as a precursor with a 24 amino acid signal peptide and a 15 amino acid C-terminal extension that presents some similarity with C-terminal propeptides known to contain a vacuolar targeting signal (Vitale and Hinz 2005). Apoplastic saporin isolated from leaf tissue has a molecular weight similar to that of the seed SO6 protein, thus suggesting that an SO6-like protein is expressed in leaves, and that it accumulates in the apoplast. Indeed, cDNA clones encoding SO6-like proteins have been isolated starting from leaf mRNA (Benatti et al. 1991; accession number DQ105520). These results raised the possibility that SO6 proteins are, at least in part, responsible for the extracellular accumulation of saporin in perisperm tissue. If this was the case, other isoforms must be responsible for the accumulation of saporin in the vacuoles of perisperm cells. Alternatively, an SO6 vacuolar targeting signal may be more effective in seeds than in leaf tissue. Indeed, partial targeting of a seed saporin isoform to the vacuole is observed when the protein is expressed in tobacco protoplasts (our unpublished observation).

Saporin L1 has been shown to accumulate intracellularly in soapwort leaves (De Angelis et al. 2001), but the sequence responsible for the intracellular retention of this protein remains unknown. The isolation of a cDNA clone (accession number DQ105519) encoding a protein whose N-terminus (after signal peptide removal) corresponds to that of saporin L1 has been reported. A comparison between the molecular mass of intracellular saporin L1 (De Angelis et al. 2001) and of that predicted by this cDNA clone suggests that L1 saporin is also synthesized as a precursor containing a C-terminal propeptide.

The biological function of saporins *in planta* is still unknown. While the ability of different RIPs (including saporin SO6 and SO9 fractions) to inhibit viral replication is well documented (Stirpe et al. 1983; Taylor et al. 1994), the

underlying mechanism(s) remains uncertain. According to the local suicide hypothesis (Ready et al. 1986; Kataoka et al. 1992), cells in which the plasma membrane is transiently breached by a virus vector would permit entry of apoplast-located toxin and be killed through ribosome inactivation. This localized cell death would block both replication and spread of the virus throughout the plant. In this model, preaccumulation of the RIP in the apoplast may be crucial. However, this model of toxin action has been criticized, since protein synthesis in damaged cells would stop anyway, independent from RIP action (Peumans et al. 2001). An intriguing, alternative possibility is that physiological mechanisms might be in place to regulate access of a particular RIP to cytosolic ribosomes, so that protein synthesis is affected only when the cell becomes infected. The observation that Iris RIPs protect plants from local but not from systemic infection indicates that their antiviral activity is effective only in initially infected cells (Vandenbussche et al. 2004). Conceivably, specific signals in these cells may lead to a change in the subcellular localization of the stored toxin, or to the degradation of a putative RIP inhibitor (Desvoyes et al. 1997). The elucidation of the mechanisms that avoid self-intoxication of soapwort tissues and the development of methods to monitor saporin subcellular localization and ribosome depurination during viral infection may help our understanding of the role played by Type I RIPs in plants.

Saporin has also been reported to be toxic to two Coleoptera species (Gatehouse et al. 1989), and a direct effect of the RIP on insects remains an interesting possibility.

4.3 Intoxication Pathways in Mammalian Cells

The intracellular transport of RIPs after internalization by mammalian cells has been studied in great detail in the case of ricin (see the chapter, "How Ricin Reaches its Target in the Cytosol of Mammalian Cells" by Spooner et al. in this volume). Briefly, ricin enters target mammalian cells by receptor-mediated endocytosis and undergoes retrograde transport to the ER where its catalytic A chain (RTA) is reductively separated from the cell-binding B chain (Spooner et al. 2004). Free RTA then enters the cytosol where it inactivates ribosomes. In order to cross the ER membrane, RTA mimics ER-associated degradation (ERAD) substrates, probably escaping proteasomal degradation thanks to its paucity of lysine residues. Once in the cytosol, RTA interacts with Hsc70 chaperones, with its destiny (folding or degradation) then depending on the cochaperones that regulate Hsc70 activity (Spooner et al. 2008).

Less attention has been paid to the pathway followed by saporin, and Type I RIPs in general, in mammalian cells during the intoxication process. Saporin cytotoxicity varies dramatically between different mammalian cell lines, with the concentrations inhibiting protein synthesis by 50% (IC_{50}) ranging from the nanomolar to the micromolar range, thus spanning three orders of magnitude (Cavallaro et al. 1995; Bagga et al. 2003b). Different lines of evidence indicate that members of the LDL family of proteins are involved in the initial stages of

saporin endocytosis. The LDL receptor family includes seven closely related family members: LDLR, the very-low-density lipoprotein (VLDL) receptor, apoE receptor 2, multiple epidermal growth factor-like domains 7 (MEGF7), glycoprotein 330 (gp330/megalin/LRP2), LRP1, and LRP1B. These proteins have been shown to be promiscuous in ligand binding (Lillis et al. 2008). The family also includes more distantly related members, such as LRP5, LRP6, and SorLa/LRP11. One of these receptor proteins, the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP1), was shown to bind saporin in vitro (Cavallaro et al. 1995; Fabbrini et al. 1997a) and is able to mediate saporin internalization in human monocytes and fibroblasts (Conese et al. 1995; Fabbrini et al. 1997b). Evidence that LRP1 mediates saporin endocytosis has also been obtained in the case of human promyelocytic U937 cells, where the downregulation of LRP nicely paralleled resistance to saporin and to a urokinase–saporin conjugate (Conese et al. 1995). Specific displacement of iodinated LRP1-receptor associated protein (RAP) with saporin in these cells was also demonstrated (Rajagopal and Kreitman 2000). Competition experiments with a large excess of antigen-purified LRP1 polyclonal antibodies indicated that cytotoxicity of both saporin and an ATF-saporin fusion could be competed in U937 cells (Fabbrini et al. 1997b). Mouse embryonic fibroblasts (MEF-2) derived from LRP1 knock-out mice were at least 10-fold less sensitive to saporin compared to MEF1 cells carrying both LRP1 and low-density lipoprotein receptor (LDLR) (Vago et al. 2005), indicating a role for LRP1 and possibly LDLR in saporin internalization in mouse fibroblasts. LB6 fibroblasts transfected with the human receptor for urokinase have been used to study internalization of a urokinase–saporin conjugate, again demonstrating a clear role for LRP1 in mediating saporin–conjugate internalization (Ippoliti et al. 2000).

While LRP1 is clearly involved in saporin uptake, at least in some cell lines, it does not appear to be essential for saporin cytotoxicity in other cases. Indeed, one study found no changes in sensitivity toward saporin between a control cell line and a CHO cell line down-regulated for LRP (Bagga et al. 2003a). This study made use of a mutant CHO cell line (CHO 13-5-1) that has no detectable LRP mRNA or protein, and exhibits a 100-fold increase in resistance to *Pseudomonas* exotoxin (PE) (Fitzgerald et al. 1995).

Saponins are low molecular weight compounds mainly produced by plants, including *S. officinalis*. They affect the plasma membrane of living cells and artificial membranes by interacting with cholesterol. Soapwort saponins have been shown to greatly enhance saporin cytotoxicity toward several different cell lines (Weng et al. 2008; Fuchs et al. 2009). This saponin-mediated cytotoxicity was affected by drugs interfering with clathrin-mediated endocytosis, while inhibitors of caveolae-mediated endocytosis had no influence (Weng et al. 2008). In both Vero and HeLa cells, chloroquine and bafilomycin A1 had no effect on saporin toxicity, indicating that saporin translocation to the cytosol is not dependent on the low pH of endosomal compartments (Vago et al. 2005). However, in the presence of saponins, saporin-mediated cytotoxicity in ECV-304 cells could be blocked by bafilomycin A1 (Weng et al. 2008). These results suggest that saporin may follow different intoxication pathways in the absence and in the presence of saponins.

The intracellular site from which saporin can escape to the cytosol remains unknown. However, several lines of evidence indicate that Golgi-mediated retrograde transport to the ER is not a prerequisite for saporin cytotoxic effect. While treatment with brefeldin A, a fungal metabolite that causes disassembly of the Golgi complex, blocks ricin and RTA cytotoxicity (Yoshida et al. 1991; Simpson et al. 1996), such an addition to saporin-treated cells fails to reduce toxicity (Vago et al. 2005). In addition, while appending the ER retrieval sequence KDEL has been shown to increase RTA cytotoxicity, presumably by promoting its retrograde transport to the ER (Wales et al. 1993), it does not enhance saporin cytotoxicity (Vago et al. 2005). Although the possibility that saporin reaches the ER via a Golgi-independent route cannot be excluded at this stage, the lack of any effect of brefeldin A and KDEL addition suggests that saporin may escape to the cytosol from a different compartment. In contrast to RTA, it seems unlikely that saporin takes advantage of the ERAD machinery to retrotranslocate to the cytosol because it does not reach the ER. ERAD mutants of Chinese hamster ovary (CHO) cells that were resistant to ricin and PE remained as sensitive to saporin as parental CHO cells (Teter and Holmes 2002; Geden et al. 2007). In addition, some other critical features of RTA are not shared with saporin. Lipid partitioning studies using Triton X-114 have demonstrated that while RTA is predominantly found in the detergent phase, the ricin B chain, ricin holotoxin and several Type I RIPs, including saporin, are instead found in the aqueous phase (Day et al. 2002). Importantly, RTA has been shown to interact with negatively charged lipid vesicles and with ER membranes, undergoing a conformational change that could make it a better substrate for the ERAD system (Mayerhofer et al. 2009). After dislocation to the cytosol, the low lysine content of RTA allows the toxin to avoid proteasomal degradation, most likely by hampering efficient ubiquitination (Deeks et al. 2002). Conversely, saporin is a very stable protein, does not stably associate with negatively charged vesicles, and is very rich in lysine residues (Santanché et al. 1997; Deeks et al. 2002; Fabbrini et al. 2003; Mayerhofer et al. 2009). Taken together, these data suggest that saporin and RTA use different strategies to reach the cytosol of mammalian cells.

Intracellular tracing of fluorescinated saporin in Vero cells and HeLa cells revealed the presence of saporin in punctuate structures. While no colocalization with early endosome and Golgi complex markers could be observed, the distribution of internalized saporin partially overlapped with that of Lamp1, a late endosome marker (Vago et al. 2005). Consistent with a role of endosome-located saporin in the intoxication process, endosomal membranes could be permeabilized using lypopolyamines or DMSO, thus increasing the access of saporin, but not of RTA, to the cytosolic compartment. In addition, two mutant CHO cell lines defective in endosomal to lysosomal transport were greatly sensitized to saporin (Geden et al. 2007). If saporin can reach the cytosol from an endosomal compartment, the translocation mechanism is not pH dependent since as mentioned above, toxicity is not affected by chloroquine or bafilomicyn A1 (Vago et al. 2005). The productive delivery route of endocytosed Type I RIPs in mammalian cells is still under investigation, and it would seem that these proteins can reach the

cytosolic compartment following as yet unidentified pathway(s). Dissecting the intracellular pathways leading to saporin cytosolic delivery will be particularly important in view of the therapeutic uses of chimeric molecules based on this plant toxin (Fabbrini et al. 2003).

The intoxication pathway followed by TC (a toxin used as an abortifacient that behaves as an invasive toxin, targeting syncythiotrophoblasts, macrophages, and T-cells) has been recently investigated (Zhang et al. 2009). TC binds cell surface receptors belonging to the LDL-related receptor family, and it has been suggested that the known abortifacient and renotoxic actions of TC are caused by LRP1-mediated uptake in trophoblasts and by LRP2/megalin-mediated uptake in proximal tubule epithelial cells (Chan et al. 2000). In agreement with this observation, Jurkatt-T cells (which are devoid of proteins belonging to the LDL receptor family) are essentially resistant to free TC, while in at least two target cell lines, JAR and K562, endocytosed TC was incorporated into "pomegrenade" vesicles deriving from multivesicular body (MVB) membranes, and was then secreted in association with these vesicles upon fusion of the MVB with the plasma membrane. These TC-loaded vesicles could mediate intercellular secretion, targeting both syngeneic and allogeneic cells, and were much more effective in delivering TC than when free toxin was administered. Whether other Type I RIPs, like saporin, are able to hijack this exosome-mediated intercellular traffic remains to be clarified.

5 Heterologous Expression of Saporin and Saporin Fusion Toxins

Initial attempts to express recombinant Type I RIPs in *E. coli* were problematic, as reported in the case of *Mirabilis* antiviral protein (Habuka et al. 1989), PAP (Chaddock et al. 1994), and saporin (Barthelemy et al. 1993). Both in vitro depurination assays (Hartley et al. 1991), and the finding that *E. coli* ribosomes are depurinated in vivo (Chaddock et al. 1994), confirmed that host toxicity was due to RIP catalytic activity. Still, recombinant saporin can be purified in active form directly from bacterial lysates or recovered after refolding from inclusion bodies, indicating that the toxic effect is not sufficient to completely compromise biosynthetic activity before a substantial amount of the toxin has been accumulated (Fabbrini et al. 1997a; Bagga et al. 2003a, b; Ghosh and Batra 2006; Pittaluga et al. 2005).

The BL21(λDE3)pLysS strain has become a popular bacterial host for RIP expression driven by T7 RNA polymerase, and has been used to produce recombinant PAP (Chaddock et al. 1994), dianthin (Legname et al. 1995), and saporin (or saporin fusions) (Lappi et al. 1994; Fabbrini et al. 1997a, b; Heisler et al. 2003; Giansanti et al. 2010). Expression in the absence of an inducer is very low due to the presence of T7 lysozyme, which represses transcription by T7 RNA polymerase. Indeed, using this bacterial host, no saporin was detectable in the absence of the

inducer, while a few mg of soluble protein per liter could be purified from bacterial lysates (Fabbrini et al. 1997a). Tight control of RIP expression may be crucial to obtain the recombinant protein in good yields. In fact, when saporin was expressed in the BL21(λDE3) strain (i.e., in the absence of T7 lysozyme), *E. coli* growth was clearly reduced even before induction of RIP expression. This toxicity was due to saporin catalytic activity, since it was not observed when a catalytic site mutant was expressed in the same host (Pittaluga et al. 2005).

Recently, a variant recombinant saporin carrying a C-terminal VSAV tetrapeptide (SapVSAV), recognized by a specific PDZ domain, has been expressed in the BL21(λDE3) host. The yield of soluble saporin improved when the corresponding PDZ domain was coexpressed. In this case, SapVSAV was most likely stabilized by the coexpressed PDZ domain (Giansanti et al. 2010).

Endotoxin contamination can be a disadvantage of bacterial expression systems (Fuchs et al. 2007). In addition, the expression of saporin fusions can be hampered by the inefficient folding of certain secretory domains in the bacterial cytosol. Therefore the development of robust eukaryotic expression systems for RIP-based immunotoxin expression would be highly desirable.

The ATF-saporin fusion provides an example of a chimeric toxin that is difficult to express at high levels and in soluble form in bacterial hosts. The urokinase system is involved in the metastatic spread of several tumors, and saporin fusions to the amino-terminal fragment of human urokinase (ATF) have several potential therapeutical applications. While only minute amounts of correctly folded fusion protein were recovered in soluble form when ATF-saporin was expressed in *E. coli* (Fabbrini et al. 1997b), the fusion protein was instead efficiently secreted in active form when targeted to the ER of *Xenopus laevis* oocytes (Fabbrini et al. 2000). However, preATF-saporin expression was found to be toxic to the oocyte, and injection of antisaporin neutralizing antibodies into the oocyte cytosol was required to eliminate host toxicity and increase the expression level (Fabbrini et al. 2000). The identification of a neutralizing antisaporin monoclonal antibody (or antibody fragment) to be used in intracellular immunization strategies may therefore be useful to counteract any toxic effect associated with saporin expression in eukaryotic cells (Fabbrini et al. 2003).

Recently, the methylotrophic yeast *Pichia pastoris* has been shown to be a suitable platform for the expression of saporin fusions (Lombardi et al. 2010). Saporin was expressed as a secretory precursor carrying the preproalpha mating factor leader sequence. Importantly, codon-optimization was found to be essential to obtain clones expressing high levels of active saporin. Although some toxicity toward the host was evident, saporin was efficiently processed by the removal of the preprosequence and accumulated in the culture medium at ∼30 mg/L, thus representing a several-fold improvement with respect to what had previously been obtained with the bacterial expression system (Fabbrini et al. 1997a). Secretion efficiency was found to be lower when saporin fusion chimeras were expressed, a fraction of the synthesized chimeric toxin being retained in the cell and degraded in the vacuole. Despite this, a model saporin immunotoxin and an ATF-saporin chimera could be expressed at several mg per liter of culture in shake flasks,

indicating that *P. pastoris* can be exploited as an expression platform for the production of therapeutic saporin chimeras (Lombardi et al. 2010).

6 Conclusions and Perspectives

Amongst the entries found while searching for RIPs in the PubMed database, a significant proportion also include the keyword "saporin." This reflects both the widespread use of saporin as a tool for the production of targeted toxins, and the increasing interest in the biochemical and functional characterization of this plant toxin. While these studies have clarified several aspects of saporin structure, catalytic activity, and intoxication processes, the role of the different saporin isoforms *in planta* is still poorly understood. Further work on the regulation of saporin biosynthesis and on saporin subcellular localization under normal and stress conditions may provide some hints on the elusive biological role of this and other plant RIPs. In addition, the identification of the subcellular compartment from which saporin escapes to the cytosol, and characterization of the translocation pathway, will contribute to our understanding of the mechanism by which certain proteins avoid the strict subcellular compartmentalization that characterizes eukaryotic cells. Finally, elucidating the molecular events responsible for saporin-mediated host toxicity will be of great help in the production of toxic chimeras for therapeutical applications.

Acknowledgments Our work is partially supported by the charity Leukaemia Busters (www.leukaemiabusters.org.uk).

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Type 1 Ribosome-Inactivating Proteins from the Ombú Tree (*Phytolacca dioica* L.)

Augusto Parente, Rita Berisio, Angela Chambery, and Antimo Di Maro

Abstract The toxicity of plant proteins, later identified as ribosome-inactivating proteins (RIPs), was described more than a century ago and their enzymatic activity was established more than 30 years ago. However, their physiological role and related biological activities are still uncertain. Therefore, despite the body of literature, research on RIPs is ongoing. This review deals with new RIPs being purified, sequenced, characterized, and cloned, and an increasing number of 3D-structures that are determined at high resolution. This is the case of the five type 1 RIPs (PD-S1-3, PD-L1/2, PD-L3/4, dioicin 1, and dioicin 2) from seeds and leaves of the ombú tree (*Phytolacca dioica* L.), native of the grassy pampas of Argentina. The data collected so far will contribute to our understanding of important issues of RIP research: (1) identifying structural determinants responsible for new enzymatic activities such as the DNA cleaving activity; (2) glycosylation and its influence on the catalytic and biological activities; (3) cellular localization of endogenous RIPs and their physiological role(s).

1 Introduction

Ribosome-inactivating proteins (RIPs; rRNA *N*-β-glycosidases; EC 3.2.2.22) have been isolated from a number of higher plants; fungi, bacteria, and at least one alga (Girbés et al. 2004). The genus *Phytolacca* (Fam. Phytolaccaceae) has several tens

A. Parente (✉), A. Chambery, and A.D. Maro
Department of Life Sciences, Second University of Naples, Italy
e-mail: augusto.parente@unina2.it
R. Berisio
Department of General Chemistry, University of Naples Federico II, Italy

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_5,

of species of herbs, shrubs, and trees.[1] American pokeweed (*Phytolacca americana* L.)[2] and Indian pokeweed (*Phytolacca esculenta* Van Houtte)[3] contain antiviral proteins whose action was described long before their recognition as inhibitors of protein synthesis (Duggar and Armstrong 1925; Kassanis and Kleczkowski 1948). Moreover, the first type 1 RIP, an antiviral protein, was identified from American pokeweed (PAP; Dallal and Irvin 1978).

This chapter will focus on the isolation and characterization of type 1 RIPs from the ombú tree[4] (*Phytolacca dioica*[5] L.; Fig. 1). The plant is very useful for this type of research, because it produces new leaves for many months, except at the end of the winter, and can be propagated by seeds, thus allowing the monitoring of RIP expression under several experimental conditions.

2 RIPs from *P. dioica* L.

The genus *Phytolacca* is a rich source of several highly conserved RIPs. The ability of PAP, isolated from *P. americana* leaves, to inhibit protein synthesis by enzymatically damaging ribosomes was initially reported by Obrig et al. (1973). Indeed, several members of this genus have been found to contain type 1 RIPs, such as PAP isoforms from *P. americana* seeds, leaves, and root cultures (Irvin 1975; Irvin et al. 1980; Rajamohan et al. 1999; Park et al. 2002), dodecandrins from leaves and cell cultures of *Phytolacca dodecandra* L'Herit[6] (Ready et al. 1984;

[1]The genus *Phytolacca* is suspected to contain a toxic saponin, which causes enteritis with vomiting, abdominal pain and diarrhea. The illness may be fatal (cfr. Saunders Comprehensive Veterinary Dictionary, 3rd edn. © 2007 Elsevier, Inc.). No information is reported of a likely involvement in the symptoms of ribosome-inactivating proteins. Poisoning of cattle and chickens from *Phytolacca dioica* L. (packalacca) or *Phytolacca dodecandra* l'Herit were reported (Storie et al. 1992; Mugera 1970).

[2]Synonym: *P. decandra* L.

[3]Synonyms: *Phytolacca acinosa* Maxim. and *Phytolacca kaempferi* (A. Gray).

[4]The ombú tree was introduced in Italy from South America. The plant (also called umbú tree) grows to a height and spread of 60 ft (20 m) or more, often with multiple trunks developing from an enormous base resembling a giant pedestal. The huge base may be 3–6 ft tall (1–2 m) and 95 ft (30 m) in circumference. Ombú tree is native of the grassy pampas of Argentina, usually widely spaced and the only trees for miles. It is dioecious, and the female tree produces large quantities of white, fleshy fruits. It is a salt-resistant species, often planted near the sea.

[5]Synonyms and common names. Synonyms (from www.hear.org/pier/species/phytolacca_dioica.htm): *Phytolacca arborea* Moq., *Phytolacca populifolia* Salisb., *Pircunia dioica* Moq., *Sarcoca dioica* Rafin. Common names (English language): belhambra, packalacca (also trade name) and phytolacca; (Spanish language) bella sombra tree, belombra, ombú and umbú (the last two also trade names).

[6]Synonym: *Phytolacca abissynica* Hoffm.

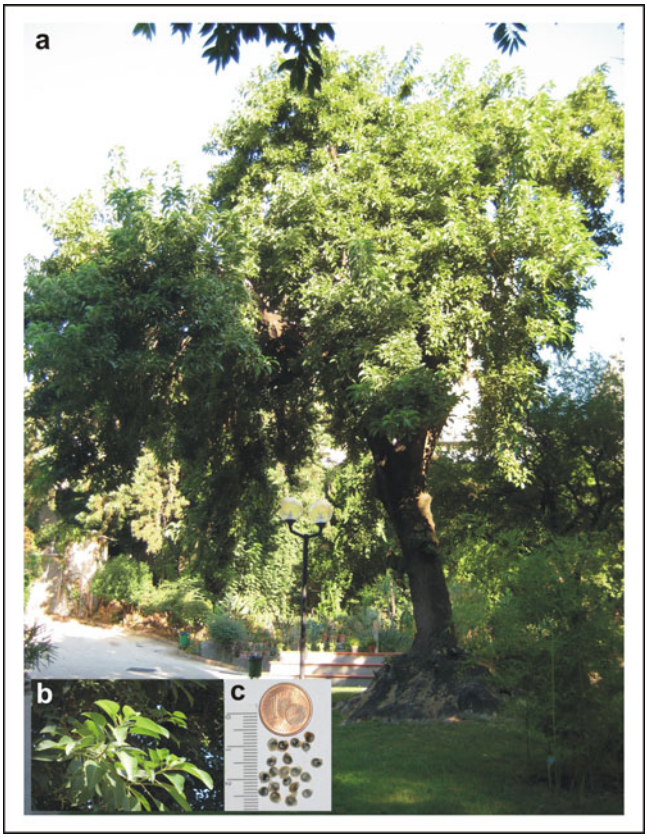

Fig. 1 (**a**) *P. dioica* tree growing in the Botanical Garden of the University of Naples Federico II, Italy. This 100-year-old plant provided leaves and seeds (**b** and **c**, respectively) for the purification of RIPs described in the chapter. *P. dioica* was introduced in Europe in 1768 and in Italy in 1840. It was propagated in the Botanical Garden of the University of Naples by seeds supplied by the French Botanist Aimé Bonplant

Thomsen et al. 1991), heterotepalins from *Phytolacca heterotepala* H. Walter (Mexican pokeweed; Di Maro et al. 2007), insularin from *Phytolacca insularis* Nakai (Song et al. 2000), and PAP-icos isoforms from *Phytolacca icosandra*[7] L. (red ink plant).

The first paper on the presence of RIPs in *P. dioica* was published by Parente et al. (1993), reporting the purification to homogeneity of three type 1 RIPs from seeds. The major form, named PD-S2 (*P. dioica*-seeds 2), accounted for about 90% of the total protein synthesis inhibitory activity of the crude seed extract (Parente et al. 1993). After this first report, four type 1 RIPs were isolated and characterized

[7]Synonym: *Phytolacca octandra* L.

both structurally and functionally from the leaves of the same plant (Di Maro et al. 1999; Parente et al. 2008).

2.1 Isolation of RIPs from Seeds and Leaves of P. dioica

Type 1 RIPs from seeds (PD-Ss; Parente et al. 1993), fully expanded leaves of adult *P. dioica* (PD-L1, PD-L2, PD-L3, and PD-L4; Di Maro et al. 1999; Parente et al. 2008), fully expanded leaves of young *P. dioica* (8–36 months old) and developing leaves of adult plants from 10 to 60-days old (dioicin 1 and dioicin 2; Parente et al. 2008) have been isolated and characterized. Preliminary data on RIP purification from the bark of summer shoots have shown the presence of the four RIPs isolated from fully expanded leaves of adult *P. dioica* PD-Ls1–4 (unpublished).

Experimental conditions employed for RIP isolation from *P. dioica* are reported in Table 1. The multi-step purification protocols provided samples with high purity, which facilitated structural studies (mass spectrometry analysis, sequence and p*I* determination, X-ray studies) and lent confidence to the enzymatic characteristics, especially the adenine polynucleotide glycosylase (APG) and DNA-cleaving activities. Indeed, some purification steps were essential for full separation of closely related RIPs (isoforms): in the case of the PD-S forms, with the same primary structure but different glycosylation pattern (see later), using the CM Sepharose resin for the second cation-exchange chromatography was essential not only for the complete separation of the three native forms, but also for nicked PD-S2 forms, one with a cut between Asn195 and Arg196 (Di Maro et al. 1995) and the other between Asp82 and Pro83 (Zacchia et al. 2009). The in vivo occurrence and the likely biological significance of these nicked seed forms are currently being investigated. Similarly, the third cation-exchange chromatography on S-Sepharose fast flow, after the CM-52 step, allowed the complete separation of PD-L2 from PD-L3. The yields of the CM-52 purified PD-L1, PD-L2, PD-L3, PD-L4, dioicin 1, and dioicin 2 were 1.54, 0.72, 2.48, 4.0, 0.08, and 0.61 mg/100 g leaves, respectively. In the case of PD-$S_{1–3}$, the yields were 7.0, 86.0, and 3.4 mg/100 g seeds, respectively.

2.2 Basic Characteristics of RIPs from Seeds and Leaves of P. dioica

The main structural characteristics (Table 2) of the purified RIPs are well within the canonical parameters of type 1 RIPs: basic p*I* (range 7.5–9.5) and a ratio Lys + Arg/Asp + Glu higher than 1, M*r* of the unglycosylated forms about 30 k, 261–266 amino acid residues, with an expected prevalence of basic amino acid residues, four half cysteines engaged in two S–S bridges (Di Maro et al. 2009; Parente et al. 2008), an average theoretical molar extinction coefficient at 280 nm 27,762.5 (corresponding to $E_{0.1\%} = 1\text{mg/mL} = 0.941$) and a GRAVY index in the range 0.343–0.559 (Table 2).

Table 1 Purification of RIPs from *Phytolacca dioica* seeds and leaves

Purification step	PD-Ss1–3 (from seeds)	PD-Ls1–4 (from fully expanded leaves)	Dioicin 1 and dioicin 2 (from both fully expanded leaves of young *P. dioica* and developing leaves of adult *P. dioica*)
Homogenate	Buffer: 5 mM Na/P, 0.14 M NaCl pH 7.2 (ratio 1:10 w:v)	Buffer: 5 mM Na/P, 0.14 M NaCl pH 7.2 (ratio 1:5 w:v)	Buffer: 5 mM Na/P, 0.14 M NaCl pH 7.2 (ratio 1:5 w:v)
Precipitation	Glacial acetic acid pH 4.0	$(NH_4)_2SO_4$ – Step 1: 40% – Step 2: at saturation	Glacial acetic acid pH 4.0
	Direct loading	Dialysis vs 10 mM NaAc, pH 4.5	Direct loading
I-Cation exchange chromatography	Streamline™ SP – equilibration: 10 mM NaAc, pH 4.5 – elution: 5 mM Na/P, 1 M NaCl, pH 7.2	Streamline™ SP – equilibration: 10 mM NaAc, pH 4.5 – elution: 5 mM Na/P, 1 M NaCl, pH 7.2	Streamline™ SP – equilibration: 10 mM NaAc, pH 4.5 – elution: 5 mM Na/P, 1 M NaCl, pH 7.2
Gel filtration	Sephacryl S-100 HR (LPLC)	Hiload 16/60 Superdex 75 (FPLC)	Hiload 16/60 Superdex 75 (FPLC)
II-Cation exchange chromatography	CM-Sepharose[a]	CM-52	CM-52
III-Cation exchange chromatography	–	S-Sepharose fast flow	–
RP-HPLC[b]	C4 column (25 × 0.5 cm) 5 μm particle size	C4 column (25 × 0.5 cm) 5 μm particle size	C4 column (25 × 0.5 cm) 5 μm particles size
Affinity chromatography on Red Sepharose[c]	–	+	+

[a]This step is required for the complete separation of the three native forms and of nicked PD-S2 forms (Di Maro et al. 1995)
[b]Used for the preparation of RIPs to be analyzed by mass spectrometry and Edman degradation
[c]Final step of the preparation of RIPs (Barbieri et al. 2000) to be assayed for the DNA cleaving activity on pBR322 (Aceto et al. 2005)

The amino acid sequences[8] of PD-S1-3; PD-L1, PD-L2, PD-L3, PD-L4, and dioicin 2 were determined using a combined approach based on Edman degradation and mass spectrometry (Fig. 2a; Del Vecchio Blanco et al. 1997; Chambery et al. 2008;

[8]The protein sequence data of *P. dioica* RIPs have been deposited in the UniProtKB with accession numbers P34967 for PD-S2, P84853 for PD-L1/2, P84854 for PD-L3/4 and P85208 for dioicin 2.

Table 2 Physicochemical properties of type 1 RIPs purified from *P. dioica* seeds and leaves. A standard plant paucimannosidic *N*-glycosylation structure (Man)3 (GlcNAc)2 (Fuc)1 (Xyl)1 or GlcNAc are indicated with their molecular masses (1,170 and 203, respectively)

RIP	Sugar moiety (M*r*)	MW (Da) (native protein)	Amino acid residues	S–S pairing	p*I*		Lys residues	Lys + Arg	Extinction coefficient		GRAVY
					Exp.	Theor.		Asp + Glu	(M)	0.1%	
PD-S1[a]	1,170 at Asn120 203 at Asn112	30,957.1[b]	265	Cys34–Cys262 Cys88–Cys110	>9.5	9.18	23	1.31	28,880	0.976	−0.343
PD-S2	1,170 at Asn120	30,753.8[b]	265	Cys34–Cys262 Cys88–Cys110	>9.5	9.18	23	1.31	28,880	0.976	−0.343
PD-S3[a]	203 at Asn112	29,785.1[b]	265	Cys34–Cys262 Cys88–Cys110	>9.5	9.18	23	1.31	28,880	0.976	−0.343
PD-L1[c]	1,170 at Asn10, 43,255	32,715[d]	261	Cys34–Cys262 Cys34–Cys258, Cys84–Cys105	nd	8.26	19	1.07	27,390	0.937	−0.444
PD-L2[c]	1,170 at Asn10, 43	31,542[d]	261	Cys34–Cys258, Cys84–Cys105	nd	8.26	19	1.07	27,390	0.937	−0.444
PD-L3[e]	1,170 at Asn10	30,356[f]	261	Cys34–Cys258, Cys84–Cys105	nd	8.54	20	1.16	27,390	0.937	−0.373
PD-L4[e]	Not glycosylated	29,185[f]	261	Cys34–Cys258, Cys84–Cys105	nd	8.54	20	1.16	27,390	0.937	−0.373
Dioicin 1[g]	Not glycosylated	30,047	nd	nd	8.74	nd	nd	1.03	nd	nd	nd
Dioicin 2	Not glycosylated	29,910[h]	266	Cys32–Cys258, Cys85–Cys102	9.37	7.73	30	1.03	27,390	0.916	−0.559

[a]RIPs from seeds, with the same primary structure, but different glycosylation
[b]From the sequence 29,586.8, considering four cysteinyl residues
[c]RIPs from leaves, with the same primary structure, but different glycosylation
[d]From the sequence 29,222.07, considering four cysteinyl residues
[e]RIPs from leaves, with the same primary structure, but different glycosylation
[f]From the sequence 29,190.16, considering four cysteinyl residues
[g]Sequence determination is ongoing
[h]From the sequence 29,914.12, considering four cysteinyl residues
nd not determined

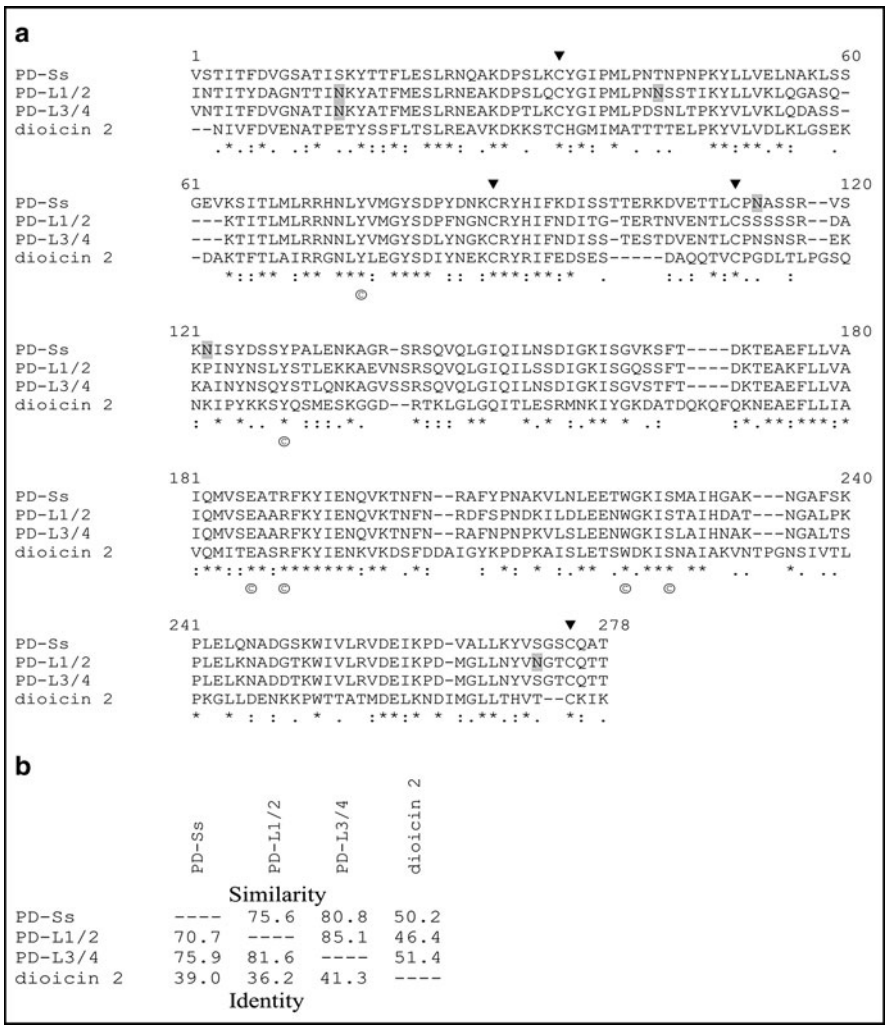

Fig. 2 (**a**) Multiple alignment of RIP sequences from *P. dioica* (PD-Ss, PD-L1/2, PD-L3/4, and dioicin 2 (accession numbers P34967, P84853, P84854, and P85208, respectively). *Asterisk* – identical, *double dots* – conserved and *single dot* – semiconserved amino acid residues. *N*-glycosylation sites have been shaded. *Copyright* indicates conserved amino acid residues found in the active site of Phytolaccaceae RIPs. (**b**) identity–similarity matrix of PD-Ss, PD-L1/2, PD-L3/4, and dioicin 2. Sequences were first aligned by the algorithm Clustal W2.0.11 and then analyzed by BOXSHADE

Parente et al. 2008; Di Maro et al. 2009). The sequence determination of dioicin 1 is ongoing. The comparative analysis of the amino acid sequences (with a consensus sequence of 278 amino acid residues) shows that PD-L1 and PD-L2 have identical primary structures, as is the case for PD-L3 and PD-L4. Hereafter, they will be

reported as PD-L1/2 and PD-L3/4. Even PD-S RIPs were found to have the same amino acid sequence each other (Chambery et al. 2008). In the case of PD-L1/2, a microheterogeneity was found by mass spectrometry at position 20, with the alternative presence of Met or Leu. The identity/similarity matrix (Fig. 2b) shows that the highest identity (81.6%; 85.1% similarity) is between PD-L1/2 and PD-L3/4, while the lowest is between dioicin 2 and PD-L1/2 (36.2%; 46.4% similarity). The identity values of dioicin 2 with PS-Ss and PD-L3/4 is 39 and 41.3%, respectively.

Figure 3a reports an identity/similarity matrix of RIP sequences from *P. dioica*, *P. americana*, *P. acinosa*, *P. heterotepala*, *P. insularis,* and *P. icosandra*.

a

Similarity

	PD-Ss	PD-L1/2	PD-L3/4	dioicin 2	PAP-S	PAP-alfa	PAP-R	PAP	PAP-I	PAP-II	PapS1-aci	Pap-aci	heterotepalin 4	PIP 2	PAP-icos	PAPII-icos
PD-Ss	----	75.7	80.5	50.0	77.5	83.8	82.4	78.7	82.0	50.0	79.0	82.4	81.6	82.0	82.0	50.4
PD-L1/2	70.4	----	85.1	45.7	89.7	78.2	70.3	85.1	79.4	45.3	90.0	79.4	80.2	77.1	79.0	45.3
PD-L3/4	75.3	81.6	----	50.4	84.7	82.8	74.8	83.2	80.9	50.0	85.4	81.3	82.1	79.0	81.3	49.3
dioicin 2	39.2	35.1	40.2	----	46.4	48.9	49.8	49.1	49.1	84.6	46.0	49.1	49.1	51.1	49.8	87.6
PAP-S	73.4	87.4	81.2	37.7	----	79.4	71.8	84.4	79.8	45.3	96.6	79.4	80.2	76.3	79.0	45.3
PAP-alfa	78.6	73.7	79.0	39.5	77.1	----	75.3	80.6	79.8	49.6	79.8	80.2	79.8	89.7	79.1	49.6
PAP-R	77.2	65.4	69.5	38.3	68.0	68.9	----	75.6	78.6	48.7	73.3	79.7	79.3	72.3	80.1	49.8
PAP	73.8	82.8	79.4	39.7	82.4	76.8	70.7	----	80.2	47.3	85.5	79.8	80.5	77.2	79.8	47.7
PAP-I	78.7	75.2	76.7	37.5	76.0	74.5	73.3	76.3	----	47.7	80.9	96.9	96.2	77.9	95.0	46.9
PAP-II	38.5	35.1	40.2	81.6	36.6	39.9	38.3	38.6	36.5	----	44.9	47.7	47.7	50.7	48.0	91.7
PapS1-aci	74.2	86.6	81.2	37.7	95.4	76.7	69.2	82.4	76.7	36.6	----	80.5	81.3	77.9	80.2	44.9
Pap-aci	79.0	74.8	76.3	37.5	76.0	75.7	74.1	76.0	95.8	37.2	76.7	----	95.8	77.9	95.8	46.9
heterotepalin 4	77.9	75.2	77.1	38.3	76.3	74.5	72.9	76.7	94.3	36.5	76.7	94.7	----	79.1	96.2	46.9
PIP 2	77.4	73.3	76.7	40.6	74.8	87.4	65.9	73.4	73.8	39.9	75.6	73.8	74.9	----	77.9	50.7
PAP-icos	79.0	74.8	77.1	37.9	76.0	74.5	74.4	76.3	94.3	36.8	76.7	95.4	95.4	74.1	----	47.3
PAPII-icos	39.2	35.5	40.6	86.1	37.3	39.5	39.7	39.0	36.8	90.2	37.3	36.8	37.5	40.2	37.2	----

Identity

b

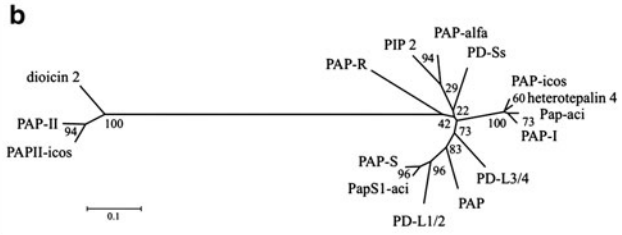

Fig. 3 (**a**) Identity/similarity matrix of type 1 RIP sequences from Phytolaccaceae. See Fig. 2 for *P. dioica* RIP sequences (PD-Ss, PD-L1/2, PD-L3/4, and dioicin 2). Sequences of RIPs other than from *P. dioica* (PAP-α, PAP, PAP-I, PAPII, PAP-R, PAP-S from *P. americana*; heterotepalin 4 from *P. heterotepala*; PIP 2 from *P. insularis*; Pap-aci and PapS1-aci from *P. acinosa*: PAP-icos and PAPII-icos from *P. icosandra*) were obtained from PubMed (*Phytolaccaceae → taxonomy search*). Sequences were first aligned by the algorithm Clustal W2.0.11 and then analyzed by BOXSHADE. (**b**) Unrooted phylogenetic tree of RIPs reported in (**a**). The tree shows the close relationship of dioicin 2 with PAP-II and PAPII-icos and their distance from other RIPs from *P. americana* and *P. dioica*, from both seeds and leaves. PD-S2 from seeds of *P. dioica* is more closely related to PAP-I or PAP-R from leaves or roots of *P. americana*, respectively, than to RIPs from seeds of *P. americana* (PAP, PAP-S, PAP-α) or PD-L4 from leaves of *P. dioica*. The neighbor-joining method was used with Poisson corrected distances. *Scale bar*, substitutions/site

Dioicin 2 has the lowest identity percentage (35.1%) with PD-L1/2 isolated from the same tissue of the same plant. The highest percentages are with PAPII-icos (86.1%) and PAP-II (81.6%). The identity values with the other RIPs are less than 41%.

The unrooted phylogenetic tree of the *Phytolacca* RIPs (Fig. 3b) clearly shows that dioicin 2, PAP II and PAPII-icos are located on a separate branch and may give rise to other RIPs. All other RIPs are grouped in four branches: (1) PAP-R; (2) PIP 2, PAP alpha, and PD-Ss; (3) PAP-icos, heterotepalin, Pap-aci, and PAP-I; (4) PD-L3/4, PAP, PD-L1/2, PapS1-aci, and PAP-S.

PD-L1/2 and PD-L3/4 from *P. dioica* leaves appear more closely related to RIPs from *P. americana* seeds than to PD-Ss from seeds of the same plant.

2.3 Differential Seasonal and Age Expression in Leaves

The expression of RIPs is developmentally regulated (Iglesias et al. 2008; Parente et al. 2008) and under transcriptional control (Kawade and Masuda 2009).

A differential seasonal expression was first found for PAP and PAP-II from *P. americana*, isolated from spring and summer leaves, respectively (Houston et al. 1983). This notion was later revisited by Rajamohan et al. (1999), reporting the expression of PAP-I (corresponding to PAP), PAP-II and PAP-III, in spring, early summer, and late summer leaves, respectively.

P. dioica has been very useful for studying RIP expression, because it produces new leaves for many months, except at the end of the winter, thus making it possible to monitor RIP activity during leaf ontogeny during most of the year. We have found that PD-L1, PD-L2, PD-L3, and PD-L4 RIPs from fully expanded leaves of adult *P. dioica* show differential seasonal and age expression. PD-L3 and PD-L4 are abundant in spring and summer leaves, decrease in autumn, and almost disappear in winter, when PD-L1 represents 80% of the RIP isoforms synthesized. On the contrary, the expression of PD-L2 remains constant throughout the year. PD-Ls 1–4 are not present in fully expanded leaves of young *P. dioica* (8–36 months old), where they appear to be replaced by two novel RIPs, dioicin 1 and dioicin 2. Furthermore, in developing leaves of adult plants (from 10- to 60- days old), PD-Ls 1–4 and dioicin 2 are always present, while dioicin 1 can be detected only at day 10 and 17. Dioicin 2 is also present in fully developed leaves of the adult plant; therefore, its expression is neither age- nor seasonally regulated.

The fact that *P. dioica* synthesizes and accumulates RIPs indirectly indicates that plant fitness benefits from these processes. In this view, the seasonal changes of RIP pattern could be due to the different and potentially adverse environmental conditions suffered, at least by the adult plant, in each season. Therefore, PD-L3 and PD-L4, mainly expressed in summer, could improve plant tolerance to drought and/or heat, whereas PD-L1, abundant in winter, may contribute to the avoidance of different abiotic stresses. Moreover, PD-Ls could be involved in the already known salt tolerance of *P. dioica* (Wheat 1977), thus conferring an adaptive, other than

functional, role. As for the prevalence of diocin 1 and dioicin 2 in young plants, this may be due to the need to protect young and perhaps more susceptible tissues to pathogen attacks, e.g., by inactivating host ribosomes after virus challenge. The presence of diocin 1 only in developing leaves of adult plants could lend support to this hypothesis. Interestingly, treatment of tobacco and bean leaves with diocin 2 greatly reduced the infection of tobacco necrosis virus (TNV), an uncapped virus without the 5′ terminal m7GpppG cap, by inducing a slight H_2O_2 burst, but without activating any cell death phenomena (Faoro et al. 2008).

All the above suggested roles for *P. dioica* RIPs are also supported by the evidence of the newly reported enzymatic properties of RIPs other than *N*-glycosidase activity, such as RNAse, DNAse, superoxide dismutase (SOD), and phospholipase activities (reviewed in Park et al. 2004a, b). It must also be taken into account that senescence may induce RIP synthesis as well (Sawasaki et al. 2008). In this context, cytological changes activated by aging, as well as by pathogen infection, could alter the RIP compartmentalization, thus exposing the host ribosomes to the their action.

2.4 Cellular Localization

Diocin 1 and dioicin 2 from fully expanded leaves of young *P. dioica* were localized in the extracellular space, in the vacuole and in the Golgi apparatus of mesophyll cells (Parente et al. 2008). The presence of RIPs in the extracellular fluid was ascertained by western blot. For immunocytochemical localization studies, antidioicin 2 IgGs gave a positive signal mainly localized in the extracellular space of mesophyll cells, where they often aggregated forming amorphous and scarcely electron opaque deposits, intensely labeled by the gold probe. Some labeling was also found in the Golgi complex, indicating that the protein traffics via this route before being sorted into the cell vacuole or secreted.

Although double localization in the vacuole and apoplast is not unusual for type 1 RIPs (Carzaniga et al. 1994; Yoshinari et al. 1996, 1997), it is interesting to note that for the highly identical PAP-II (81.6%), an exclusive extracellular localization has been reported (Ready et al. 1986).

2.5 Glycosylation of P. dioica *RIPs*

The structure of glycan moieties present in PD-Ss and PD-Ls was determined by a fast and sensitive mass spectrometry-based approach, applying a precursor ion discovery mode on a Q-TOF mass spectrometer (Di Maro et al. 2009; Chambery et al. 2008). The MS analysis confirmed that PD-Ls 1–3 were glycosylated at

different sites. In particular, PD-L1 contained three glycidic chains, with the well-known paucimannosidic structure (Man)3 (GlcNAc)2 (Fuc)1 (Xyl)1, linked to Asn10, Asn43, and Asn255. PD-L2 was glycosylated at Asn10 and Asn43, and PD-L3 was glycosylated only at Asn10. PD-L4, dioicin 1, and dioicin 2 were not glycosylated.

The standard plant paucimannosidic *N*-glycosylation pattern was found for PD-S1 and PD-S2 on Asn120, while in PD-S1 and PD-S3 Asn112 was shown to link an HexNAc residue, probably *N*-acetyl-D-glucosamine (GlcNAc) (Chambery et al. 2008). The glycosylation patterns of PD-Ss, PD-L1/2, and PD-L3/4 RIPs help explaining their different chromatographic behavior (Table 1).

Basic understanding of protein glycosylation is still an area of intense investigation. Several roles have been ascribed to *N*-linked glycans, such as prevention of proteolytic degradation, induction of the correct folding and influence on protein conformation, stability and biological activity, involvement in protein recognition, and cell–cell adhesion processes (Ceriotti et al. 1998; Elbein 1991; Lis and Sharon 1993; Sharon and Lis 1993; Varki 1993). Regarding the protein folding and stability, a direct contribution of *N*-glycans has also been related to the increase of protein solubility, the reduced tendency to aggregate, and to the presence of additional hydrogen bonds and hydrophobic interactions between the oligosaccharide and the protein (O'Connor and Imperiali 1996; Wyss and Wagner 1996). In this context, the four PD-Ls forms constitute an excellent experimental model suitable to further investigate the role of glycosylation in the modulation of the biological activity on different substrates.

The primary structure of PD-L1 and PD-L2 are identical, as well as those of PD-L3 and PD-L4 (see Sect. 10.2.2). Therefore, biological differences between each protein couple could be ascribed to the presence of the glycan moieties. The comparative modeling of PD-L1, PD-L2, PD-L3, and PD-L4 showed an overall high structural similarity, but also potential influences of the glycan chains on their APG activity on different substrates (Di Maro et al. 2009), possibly related to the bending of the glycan chain linked to Asn255. The observed catalytic activity decrease is much more evident with the poly(A) (41%), but it is repeatedly observed also with the rRNA (24%) and hsDNA (4%), suggesting that it is associated with an acquired impairment of adenine interaction with the enzyme when Asn255 is glycosylated. Indeed, the relatively lower activity on DNA and rRNA could be explained in terms of obvious lower frequency-abundance of adenines in these substrates. The same trend is also observed when the activities of PD-L4 are compared with those of PD-L3.

Of particular interest was the DNA cleaving activity shown by PD-L1 (and PD-L2), both native (with sugars) and recombinant (without sugars and likely without contaminating DNases), dioicin 1 and dioicin 2 on ds pBR322 DNA (see Sect. 10.3.5 later), while PD-L3/4 does not possess this activity. First, it was ascribed to differences in glycosylation; it has been later attributed solely to the differences of the protein sequences (see Sects. 3.5 and 4.3 below for PD-L1; Ruggiero et al. 2009).

3 Enzymatic and Biological Characteristics

3.1 N-β-Glycosidase and APG Activities

RIPs from *P. dioica* are *N*-β-glycosidases as shown by the appearance of the "aniline fragment" in the RNA from ribosomes treated with the RIPs (Parente et al. 1993, 2008). When assayed for the inhibition of protein synthesis on a cell-free system, they gave IC_{50} values in the pmolar range (Table 3), comparable to those of other type 1 RIPs. PD-S2 inhibited protein synthesis by cells at a much higher concentration (120 pM in the reticulocyte lysate against >3,310, 2,950, 6 and 90 nM for 3T3 fibroblasts, HeLa, NB 100, and BEWO cells, respectively; Parente et al. 1993). The maximum release of adenine from purified rat liver ribosomes in the case of PD-S2 was ~0.5 mol/mol of ribosomes (as in the case of PAP-S). PD-S2 also inhibited phenylalanine polymerization by purified rat liver ribosomes. The inhibition was not complete, with a residual ~40% of polymerization even at the highest concentrations of RIPs tested (Parente et al. 1993). This resistance was observed previously with abrin (Battelli et al. 1984) and with an RIP from *Petrocoptis glaucifolia* (Arias et al. 1992). These results suggest that part of the ribosomes escape inactivation, and this was confirmed by treating ribosomes with PD-S2 RIP, and incubating them again, after washing, with or without the same RIP. In this second incubation, pretreated ribosomes polymerized phenylalanine to the same extent (~30% of controls) independently of the addition of

Table 3 Enzymatic characteristics and cellular localization of Type 1 RIPs purified from *P. dioica* seeds and leaves

RIP	IC_{50}[a] (pM)	APG[b] activity	DNA cleaving activity	Cellular localization
PD-S1[c]	120	nd	nd	nd
PD-S2[c]	60	nd	nd	nd
PD-S3[c]	80	nd	nd	nd
PD-L1[d]	102	+	+	nd
PD-L2[d]	110	+	+	nd
PD-L3[e]	228	+	Absent	nd
PD-L4[e]	134	+	Absent	nd
Dioicin 1	658	+	+	Extracellular space Vacuole Golgi apparatus
Dioicin 2	229	+	+	Extracellular space Vacuole Golgi apparatus

[a]Protein synthesis inhibition
[b]Adenine polynucleotide glycosylase (APG) activity on substrates such as RNA, poly(A) and DNA (Stirpe and Battelli 2006)
[c]RIPs from seeds, with the same primary structure, but different glycosylation
[d]RIPs from leaves, with the same primary structure, but different glycosylation
[e]RIPs from leaves, with the same primary structure, but different glycosylation
nd not determined

PD-S2 RIP. Addition of supernatant from a rabbit reticulocyte lysate (22 μg of protein/sample) did not modify the inhibition. A similar incomplete inhibition was observed with ricin added to ribosomes at 1:1 molar ratio.

RIPs from *P. dioica* show APG activity, determined by measuring the adenine amount released from herring sperm DNA at 260 nm. This APG activity appears to be more variable among RIPs and related to amino acid residue(s) present in the active site other than the ones already known to be part of it (i.e. Tyr76, Tyr129, Glu186, Arg189, Trp220, numbering of the consensus sequence of *P. dioica* RIPs, Fig. 2a). Indeed, a conserved seryl residue identified by multiple sequence alignment analysis and located in the proximity of the catalytic tryptophan, appears to play a role in this activity. Its involvement in the enzymatic mechanism of RIPs was investigated in PD-L4 by site-directed mutagenesis (Chambery et al. 2007). The replacement of Ser211 (numbering of the PD-L4 sequence or Ser224, numbering of the aforementioned consensus sequence) with Ala apparently does not influence the inhibition of the protein synthesis (determined as IC_{50} in a cell-free system), but it reduces the APG activity, assayed spectrophotometrically on other substrates such as DNA, rRNA, and poly(A). The ability of PD-L4 to deadenylate polynucleotides appears more sensitive to the Ser211Ala replacement when poly(A) is used as substrate, as only 33% activity is retained by the mutant, while with more complex and heterogeneous substrates such as DNA and rRNA, its APG activity is 73% and 66%, respectively. While the mutated protein shows a conserved secondary structure by CD, it also exhibits a remarkably enhanced tryptophan fluorescence. This indicates that although the overall protein 3D structure is maintained, removal of the hydroxyl group locally affects the environment of a Trp residue. Modeling, docking analyses, and 3D structure (Sect. 10.4.2) confirmed the interaction between Ser211 and Trp207, which is located within the active site, thus likely affecting the PD-L4 APG activity (Chambery et al. 2007).

3.2 Toxicity to Mice

PD-S2 RIP was toxic to mice with an LD_{50} of 1.12 mg/kg of body wt (Parente et al. 1993). The pathology of dead animals was similar to that observed in mice poisoned with other RIPs (Battelli et al. 1990), with necrotic lesions in the liver and kidneys.

3.3 Immunotoxin

The PD-S2 RIP could be derivatized with 2-iminothiolane and subsequently linked to monoclonal antibodies retaining good inhibitory activity on protein synthesis by the reticulocyte lysate system (IC_{50} 18 nM and 26 nM, respectively, after derivatization and after conjugation to the antibody). An immunotoxin prepared with the

anti-CD30 monoclonal antibody and containing 2.5 mol of RIP per mol of antibody inhibited protein synthesis by target L540 cells with an $IC_{50} < 50$ pM. A similar immunotoxin made with a control LS3 antibody was much less toxic to the same cells (IC_{50} 45 nM) (Parente et al. 1993). The toxicity of this immunotoxin to target cells was comparable to that of an immunotoxin prepared with the same antibody and saporin (Tazzari et al. 1992).

Consistent with the results obtained with other RIPs, the effects of PD-S RIPs on different cells were highly variable, BeWo and NB 100 being more sensitive than HeLa cells and fibroblasts (Battelli et al. 1992).

PD-S2 RIP is immunologically distinct from most RIPs and appears to be suitable for the preparation of immunotoxins. Thus PD-S2 RIP could be useful to overcome the immune reaction which would follow the administration of immunotoxins prepared with other RIPs.

3.4 Cross-Reactivity

The PD-S2 RIP gives a significant cross-reaction only with antibodies against dianthin 32 and PAP-R, and a weak or no cross-reactivity with antibodies against other RIPs, including saporin 6, momordin, momorcochin-S, and trichokirin (Parente et al. 1993). This immune-response pattern was somewhat unexpected, because of the many identities in the amino acid sequences of PD-S2 RIP and PAP-S (73.4% identity; Fig. 3a), and since RIPs from plants belonging to the same family often give a strong cross-reaction with the respective antisera (Strocchi et al. 1992).

Cross-reactivity data have also been obtained for dioicin 1 and dioicin 2. These two RIPs were localized, by immunoblot analysis, in the extracellular fluid proteins of fully expanded leaves of young *P. dioica* plants. Antidioicin 1-specific IgGs cross-reacted with dioicin 2, as they showed up both RIPs, while antidioicin 2-specific IgGs did not react with dioicin 1. When used for immunocytochemical localization studies, antidioicin 1 IgGs gave only faint or no staining, while antidioicin 2 IgGs gave a positive signal mainly localized in the extracellular space of mesophyll cells (Parente et al. 2008).

3.5 Activity on Double-Stranded pBR322 DNA

PD-L1/2, dioicin 1, and dioicin 2 purified on Red Sepharose® not only showed *N*-β-glycosidase and APG activity but cleaved supercoiled pBR322 dsDNA, generating relaxed and linear molecules. PD-L3/4, purified in the same way, did not produce the same effect. The DNA cleaving activity of PD-S2 could not be determined because of a very tight interaction with the substrate DNA (supercoiled pBR322 dsDNA), with the resulting complex migrating towards the cathode (Delli Bovi, personal communication). An extensive study has been performed with PD-L1, the most glycosylated *P. dioica* RIP isoform. This RIP produced both free

3′-OH and 5′-P termini randomly distributed along the DNA molecule, as suggested by labeling experiments with [α-^{32}P]dCTP and [γ-^{32}P]dATP. Moreover, when the reaction was carried out under low-salt conditions, cleavage was observed mainly at a specific site, located downstream of the ampicillin resistance gene (close to position 3200), ending with the deletion of a fragment of approximately 70 nucleotides. This cleavage pattern is similar to that obtained under the same conditions with mung bean nuclease, a single-strand endonuclease. Furthermore, pBR322 DNA treated with PD-L1 showed reduced transforming activity with *Escherichia coli* HB101 competent cells in comparison to untreated control plasmid DNA.

Semiquantitative analysis of the effect of PD-L1/2, dioicin 1 and 2 showed that pBR322, pGem-3, PM2, and ΦX174 replication form DNA were cleaved under standard experimental conditions (50 mM Tris–Cl, 12.5 μM EDTA, pH 7.5, 37°C), producing linearized and relaxed forms. However, an extensive study, using several experimental conditions and methodologies, was performed with PD-L1 on pBR322 dsDNA. These include (1) analyzing the effect of temperature, salt, and divalent metal ions; (2) mapping the preferential RIP cleavage site; (3) performing "nick-translation-like" experiments; (4) assessing the endonucleolytic activity, under low-salt conditions, on pBR322 DNA by low amounts of S1, DNase I, or mung bean endonucleases to map the pBR322 linearization cleavage site; (5) performing substrate competition experiments with pBR322-oligonucleotides and transformation assays.

Overall, the results suggest that PD-L1 RIP from *P. dioica* leaves induce the cleavage of phosphodiester bond(s) on pBR322 DNA. This action is similar to that previously reported for either type 1 or type 2 RIPs extracted from different sources (Ling et al. 1994; Roncuzzi and Gasperi-Campani 1996). The experiments have shown that the nicking activity on supercoiled pBR322 DNA results in the production of predominantly circular and linear forms. Furthermore, the nicking activity and the linearization cleavage(s) were dependent on (1) temperature, (2) ionic strength, with inhibition on increasing the NaCl concentration, and (3) divalent cations, such as Mg^{2+}, Mn^{2+}, Zn^{2+}, and even more so Co^{2+}. Their presence under standard conditions potentiated the capacity to produce linear and circular pBR322 forms. However, the activity was completely abolished in the presence of 10 mM EDTA. This result suggests that PD-L1 could be endowed with or contaminated by cation-dependent endonuclease activity because, as reported for the most well-known endonucleases, the addition of a chelating agent blocks the activity, even though PD-L1 does not seem to be strictly dependent on the presence of the divalent cations tested so far. However, the recombinant PD-L1 (rPD-L1; Ruggiero et al. 2009) exhibits the same activity. We can hypothesize that the addition of EDTA abolishes the activity because it might chelate metal ions present in trace amounts that are important for maintenance of the structure. It cannot be excluded that the activity is abolished merely because the ionic strength of 10 mM EDTA is high enough to compact the DNA structure and dramatically reduce any endonucleolytic activity on it.

Under low-salt conditions, PD-L1 exerted its nicking activity predominantly at a major site. In fact, the experiments in which the linearized and circular pBR322

forms, obtained after RIP incubation in the absence of salts, were digested with various restriction enzymes, showed fragments of different sizes according to the preferential cleavage downstream of the ampicillin resistance gene of the plasmid. It has already been reported that this region is sensitive to the single-strand-specific mung bean endonuclease (MBN; Sheflin and Kowalski 1985). Moreover, the linearization cleavage by the PD-L1 nicking activity under low-salt conditions generates ligatable blunt termini and a deletion of an approximately 70-bp DNA fragment at a specific site, as suggested by the sequence analysis of the mutant clones. The same deletion was present in clones obtained from both blunted and unblunted linear pBR322 DNA produced by PD-L1 cleavage. Furthermore, once this region was eliminated, as in the case of mutant clones with the deletion, the nicking activity under low-salt conditions was mainly restricted to a second preferential site, which is another structurally unstable sequence, described as a preferential site for topoisomerase II (Amir-Aslani et al. 1995) and single-strand endonucleases such as S1 nuclease and MBN (Sheflin and Kowalski 1985). These mapped preferential sites are the only two regions rich in A–T sequences that could assume a hairpin conformation, as found after analysis of the complete pBR322 sequence by the PC-GENE software hairpin option. Furthermore, analysis of the entire pBR322 sequence by Web-Thermodyn (the sequence analysis software for profiling DNA helical stability; Huang and Kowalski 2003), revealed that these two regions require the lowest free energy to unwind and separate the strands of the double helix under our low-salt conditions at 37°C (http://wings.buffalo.edu/gsa/dna/dk/WEBTHERMODYN). As described earlier, these two regions are very rich in A–T sequences, and the stable unwinding of these regions may be important for single-strand-specific nuclease hypersensitivity (Kowalski et al. 1988). PD-L1 activity on supercoiled DNA produced free 3′-OH and 5′-P, as shown by labeling experiments, in common with other endonucleases. However, the endonucleolytic effect, as in the case of MBN (Sheflin and Kowalski 1985), is mainly dependent on the ionic environment. In fact, PD-L1 endonucleolytic activity on pBR322 DNA was more pronounced at low-salt concentrations and was almost absent under high-salt conditions. It has often been suggested that the nicking activity of RIPs can be attributed to contaminating endonucleases (Barbieri et al. 2000; Day et al. 1998). We addressed this question by incubating pBR322 DNA with endonucleases such as DNase I, S1 and MBN, using the same experimental conditions as for PD-L1. Our experiments performed with DNase I and S1 showed that in the presence of very low amounts of these enzymes, the cleaving action appeared to be limited and largely resulted in linear and circular plasmid forms, as obtained with PD-L1 treatment. However, it should be noted that in both cases this result was achieved with the addition of a minimum amount of divalent cations (Mg^{2+} for DNase I and Zn^{2+} for S1, not necessary for PD-L1) and that the linearized form was a consequence of random cleavage of pBR322 DNA. The pattern obtained with PD-L1, conversely, seemed to be very similar to that obtained with MBN. Thus, if a contaminant is present, it should be an endonuclease (the orthologue of MBN in *P. dioica*?) with functional and structural properties very similar to the well-known properties of MBN, even though its complete amino acid sequence has not yet been

determined (Di Maro et al. 2008). If this is the case, the contaminating MBN-like protein should be present in very small amounts because, apart from PD-L1 RIP, no traces of other proteins were detected using the purification protocol reported in Table 1. It has also been suggested that the contamination could not be detected by the analytical procedures used. However, contamination from DNases could also be excluded on the basis of the following considerations: (1) our purification procedure for PD-Ls and dioicins (Table 1) includes steps that have been reported to be capable of removing the contaminating DNase activities (Barbieri et al. 2000); (2) the contaminating DNase activities should be present in almost all our RIP preparations eluted over a wide range of ionic strength (from 20 to 120 mM NaCl) necessary to elute proteins with high p*I*. Indeed, p*I* values for RIPs (>8.5) used in this study appear to be higher than those reported for commercial DNase I (p*I* 4.5) and S1 (theoretical p*I* 4.26); (3) contamination by DNase II (p*I* between pH 6.0 and 8.0) is not likely because this enzyme acts in the presence of high-salt concentration (Adams et al. 1986), while RIP endonucleolytic activity is inhibited under these conditions; (4) finally, the purification and storage conditions (low pH, water, and the absence of metal ions) would cause a loss in activity of such contaminating endonucleases. It is well known that MBN stability and activity are Zn^{2+}-dependent at pH 5.0. In fact, its presence is essential during the purification procedure and over 90% of the activity can be lost or restored by zinc deprivation or its addition after dialysis at pH 5.0 in the absence of EDTA (Kowalski et al. 1976). Hence, if MBN endonucleolytic activity is greatly potentiated in the presence of Zn^{2+}, it cannot be explained why PD-L1 is only slightly potentiated by this ion and can exert its activity without the addition of any divalent cations. Moreover, as mentioned above, our purification procedure involves steps (dialysis at pH 4.5; Di Maro et al. 1999) that could inactivate the MBN-like protein and exclude copurification of a protein with MBN endonuclease properties (i.e., stepwise elution at pH 7.2, which allows the elution of proteins with p*I* in the range 5.0–7.0, such as MBN; Kowalski et al. 1976). In conclusion, our results suggest that the activity of PD-L1 on DNA is an intrinsic property of this RIP form and is exerted mainly at low ionic strength, where secondary single-stranded structures may be formed and unpaired bases are present. Similar behavior is shown by other known single-strand-specific endonucleases (Desai and Shankar 2003). The competition experiments performed at various single- and double-stranded oligonucleotide/substrate excesses support this hypothesis and show that the nicking RIP activity persisted even at a 1,000-fold molar excess of the scavenger oligos over pBR322. This suggests that the nicking activity is dependent more on the single-stranded secondary structure with unpaired bases than on the occurrence of a specific sequence. Recent reports are in agreement with this hypothesis (Park et al. 2004a, b). Experiments showing a decrease in the transformation capacity of plasmid DNA after PD-L1 treatment suggest that damage to DNA occurred. This could be considered the mechanism responsible for the additional biological activity of RIPs according to results described in a different system (Nicolas et al. 1997). The fact that some RIPs may be endowed with enzyme activity against DNA, even though it may occur only at high concentrations, helps to explain some of their different biological properties. In fact, direct or indirect RIP

activity against DNA has been reported in several papers hypothesizing different roles and biological significance: (1) RIP binding DNA (Hao et al. 2001); (2) nuclear DNA damage (Brigotti et al. 2002); (3) internucleosomal DNA fragmentation activity (Bagga et al. 2003); and (4) a role in transforming mammalian cells (Barbieri et al. 2003). These different capabilities may be necessary for these proteins to perform different biological roles: (1) resistance to pathogenic microorganisms or viruses; (2) implication in the mechanism of apoptosis and in metabolic regulation; and (3) activity as gene expression regulators. There remains a need to elucidate the mechanism of DNA cleavage exerted by PD-L1 and the structural determinants involved in this activity, questions not yet fully resolved for other RIPs showing the same activity, although it has been suggested that RIPs may act as DNA glycosylase/AP lyases (Wang et al. 1999a). This mechanism proposed for MAP30 is based on the presence of a lysyl residue (K195) close to a triptophanyl residue, the side-chain amino group of which would function as a nucleophile that attacks the C19 of the ribose of the abasic site (Wang et al. 1999b). However, PD-L4, which contains a lysyl residue corresponding to K195 of MAP 30 in the 3D structure, does not show nicking activity under the experimental conditions used here.

4 X-ray Crystal Structure of *P. dioica* RIPs

X-ray structures of RIPs from *P. dioica* have recently been determined (Table 4) (Ruggiero et al. 2007a, b, 2008, 2009). These structures describe three of the four RIPs isolated from fully expanded leaves of adult *P. dioica* leaves (PD-L1-4). Crystallographic studies of PD-L4, in its unliganded and adenine-bound states (Table 4), have provided atomic resolution structural information. As such, they constitute reference structures for this class of proteins (Ruggiero et al. 2008).

4.1 Atomic Resolution Studies of PD-L4: A Reference RIP Structure

Analogous to other RIPs (ricin A-chain, trichosanthin, PAP-I), *P. dioica* RIPs are composed of two domains and possess a well-defined secondary structure (Fig. 4a).

Table 4 Available crystal structures of RIPs from *P. dioica*

Description	Ligand	Resolution (Å)	PDB code	Ref.
PD-L4	–	1.10	2Z4U	Ruggiero et al. 2007a, b, 2008
PD-L4	Adenine	1.24	2QES	Ruggiero et al. 2007a, b, 2008
PD-L4 mutant S211A	–	1.29	2Z53	Ruggiero et al. 2007a, b, 2008
PD-L4 mutant S211A	Adenine	1.24	2QET	Ruggiero et al. 2007a, b, 2008
PD-L1	–	1.45	3H5K	Ruggiero et al. 2007a, b, 2009
PD-L3	–	1.80	Ongoing	–

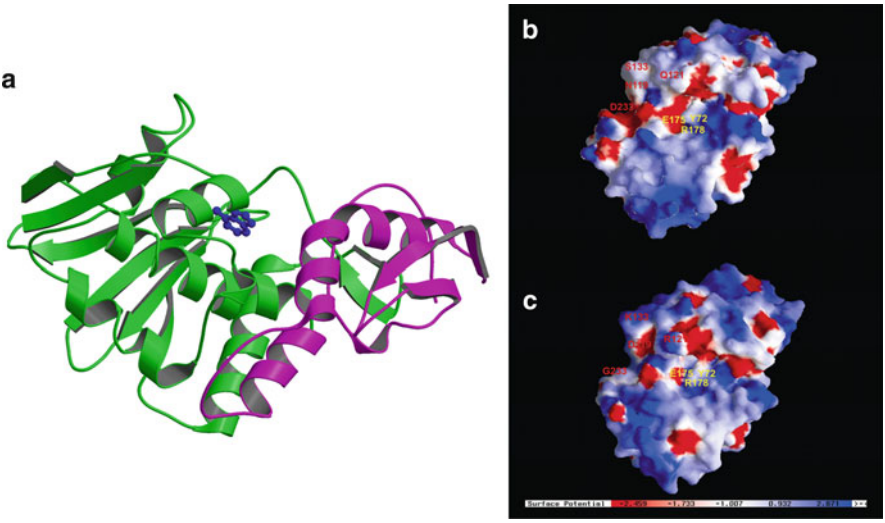

Fig. 4 (**a**) Ribbon structure of PD-L4. N-terminal and C-terminal domains are drawn in *green* and *magenta*, respectively; adenine is drawn in *blue*. The figure has been drawn using MOLSCRIPT (Esnouf 1999). (**b**) Surface electrostatic potential distributions of PD-L4 and 8C9 of PAP. Positive (*blue*) and negative (*red*) electrostatic potential is mapped on the molecular surfaces by GRASP. PD-L4 residue numbering has been used

Despite a structural similarity with pokeweed antiviral protein (PDB code 1qcg), with an average r.m.s.d. value after superposition of 261 equivalent Cα atoms of 0.65 Å, significant differences exist in the electrostatic potential surface of the two proteins (Fig. 4b, c). These differences are particularly evident in the putative RNA binding site cleft, where the electrostatic potential surface of PD-L4 is more negatively charged (Fig. 4b, c). This behavior is predictive of a different activity/specificity of the two proteins.

4.2 An Insight into the Active Site of PD-L4: Tyr72 as a Substrate Carrier Through π–π Stacking Interactions with Adenine

Although high resolution structures of various RIPs have been determined (Ago et al. 1994; Fermani et al. 2005; Hou et al. 2007; Kurinov and Uckun 2003; Kurinov et al. 1999; Savino et al. 2000; Touloupakis et al. 2006; Zeng et al. 2003), the mechanism by which they inhibit cell growth is still not fully understood. The currently accepted reaction mechanism involves the protonation of the adenine to be cleaved and the successive hydrolysis by a water molecule of the positively charged oxicarbonium intermediate. However, the residues which are involved in the protonation of the adenyl group are not known and contrasting hypotheses have been proposed (Guo et al. 2003; Huang et al. 1995; Ren et al. 1994).

The well-conserved active site residues of PD-L4 (Tyr72, Glu175, Arg178, Trp207) are located in the central part of the long concave presumed RNA-binding region (Fig. 4a). Analysis of the refined model of PD-L4 in the unliganded state shows that two well-defined conformations exist for the active site Tyr72 (Fig. 5a).

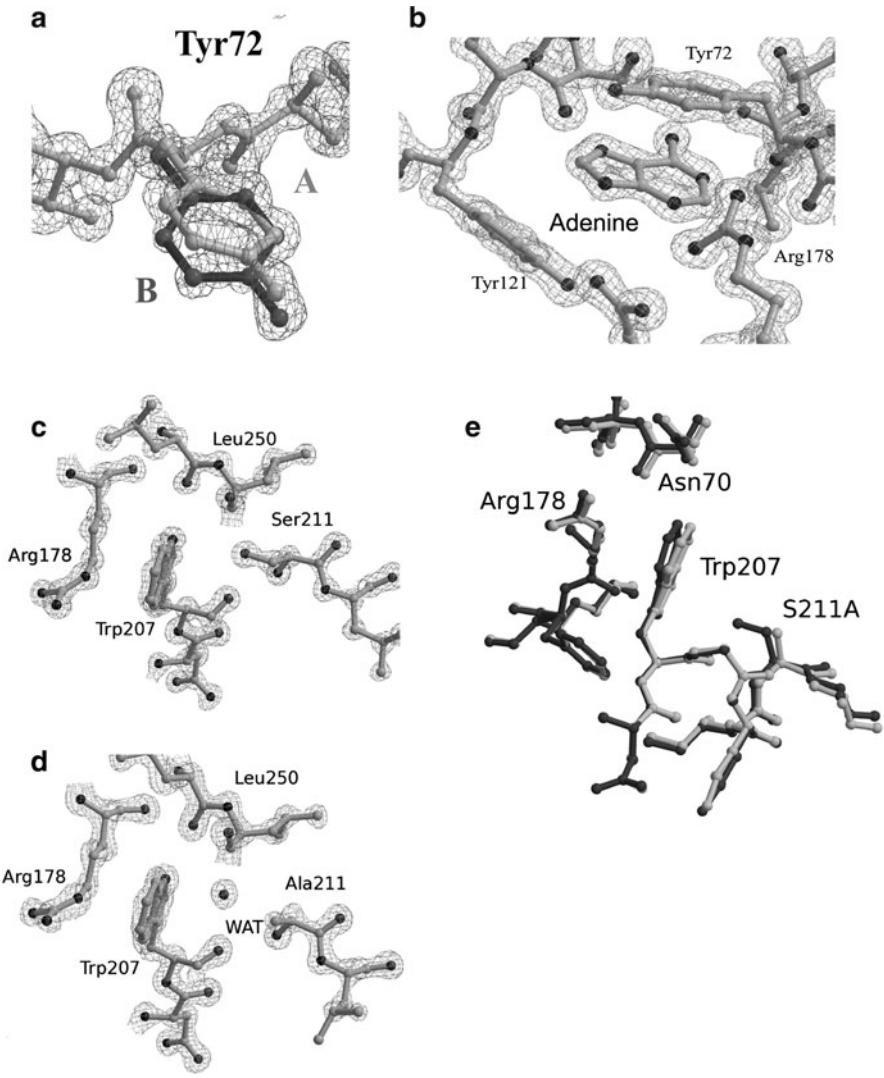

Fig. 5 (**a**) (2Fo–Fc) electron density map, contoured at 2σ, of A and B conformations of Tyr72 in PD-L4. (**b**) (2Fo–Fc) electron density map, contoured at 2σ, of PD-L4 in complex with adenine. (**c**) (2Fo–Fc) electron density map, contoured at 2.5σ, of (A) PD-L4. (**d**) PD-L4 mutant S211A at the mutation site. (**e**) Superposition of residues close to the mutation site in PD-L4 (*dark gray*) and the PD-L4 mutant S211A (*light gray*). Figures have been drawn using BOBSCRIPT and MOLSCRIPT (Esnouf 1999)

In contrast, only one of the two Tyr72 conformations, the less abundant in the unliganded state (occupancy factor 0.15), is observed in the adenine complex. In this conformation, the phenoxy plane of Tyr72 is almost parallel with that of the adenine base (Fig. 5b). Many different protein systems, beside RIPs, employ Tyr residues in the recognition of the adenine ring, through a π–π stacking interaction (Boehr et al. 2002). Also, the orientation of Ade and Tyr72, with the Tyr OH group pointing at the N9 atom of adenine, has been found to be the most frequent in proteins; this has been attributed to the occurrence of favorable electrostatic interactions between the two rings (Boehr et al. 2002). The observed tight interaction of the Tyr72 ring with adenine (with a distance of 3.75 Å between their centroids) as well as the existence of two conformations in the unliganded PD-L4 are likely to be a requirement for the catalytic role of the adenine interaction, attributed to Tyr72 (Huang et al. 1995). It is worth noting that the A conformation of Tyr72 (Fig. 5a) is nearly superposable to that observed in the complex of inactive mutants of trichosanthin with AMP (Guo et al. 2003). Consistent with these findings, it has been proposed that Tyr72 in the A conformation (Fig. 5a) is devoted to the binding of the adenyl group in the early stages of the reaction mechanism. Modeling of AMP with Tyr72 in the A conformation shows that adenine is in the near proximity of Asp91, likely to be the protonating moiety (Huang et al. 1995). Consistently, the equivalent residue of Asp91 in trichosanthin (Glu85) has been shown to strongly affect catalysis (Guo et al. 2003). Most probably, after protonation, the adenyl group remains bound to the A conformation of Tyr72 (Fig. 5a) since it is unlikely that a positively charged adenine could be hydrogen bonded to the positively charged Arg178 (Fig. 5b). Finally, only when the *N*-glycosidic bond has been cleaved, the product adenine is accompanied to its final destination by Tyr72 (B conformation, Fig. 5a) and establishes the hydrogen-bonding interactions (to Arg178, Val73, Ser120) observed in the structures of the adenine complex of PD-L4 (Fig. 5b).

Sequence alignment of all known RIPs clearly evidences that a high sequence conservation characterizes residues which are not traditionally considered as catalytically relevant, since they are located outside the active site cleft. As a part of a systematic study of the impact of these residues on function and structure of RIPs, PD-L4 was used as a model for mutational studies (Chambery et al. 2007). This study provided evidence that the mutation of the invariant Ser211 to Ala causes a significant decrease in APG activity, with the major extent of reduction for poly(A) substrates (Chambery et al. 2007). Structural bases of the reduced activity of the PD-L4 S211A mutant were obtained by determining the X-ray structure of this mutant both in its unliganded state and in complex with adenine, the major product of their enzymatic reaction (Table 4). In the crystal structure of the PD-L4 S211A mutant, the formation of a cavity formed by the lack of the Ser211 OH group is not compensated by a reorganization of the local enzyme structure, but the OH is replaced by a water molecule (Fig. 5c, d). This resulted in subtle conformational changes of residues which play a fundamental role in substrate binding, like Trp207 and Arg178 (Fig. 5e; Ruggiero et al. 2008). These data point to the importance of precise catalytic residue positioning for substrate binding. Furthermore, these studies show that subtle, albeit significant, structural changes are responsible for

significant differences in the enzymatic activity. This highlights the importance of atomic resolution studies for the understanding of enzymatic properties (Schmidt and Lamzin 2002; Vrielink and Sampson 2003).

4.3 *PD-L1 and PD-L4 – Two Homologous Proteins with Distinct Functional Properties*

As previously reported, leaves of *P. dioica* express four type 1 RIPs, named PD-L1, PDL-2, PD-L3, and PD-L4. PD-L1–3 isoforms exhibit different degrees of glycosylation, whereas PD-L4 is not glycosylated (see Sect. 10.2.5). Despite the high sequence identity of these proteins, PD-L1 (and PD-L2) induce the cleavage of supercoiled double-stranded pBR322 DNA, whereas PD-L4 (and PD-L3) do not (see Sect. 10.3.5, Aceto et al. 2005). The structural basis of the different functional behavior of PD-L1 and PD-L4 was identified by determining the X-ray structure of native PD-L1 and by evaluating the role of glycosylation on DNA cleaving activity (Ruggiero et al. 2009). The crystallographic structure of PD-L1 evidenced that the protein catalytic cleft is not large enough to host double-strand DNA. This suggested that DNA cleavage occurs at unstable sites, where the double helix is locally unfolded. Consistently, regions of the *E. coli* plasmid pBR322 identified as PD-L1 cleavage sites, are rich in adenine and thymine (AT-rich) (Aceto et al. 2005), and therefore characterized by a lower thermal stability. It is likely that the stress present within supercoiled DNA destabilizes double helices in AT rich regions, thus making them accessible to the action of RIPs (Ak and Benham 2005; Benham and Bi 2004). The structure of PD-L1 provided evidence for the flexible nature of its glycan chains, a result which suggested that glycan chains provide little, if any, contribution to the formation and stabilization of the enzyme–substrate complex prior to catalysis. Consistently, DNA cleavage assays on the *E. coli* plasmid pBR322 clearly showed that native and recombinant (nonglycosylated) PD-L1 were able to cleave the plasmid pBR322, as linearized forms were clearly detectable for both proteins (Ruggiero et al. 2009). By contrast, no cleavage of pBR322 was observed upon treatment with PD-L4, purified by the same procedure as native PD-L1. Altogether, these data unambiguously showed that the different behavior of PD-L1, compared to its homologue PD-L4, is not due to the protein glycosylation, but to differences in their protein sequences. When the structures of PD-L1 and PD-L4 are compared, most significant structural variations are observed in loop regions. Among these, a conformational change of the loop including Asp91 (Fig. 6) was identified. Asp91 has been proposed to play an important role in catalysis, as the equivalent amino acid residue in trichosanthin (Glu85) has important implications for *N*-glycosidase activity (Guo et al. 2003). Compared to the PD-L4 structure, the entire loop embedding Asp91 is pulled back in the PD-L1 structure (Fig. 6). This conformational change, induced by the presence of arginine at position 97, a serine in PD-L4, opens the active site cleft by about 2.5 Å. (Fig. 6). Notably, this same loop conformation is observed in the structure of the PAP, which

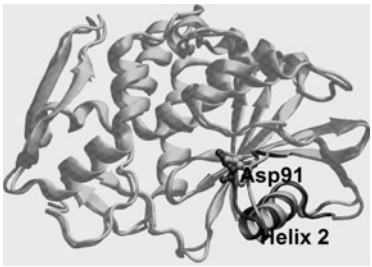

Fig. 6 Superposition of PDL1 (*light gray*) and PD-L4 (*dark gray*) structures. The entire protein structures are represented as *transparent cartoons*, whereas the loop 88–106 and α-helix 2 is shown as *solid cartoons*. Asp91 in the two structures is shown in *ball-and-stick* representation

displays a similar ability to induce DNA cleavage. In addition, other RIPs exhibiting DNA cleaving activity, like saporin 6 (Savino et al. 2000) and dianthin 30 (Fermani et al. 2005), are characterized by a two-residue shorter loop. The observed catalytic cleft opening may allow the binding of regions of the to-be-cleaved supercoiled DNA, whose binding is hampered by the obstructing loop in PD-L4. Following DNA binding and deadenylation by PD-L1, a spontaneous breakage of phosphodiester bonds was proposed. Consistently, thermodynamic studies have shown that abasic sites impact the stability, conformation, and melting behavior of a DNA duplex (Vesnaver et al. 1989). Consequently, phosphodiester bonds in extensively deadenylated regions of supercoiled DNA likely become liable because of the existence of tension in supercoiled DNA.

Overall, structural studies on PD-L1 confirmed that DNA cleaving activity is not to be attributed to nuclease contaminations during RIP preparation, as previously proposed (Ruggiero et al. 2009). In this framework, DNA cleavage is proposed to be a consequence of PD-L1 catalytic action, although not directly catalyzed by the enzyme. This interpretation is in line with evidences that (1) various RIPs which exhibit DNA relaxing activity are also able to depurinate supercoiled double-stranded DNA (Wang et al. 1999) and that (2) mutants of PAP that inhibit *N*-glycosidase activity also inhibit the cleavage of supercoiled double-stranded DNA (Bagga et al. 2003). In this study, PD-L1 and PD-L4, which share a sequence identity of 81.5%, offered a good opportunity for understanding the structural basis of DNA cleavage, given the limited number of diverse residues in their sequences.

5 Concluding Remarks

Structure and function studies of type 1 RIPs from *P. dioica* have provided fundamental knowledge on these plant toxins. These include (1) phylogenetic relationships among *Phytolacca* RIPs; (2) confirmation that RIP expression is developmentally regulated; (3) double localization, both in the extracellular spaces and in the cell vacuoles of leaf tissues. This offers a view of the physiological role of RIPs. Furthermore, detailed structural information obtained by high resolution X-ray studies, contribute to our knowledge of the role of single amino acid residues

and of the glycan moieties, shedding new light on the discussed DNA cleaving activity reported for RIPs. However, there is still much to be learned about this family of enzymes: such as gene number and organization; complete screening of plant tissues; detection of the factor(s) regulating RIP expression; biosynthesis and characterization of their biological activity in plant. An even better knowledge on the intracellular trafficking and protein target(s) upon intoxication of these type 1 RIPs will extend the utility of theses enzymes for better targeted biotechnological applications.

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Sambucus Ribosome-Inactivating Proteins and Lectins

José Miguel Ferreras, Lucía Citores, Rosario Iglesias, Pilar Jiménez, and Tomás Girbés

Abstract Plant ribosome-inactivating proteins (RIPs) are inhibitors with RNA-*N*-glycosidase activity that irreversibly inactivate eukaryotic ribosomes, thereby impairing protein synthesis. In recent years, more than 40 RIPs and lectins belonging to the *Sambucus* genus have been isolated and characterized to varying degrees. The type 2 RIPs isolated from *Sambucus* have the peculiarity that although they are enzymatically more active than ricin, they lack the high toxicity of ricin to intact cells and animals. The presence in the same tissue of heterodimeric and tetrameric type 2 RIPs, structurally related monomeric, and homodimeric lectins together with unrelated type 1 RIPs make *Sambucus* an ideal model for studying these special proteins whose biological role is unknown at present. In the light of the accumulated results on the *Sambucus* RIPs and lectins, we present here the main findings about structural features and biological activities of these proteins as well as the evolutionary relationship between them and some of their potential uses.

1 Ribosome-Inactivating Proteins

The term RIP (ribosome-inactivating protein) is used to refer to certain proteins present in some species of the angiospermae and bacteria that inhibit protein synthesis through a catalytic mechanism (Barbieri et al. 1993; Girbes et al. 2004; Stirpe 2004; Obrig 1997; Hartley and Lord 2004).

J.M. Ferreras, L. Citores, and R. Iglesias
Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, E-47005 Valladolid, Spain
e-mail: rosario@bio.uva.es; luciac@bio.uva.es; riglesia@bio.uva.es

P. Jiménez and T. Girbés (✉)
Nutrición y Bromatología, Facultad de Medicina and CINAD, Universidad de Valladolid, E-47005 Valladolid, Spain
e-mail: pilarj@bio.uva.es; girbes@bio.uva.es

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_6,

In view of their structure and biological activity, plant RIPs have traditionally been classified into two categories (Barbieri et al. 1993; Girbes et al. 2004; Stirpe 2004), namely type 1 RIPs and type 2 RIPs. Type 1 RIPs (such as PAP or saporin) consist of a single polypeptide chain that displays the enzymic activity, whereas type 2 RIPs (such as ricin or abrin) contain two different polypeptide chains linked by a disulphide bridge: an A chain (the active chain, with enzymic activity) and a B chain (the binding chain which is able to bind to cell surface receptors). The B chain allows rapid internalization of the type 2 RIP into the eukaryotic cell, translocation of the A chain into the cytosol, and inactivation of the ribosomes. This is the reason why current type 2 RIPs, such as ricin and related proteins, are the most cytotoxic agents known to date (IC_{50} values in HeLa cells in the 1–10 pM range; LD_{50} values in mice are in the 0.5–33 μg/kg body weight range). Some type 2 RIPs, such as *Ricinus communis* agglutinin, contain two A chains and two B chains (Citores et al. 1993; Girbes et al. 2004; Olsnes et al. 1974; Roberts et al. 1985; Hartley and Lord 2004).

In our laboratory, a number of nontoxic type 2 RIPs were found in some species from the genus *Sambucus* (Girbes et al. 1993a, b, 1996a; Citores et al. 1994, 1996a, 1997, 1998; de Benito et al. 1997; Rojo et al. 1997). The reason for and the importance of the lack of toxicity will be discussed later.

Despite the intense research carried out on ricin since its discovery in 1889, it was only in 1987 that Endo found an enzymic activity associated with the ricin A chain (Endo et al. 1987). RIPs are rRNA *N*-glycosidases (or *N*-glycosylases) (EC 3.2.2.22) that depurinate the adenine No. 4324 from the 28S rRNA of the large subunit of rat ribosomes (or the equivalent adenine in sensitive ribosomes from other animals) (Barbieri et al. 1993; Girbes et al. 2004; Stirpe 2004). This adenine is located in a loop involved in the interaction of the ribosome with elongation factor 2 (EF-2) in eukaryotes and elongation factor G (EF-G) in prokaryotes (Barbieri et al. 1993; Stirpe 2004; Girbes et al. 2004). RIP-dependent ribosome inactivation arrests protein synthesis by preventing EF-dependent polypeptide chain translocation (Brigotti et al. 1989).

Some RIPs also inactivate ribosomes from fungi and certain plants and bacteria. In all these cases, the mechanism of action is the same as that acting on the ribosomes from animals (Barbieri et al. 1993; Stirpe 2004; Girbes et al. 2004). Some RIPs also display *N*-glycosidase activity on other adenines from ribosomal RNA (Barbieri et al. 1992; Iglesias et al. 1993), on viral RNA (Girbes et al. 1996b; Barbieri et al. 1994) and on genomic DNA (Barbieri et al. 1994, 2004). Other activities reportedly associated with RIPs are chitinase activity (Shih et al. 1997), topological activity on DNA (Huang et al. 1992), HIV integrase inhibitory activity (Lee-Huang et al. 1995), superoxide dismutase activity (Li et al. 1997), DNase activity (Ruggiero et al. 2007), and lipase activity (Lombard et al. 2001; Morlon-Guyot et al. 2003). In particular, the *N*-glycosidase on both viral RNA and genomic DNA could have a special biological role. In fact, the *N*-glycosidase activity on viral RNA has been related to the antiviral role found in many RIPs (Barbieri et al. 1993; Girbes et al. 2004; Stirpe 2004). On the other hand, *N*-glycosidase activity on genomic plant DNA could be related to a role in plant senescence (Stirpe et al. 1996).

No precise biological role has yet been assigned to RIPs. It has been postulated that they could have a role in the defense of plants against predators, fungi, and viruses (Barbieri et al. 1993; Stirpe 2004; Girbes et al. 2004). Furthermore, it is believed that some RIPs could play a hitherto undefined role in plant senescence (Stirpe et al. 1996). Indirect evidence favors the antiviral proposal. Almost all the RIPs tested to date have some antiviral activity. Furthermore, some RIPs were first recognized as antiviral proteins prior to the discovery of their activity as translational inhibitors (Barbieri et al. 1993; Stirpe 2004). Strong support of the antiviral hypothesis came from research on sugar beet RIPs. Sugar beet contains two type 1 RIPs that we named beetins (Girbes et al. 1996b). Both beetins were found to be present in the leaves of highly infected plants and were induced in healthy plants grown in the laboratory either by infection with plant viruses or molecular mediators of the defense against viral attack such as hydrogen peroxide or salicylic acid (Iglesias et al. 2005, 2008).

Research on RIPs is expanding because of the interest in their application in human therapy; in particular, cancer, AIDS, and autoimmune diseases (Girbes et al. 2004; Stirpe 2004). The goal of the present review is to comment on the genus *Sambucus* (Caprifoliaceae), in which about 40 RIPs and structurally related lectins have been found in the last few years. The complexity and the large number of RIPs found in *Sambucus* makes this special family an ideal model for studying the expression, distribution, and seasonal variations of these proteins with the aim of gaining information useful for defining their biological role.

2 Occurrence and Structural Diversity of *Sambucus* Proteins

The genus *Sambucus* L. is composed of about 20 species (Guangwan et al. 2008). They are small trees, shrubs, or herbs (in few cases) located in North America, Asia, Europe, northern Africa, the West Indies, and the Andean region of South America.

The presence of RIPs and lectins has been studied mainly in *Sambucus ebulus* L. (Dwarf elder), *Sambucus nigra* L. (European elder), *Sambucus sieboldiana* Blume ex Graebn. (Japanese elder), and *Sambucus racemosa* L. (Red elder). Type 2 RIPs and structurally related lectins have been found in all *Sambucus* spp. (Table 1). In addition, type 1 RIPs have also been found in *S. ebulus* and *S. nigra*.

To better classify and review all the proteins found to date in *Sambucus*, we divide them into three general groups, type 1 RIPs, type 2 RIPs, and pure homolectins, based on their structure and biological activity. Type 1 RIPs consist of a single polypeptide chain (A chain) of ~30 kDa, which displays the enzymic activity. Type 2 RIPs contain two different A and B polypeptide chains of ~30 kDa linked by a disulphide bridge. Type 2 RIPs can be heterodimeric (type A–B) or tetrameric (A–B–B–A). Tetrameric type 2 RIPs are four-chain proteins, consisting of two dimers of the type (A–B) linked also by a disulphide bridge. The third group corresponds to the lectins, which do not show enzymic activity and present only lectin activity. They can be homodimeric (two-chain proteins of the

Table 1 RIPs and lectins from *Sambucus* species

Species	Tissue	Structure	Protein
Sambucus ebulus L.	Leaves	Type 1 RIP	Ebulitins α, β and γ
		Heterodimeric	Ebulin l
		Homodimeric	SELld
		Monomeric	SELlm
	Fruits	Heterodimeric	Ebulin f
		Homodimeric	SELfd
	Rhizome	Heterodimeric	Ebulins r1 and r2
		Tetrameric	SEA
		Monomeric	SEA II
Sambucus nigra L.	Bark	Heterodimeric	Nigrin b, basic nigrin b, SNA I′, SNLRPs 1 and 2
		Tetrameric	SNA I
		Monomeric	SNA II
	Leaves	Heterodimeric	Nigrins l1 and l2
		Homodimeric	SNAld
		Monomeric	SNAlm and SNAIVl
	Fruits	Type 1 RIP	Nigritins f1 and f2
		Heterodimeric	Nigrin f
		Tetrameric	SNAIf
		Monomeric	SNA IV
	Seeds	Heterodimeric	Nigrin s
		Monomeric	SNA III
	Flowers	Tetrameric	SNAflu-I
	Pollen	n.d.	SNApol-I and SNApol-II
Sambucus sieboldiana Blume ex Graebn.	Bark	Heterodimeric	Sieboldin b
		Tetrameric	SSA
		Monomeric	SSA-b-3 and SSA-b-4
Sambucus racemosa L.	Bark	Heterodimeric	Basic racemosin b
		Tetrameric	SRA
		Monomeric	SRAbm

Species indicated in the table have been shown to contain type 1 RIPs, heterodimeric type 2 RIPs (one A chain and one B chain), tetrameric type 2 RIPs (two A chains and two B chains), and monomeric and homodimeric lectins (one or two B chains). The references are indicated in the text

type B–B held together by a disulphide bridge) or monomeric (one-chain proteins of the type B). As discussed below, from a structural and functional point of view these chains are closely related to the B chain of type 2 RIPs.

Type 1 RIPs have been found in *S. ebulus* leaves (ebulitins α, β, and γ) (de Benito et al. 1995) and *S. nigra* fruits (nigritins f1 and f2) (de Benito et al. 1998). However, this does not mean that they are not also present in other tissues or other *Sambucus* species. Nigritin f2 is only present in mature fruits, whereas nigritin f1 is present at the same proportions in both green and mature fruits. Thus, nigritin f1 seems to be a constitutive protein whereas nigritin f2 could be induced by signals related to the onset of maturation (de Benito et al. 1998).

Heterodimeric type 2 RIPs have been found in several parts of *S. ebulus*, *S. nigra*, *S. sieboldiana*, and *S. racemosa* (Table 1). *S. ebulus* contains type 2 RIPs in the leaves (ebulin l) (Girbes et al. 1993b), rhizome (ebulins r1 and r2) (Citores et al. 1997), and fruits (ebulin f) (Citores et al. 1998) although they seem to

be absent in seeds. *S. nigra* contains type 2 RIPs in all parts of the plant studied: bark (nigrin b, SNAI′, SNLRP1, SNLRP2, and basic nigrin b) (Girbes et al. 1993a; Van Damme et al. 1997a, b; de Benito et al. 1997), leaves (nigrin l1 and l2) (unpublished results), fruits (nigrin f) (Citores et al. 1996a; Girbes et al. 1996a) and seeds (nigrin s) (Citores et al. 1994). The bark of *S. sieboldiana* contains also a type 2 RIP (sieboldin b) (Rojo et al. 1997). Very few studies have been carried out with other *Sambucus* species. Some of them indicate that the bark of *S. racemosa* lacks D-galactose-binding type 2 RIPs but exhibits RIP activities in the D-galactose-unbound protein fraction (unpublished results). This RIP activity has been attributed to a 58 kDa protein composed by two subunits of 27.5 and 29.5 kDa, thus indicating that *S. racemosa* contains a protein equivalent to basic nigrin b from *S. nigra* (Rojo et al. 2003b; Ferreras et al. 2000). The most representative and studied members of this group are nigrin b and ebulin l (Girbes et al. 1993a, b). The name SNAV to refer to nigrin b has also been used because of the order of isolation of the lectins from elder (Van Damme et al. 1996a).

Tetrameric type 2 RIPs have been found in plant tissues together with heterodimeric RIPs. SNAI in *S. nigra* (Van Damme et al. 1996b) and SSA in *S. sieboldiana* (Kaku et al. 1996) are present in the bark of the perennial trunk. SNAIf has been isolated from fruits of *S. nigra* (Peumans et al. 1998). SEA previously described merely as a lectin (Nsimba-Lubaki et al. 1986) has now been found to be a tetrameric RIP and is found in the perennial root system of *S. ebulus* (Iglesias et al. 2010). SRA, from the bark of *S. racemosa* (Rojo et al. 2003b; Nsimba-Lubaki et al. 1986), has been included in this group because of its tetrameric character although it is not known whether the protein has an A chain with enzymic activity equivalent to the one in SNAI, SNAIf, SSA, and SEA. In addition, a tetrameric protein from *S. nigra* inflorescences (SNAflu-I), which might be the equivalent of SNAI in flowers, has been found (Karpova et al. 2007); the presence of two more proteins (SNApol-I and SNApol-II) has also been reported in pollen, but their structural characteristics have not been stated (Karpova et al. 2007). The lectin subunit of all the tetrameric type 2 RIPs from *Sambucus* specifically binds to the Neu5Ac(α-2,6)Gal/GalNac sequence (Shibuya et al. 1987; Kaku et al. 1996). This fact makes these lectins unique and different from other type 2 RIPs either from *Sambucus* or other families.

Lectins devoid of *N*-glycosidase activity and specific for Gal/GalNac, together with type1 and type 2 RIPs are also found. Monomeric lectins are present in the leaves (SELlm) (Citores et al. 2008) and rhizomes (SEAII) (Citores et al. 1997) of *S. ebulus*; in the leaves (SNAlm, SNAIVl) (unpublished results), bark (SNAII) (Kaku et al. 1990), fruits (SNAIV) (Van Damme et al. 1997c) and seeds (SNAIII) (Peumans et al. 1991) of *S. nigra*; in the bark of *S. sieboldiana* (SSA-b-3 and 4) (Rojo et al. 2004) and in the bark of *S. racemosa* (SRLbm) (Rojo et al. 2003b). The homodimeric lectins are found in leaves (SELld) (Rojo et al. 2003a) and fruits (SELfd) (Citores et al. 1998) from *S. ebulus* and leaves (SNAld) (unpublished results) from *S. nigra*. There is also evidence for the occurrence in *S. nigra* bark and fruits of small lectins consisting of a truncated part of the B chain of the Neu5Ac(α-2,6)Gal/GalNac-specific type 2 RIP SNAI found in the same tissues (Peumans et al. 1998).

The presence of these proteins in the different tissues is subject to seasonal and developmental variations. The concentration of the RIP ebulin l in leaves decreases with the development of *S. ebulus* and almost disappears in senescence while the content in the lectin SELld changes in the opposite way (Rojo et al. 2003a). In addition, the occurrence of SELlm in the same tissue appears to be restricted to young shoots since upon shoot development the lectin rapidly disappears (Citores et al. 2008). According to this, the type 2 RIPs nigrin l1 and nigrin l2 from *S. nigra* accumulate in shoots, decay in mature leaves, and are completely absent in senescent leaves (unpublished results). On the other hand, ebulin f from *S. ebulus* accumulates in the green fruits and disappears completely with maturation. Interestingly, in green fruits ebulin f can be polymerized with other ebulin f molecules and even with lectins to form high molecular weight aggregates that coexist with free forms of both ebulin f and lectins (Citores et al. 1998). Unlike the fruits of *S. ebulus*, both green and mature fruits of *S. nigra* contain nigrin f. However, fruit maturation leads to a substantial reduction in the concentration of nigrin f (Citores et al. 1996a). *S. nigra* bark also shows large changes in the amount of nigrin b throughout the year. The highest concentration of the RIP was found in the spring and summer, and the lowest in the winter. SNAI content slightly increased in summer and decreased in autumn–winter (unpublished results). There was no comparable change in the amount of the lectin SNAII since it increased in autumn, accumulated in winter, and considerably decreased in spring (unpublished results).

The different concentrations and localization in plant tissues suggest different biological roles played by these proteins. The results point to a possible protective role of the RIPs against plant pathogens in the early growth stages. Both, RIPs and lectins, might also play an important role in development, perhaps in some cases together with a storage protein role. It has been suggested that abundant type 2 RIPs from *S. nigra* bark (SNAI and nigrin b) can behave as storage proteins that may be used as specific defense proteins if the plant is attacked by insects or higher animals (Van Damme et al. 1996a). The presence in the same tissue of related proteins with different biological activities suggests a specialized expression pattern of their genes. Recent results indicated that the expression of several type 2 RIPs from *Sambucus* including nigrin b (SNA V) in tobacco plants could protect such transgenic plants from viral infection by an unknown mechanism (Vandenbussche et al. 2004).

3 Similarity and Processing

The complete primary sequences of some of the RIPs and lectins from *Sambucus* have been deduced from the nucleotide sequence of cloned cDNAs and genes. Sequence comparisons among the known type 2 RIPs and lectins revealed a high level of conservation although they display different molecular structures and sugar-binding specificities.

Analysis of the sequences indicates that the heterodimeric type 2 RIPs, such as nigrin b, ebulin l, and sieboldin b, derive from a single precursor comprising

a signal peptide and two different domains separated by a linker sequence (Van Damme et al. 1996a; Rojo et al. 1997; Pascal et al. 2001). After posttranslational processing, the N-terminal region of the precursor eventually yields the A chain with *N*-glycosidase activity whereas the C-terminal region is converted into the carbohydrate-binding B chain. Both chains remain linked by a disulphide bridge (Fig. 1a). Similarly, genes encoding tetrameric type 2 RIPs such as SNAI, SSA, and SEA have been cloned (Van Damme et al. 1996b; Kaku et al. 1996; Iglesias et al. 2010) and analysis of their sequence reveals that at the N-terminus these polypeptides have a signal peptide followed by an amino acid sequence containing the A chain, the linker peptide, and the B chain. This polypeptide is processed and, upon proteolytic removal of the linker peptide, produces a heterodimer that contains an A chain and a B chain linked by a disulphide bridge. The union of two heterodimers by another disulphide bond between the two B chains yields the tetrameric protein (Fig. 1a). Analysis of the deduced amino acid sequence of some of the monomeric lectins, such as SNAIV and SELlm (Van Damme et al. 1997c; Citores et al. 2008), indicated that the precursors of these lectins display a striking sequence identity with type 2 RIPs in the signal peptide in the first amino acid residues of the A-chain and in the linker region between the A and B chains of type 2 RIPs. The lectin precursor is converted into the mature protein through a processing mechanism where the signal peptide, a small part of the A chain precursor, the connecting peptide and in some cases few residues of the N-terminal amino acid sequence of the B chain are lost (Fig. 1a). The data therefore suggest that these lectins could be encoded by a truncated type 2 RIP gene which lost a substantial part coding for the A chain. The same processing mechanism has been found for the homodimeric lectins SNAld and SELld (Rojo et al. 2003a; unpublished results). In these cases, a new Cys appeared which is most probably responsible for the dimerization of the lectin polypeptide chain through an interchain disulphide bridge (Fig. 1a). The mature monomeric lectins SSA-b-3 and b-4 are processed from a precursor containing a signal peptide followed only by the mature polypeptide. The structure of these lectins also showed a striking homology with that of the B chain of type 2 RIPs (Rojo et al. 2004).

RIPs and lectins from *Sambucus* are structurally related to ricin and the other toxic type 2 RIPs. A comparison of the amino acid sequence of ebulin l with ricin indicates that the A chains share 34% amino acid identity and the B chains share 48% identity (Pascal et al. 2001). Figure 1b shows the primary sequences of heterodimeric (nigrin b) and tetrameric (SNAI) type 2 RIPs and monomeric (SNAlm) and homodimeric (SNAld) lectins isolated from *S. nigra*. Comparison of the sequences indicates that there is considerable sequence homology within this class of proteins. The tetrameric SNAI shares approximately 53, 54, and 51% amino acid identity with nigrin b, SNAlm, and SNAld, respectively. Nigrin b shares with SNAlm and SNALd a sequence homology of 86 and 72%, respectively, and SNAlm displays 70% homology with SNAld.

The level of sequence identity between the A chains is highest in the regions corresponding to the active site sequences. The invariant residues within this site which have been reported to be required for *N*-glycosidase activity are conserved

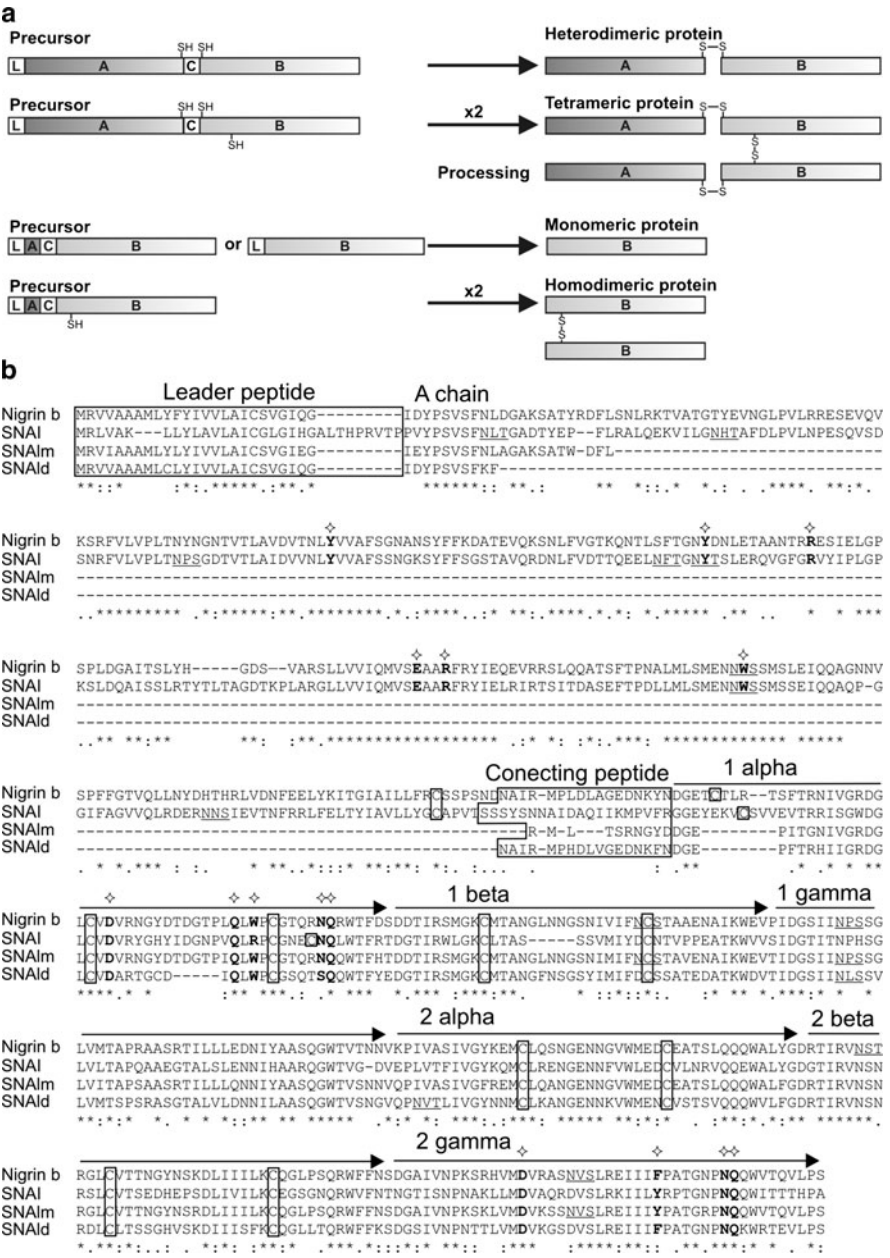

Fig. 1 (**a**) Graphical representation of the sequences of the precursors encoding heterodimeric, tetrameric, monomeric, and homodimeric proteins from *Sambucus*. The leader (L), the connecting (C) peptide, the A (or A fragments) and B chains and the sulfhydryl groups involved in the intermolecular disulphide bridge formation between the chains are indicated. (**b**) Amino acid sequences alignment of nigrin b (GenBank accession no. AAB39475, P33183), SNAI (AAC49158),

together with residues that maintain the site in an active catalytic conformation (Pascal et al. 2001; Kaku et al. 1996; Rojo et al. 1997; Van Damme et al. 1996a, b, 1997a, b). The B chain of the type 2 RIPs and lectins consists of two homologous β-trefoil domains each of which hold a carbohydrate-binding site and thus exhibits lectin activity. Each domain comprises three subdomains (1α, 1β, and 1γ for domain 1; and 2α, 2β, and 2γ for domain 2). The B chains contain eight conserved cysteinyl residues which could form four intrachain disulphide bridges. In addition, in type 2 RIPs there is a cysteine at the N-terminal of the B chain which forms a disulphide bond with a conserved cysteine at the C-terminal of the A chain (Pascal et al. 2001; Kaku et al. 1996; Rojo et al. 1997; Van Damme et al. 1996a, b, 1997a, b). A free cysteine residue in the B chain of tetrameric type 2 RIPs responsible for the disulphide linkage between the two heterodimers (A–B–B–A) is present and is not found in the heterodimeric RIPs (Kaku et al. 1996; Van Damme et al. 1996b; unpublished results). There is a cysteine in the homodimeric lectins which is responsible for the dimerization of these lectins (Rojo et al. 2003a).

The tetrameric and dimeric type 2 RIPs contain B chains that recognize Neu5Ac (α-2,6)Gal/GalNAc and Gal/GalNAc, respectively. However, all the lectins found are specific for Gal/GalNac. Recently, it has been reported for the tetrameric type 2 RIP from *S. sieboldiana* SSA that three amino acid residues (S505, A541, and Q542) in the C-terminal subdomain of the SSA-B chain are critical for binding to the sialic acid in the Neu5Ac(α-2,6)GalNAc sequence to occur (Kaku et al. 2007). The bark of *S. nigra* also contains atypical heterodimeric type 2 RIPs named SNLRP1 and SNLRP2 (Van Damme et al. 1997a). These proteins exhibit rRNA *N*-glycosidase activity, but are devoid of carbohydrate-binding activity displaying striking differences in those amino acids that participate in sugar binding. Molecular modeling of the protein confirmed that the A chain is fully active, whereas the B chain contains two functionally inactive carbohydrate-binding sites and hence lacks the ability to agglutinate red blood cells (Van Damme et al. 1997a). Basic nigrin b, also isolated from *S. nigra* bark, does not agglutinate human red blood cells (de Benito et al. 1997). Tryptic peptide sequences obtained from basic nigrin b indicate that this protein has a high sequence homology with SNLRPs (close to 90%). However, there must be crucial differences between basic nigrin b and both SNLRPs since the inhibitory activity on protein synthesis of basic nigrin b is nearly 30,000 times greater than those of both SNLRPs. In fact, basic nigrin b is the most active RIP known to date (de Benito et al. 1997).

Fig. 1 (*continued*) SNAlm (AAN86132), and SNAld (AAN86131). Alignment of the sequences was done using ClustalW (Thompson et al. 1994). Gaps have been introduced to optimize the identity between sequences. Identical residues (*asterisk*), conserved substitutions (*colon*), and semiconserved substitutions (*dot*) are reported. The boxes enclose the leader and the connecting peptides. Key residues of the A chain active site and the B chain sugar-binding sites are identified in bold face type with diamonds. The *arrows* indicate the 1α, 1β, 1γ, 2α, 2β, and 2γ domains. The cysteines involved in the intra- and intermolecular disulphide bridges of the A and B chains are also enclosed in boxes. The *N*-glycosilation signals are underlined

4 Structure

The structure of ebulin l has been resolved by X-ray diffraction analysis and the tertiary structure closely resembles that of ricin (Pascal et al. 2001) (Fig. 2).

In the A chain, ebulin l has roughly the same positioning of key active site residues as ricin (Fig. 2). One exception is that the side chain of the Tyr 77 (Tyr 80 in ricin) of ebulin is rotated out of the binding site pocket. This orientation is similar

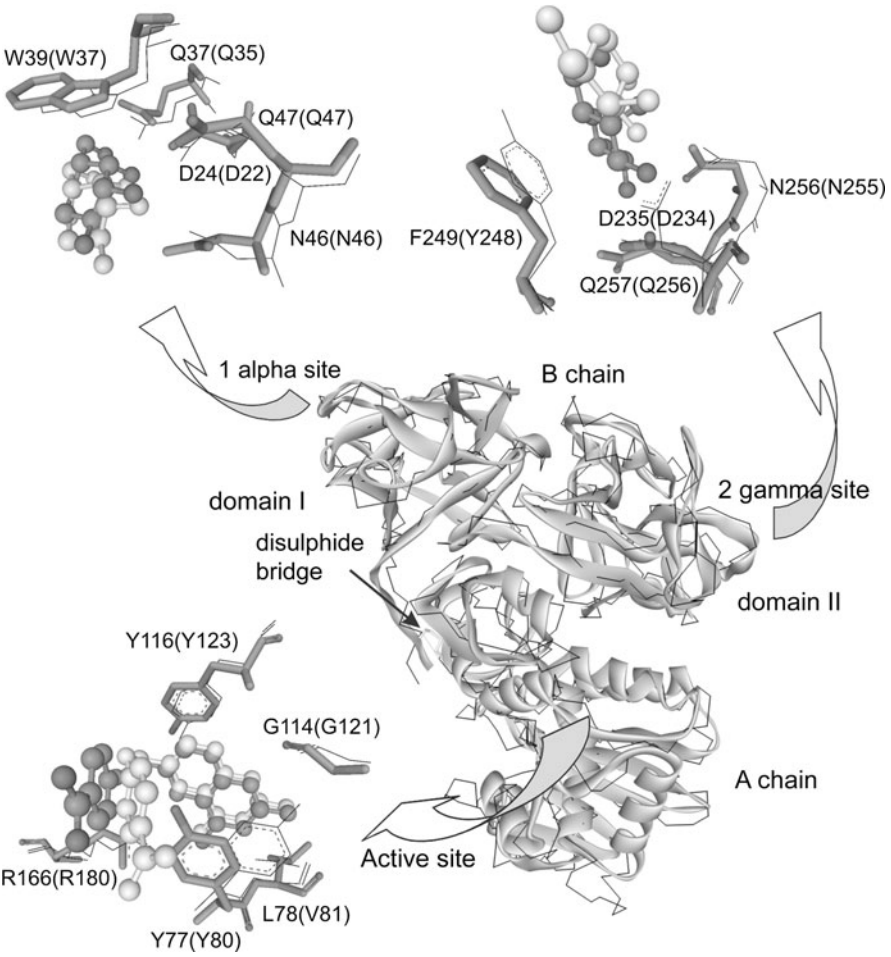

Fig. 2 Superimposition of the three-dimensional models of ebulin l (*solid ribbon*) and ricin (Cα wire). The disulphide bridge between the A and B chains is indicated. The active sites of ebulin l (*sticks*) and ricin (*lines*) bound to the substrate analog pteroic acid (*dark balls* and *sticks* for ebulin l and *light balls* and *sticks* for ricin) and the sugar-binding sites 1α and 2γ bound to D-galactose are emphasized. The key residues in ebulin l and the corresponding residues of ricin (parentheses) are indicated

to that seen in PAP (a type 1 RIP from *Phytolacca Americana* L.). Pteroic acid, an A chain substrate analog, binds in a similar way to both active sites. The pterin ring stacks with the side chain of Tyr 77 and forms hydrogen bonds with the backbone of Leu 78 (Val 78 in ricin) and Gly 114 (Gly 121 in ricin). Arg 166 (Arg 180 in ricin) donates a hydrogen bond to N5 of the pterin. This is consistent with the fact that both proteins have a similar inhibitory activity of protein synthesis (Table 2).

The overall fold of the ebulin B chain is very similar to that of the ricin B chain (Fig. 2). Ricin binds both galactose and lactose in the subdomains 1α and 2γ. Ebulin l binds to galactose and lactose by its 1α subdomain in a nearly identical fashion to ricin and uses the same sugar-binding residues as ricin: Trp 39 (Trp 37 in ricin), Asp 24 (Asp 22 in ricin), Gln 37 (Gln 35 in ricin), Asn 46 (Asn 46 in ricin), and Gln 47 (Gln 47 in ricin) (Figs. 1b and 2). Although ricin and ebulin l have very similar 2γ binding site geometries, lactose does not bind to the ebulin 2γ subdomain. The mode of galactose binding in the subdomain 2γ of ebulin is somewhat different

Table 2 Effect of type 2 RIPs on protein synthesis by a rabbit reticulocytes lysate and toxicity to intact cells and animals

Species	RIP	Rabbit lysate IC_{50} (nM)[a]	HeLa cells IC_{50} (nM)	Mouse LD_{50} (mg/kg)
Abrus precatorius (L.) Wright				
	Abrin	0.5	0.0037	0.00056
Adenia digitata (Harv.) Engl.				
	Modeccin	2.3	0.0003	0.0023
Adenia volkensii Harm.				
	Volkensin	0.37	0.0003	0.00138
Ricinus communis L.				
	Ricin	0.1	0.00067	0.003
Viscum album L.				
	Viscumin	3.5	0.008	0.0024
Sambucus ebulus L.				
	Ebulin f	0.03	17	>1.6
	Ebulin l	0.15	64.3	2
Sambucus nigra L.				
	Nigrin b	0.03	27.6	12
	Basic nigrin b	0.0003	>15,000	>40
	Nigrin f	0.03	2.9	>1.6
	Nigrin l1	0.04	n.d.	>65
	Nigrin l2	0.03	n.d.	>65
	SNAI	1.65	>400	n.d.
	SNLRP	5.74	>1,500	n.d.
Sambucus sieboldiana Blume ex Graebn.				
	Sieboldin b	0.015	11.8	>1.6
	SSA	16.4	917	>4.2

[a]Reduced toxin

References: abrin, modeccin, and viscumin (Stirpe and Barbieri 1986); volkensin (Stirpe et al. 1985); ricin (Barbieri et al. 2004; Citores et al. 1996b; Gayoso et al. 2005); ebulin f (Citores et al. 1998; Ferreras et al. 2000); ebulin l and nigrin b (Citores et al. 1996b; Ferreras et al. 2000; Girbes et al. 1993a, b); basic nigrin b (de Benito et al. 1997; Ferreras et al. 2000); nigrin f (Citores et al. 1996a; Ferreras et al. 2000); nigrin l1 and l2 (Ferreras et al. 2000); SNAI and SNLRP (Barbieri et al. 2004; Battelli et al. 1997a); Sieboldin b and SSA (Rojo et al. 1997)

(Fig. 2). The orientation and the positioning of galactose within the binding cleft are shifted as compared with ricin, galactose bound to ebulin is located further into the binding cleft than the galactose moiety bound to ricin. This would cause steric interference for any sugar attached to the C1 hydroxyl and for this reason lactose does not bind to the 2γ site of ebulin l. This altered mode of galactose binding in the 2γ site of ebulin l may indicate a weaker binding to complex sugars. In fact, it was found that ebulin l, nigrin b, and the lectins SELlm and SELld have different binding properties to D-galactose containing matrix than ricin (Pascal et al. 2001; Citores et al. 2008). Notably, and unlike ricin, the binding to this matrix was dependent on temperature, being maximum in the range of 0–10°C and abolished at 20°C (Citores et al. 2008). This may alter or diminish binding to cell surfaces.

5 Enzymic Activity

Both type 1 and 2 RIPs from *Sambucus* are *N*-glycosidases that depurinate the rRNA in the same way as ricin (Girbes et al. 1993a, b; de Benito et al. 1995, 1997, 1998; Citores et al. 1996a, 1997, 1998; Battelli et al. 1997a; Rojo et al. 1997; Hartley and Lord 2004) The effect of *Sambucus* RIPs on mammalian ribosomes has been evaluated by assaying their inhibitory activity on protein synthesis by cell-free systems from rat brain and liver and rabbit reticulocytes lysate (Girbes et al. 1993a, b; de Benito et al. 1995, 1997, 1998; Citores et al. 1996a, 1997, 1998; Battelli et al. 1997a; Rojo et al. 1997). All RIPs assayed inhibit protein synthesis in rabbit reticulocyte lysates at similar concentrations to ricin (IC_{50} values of 0.15 and 0.10 nM for ebulin l and ricin, respectively; Table 2). By contrast, they do not inhibit protein synthesis in plant-derived cell-free systems such as *Vicia sativa* L., *Cucumis sativus* L. and *Triticum aestivum* L. nor in an *Escherichia coli* cell-free system (Girbes et al. 1993a, b; de Benito et al. 1995, 1997, 1998; Citores et al. 1996a, 1997, 1998; Battelli et al. 1997a; Rojo et al. 1997). Although all RIPs inhibit protein synthesis in mammalian systems, they differ in their potency; the most active being basic nigrin b (IC_{50} of 0.0003 nM in rabbit reticulocytes lysates) (de Benito et al. 1997), sieboldin b (IC_{50} of 0.015 nM) (Rojo et al. 1997), ebulin f, nigrins b, f, and l2 (IC_{50} values of 0.03 nM) (Girbes et al. 1993a; Citores et al. 1996a, 1998) and ebulin r2, nigrin l1 and basic racemosin b (IC_{50} values of 0.04 nM) (Citores et al. 1997; unpublished results). The less active are nigritin f2 (IC_{50} of 8.06 nM) (de Benito et al. 1998), the SNLRPs (IC_{50} of 5.74 nM) (Barbieri et al. 2004), SSA (IC_{50} of 16.4 nM) (Rojo et al. 1997) and SNAI′ (IC_{50} of 2.24 nM) (Van Damme et al. 1997b). RIPs have also been found to remove adenine from various polynucleotides other than rRNA; thus, they may be considered as polynucleotide:adenosine glycosidases (Barbieri et al. 1997). It has been reported that SNAI and SNLRP remove several adenines from ribosomes, herring sperm DNA, poly(A), and isolated ribosomal RNA (Battelli et al. 1997a). Nigrin b and basic nigrin b are able to multidepurinate genomic tobacco mosaic virus (TMV) RNA, which after acid aniline treatment undergoes complete degradation (de Benito et al. 1997).

In addition, SNLRP has been found to weakly multidepurinate TMV genomic RNA, whereas SNAI showed no activity (Vandenbussche et al. 2004). Some type 1 and 2 RIPs from *Sambucus* display other enzymatic activities. Nigritins f1 and f2 and basic nigrin b have topological activity turning supercoiled circular DNA into linear and relaxed circular DNA forms (de Benito et al. 1997, 1998). This activity has been described also for other RIPs, i.e., dianthin (Huang et al. 1992) and could be a consequence of an *N*-glycosidase activity on the DNA strand (Barbieri et al. 1997). Recent studies have suggested that RIPs are also capable of inducing cell death by apoptosis (Narayanan et al. 2005). Nigrin b, ebulin l, and SEA have been found to induce apoptosis by promoting DNA fragmentation in COLO 320 human colon adenocarcinoma cells at the same concentrations seen to be cytotoxic (Gayoso et al. 2005; Iglesias et al. 2010). Nonetheless, this does not mean that apoptosis would be a consequence of their direct interaction with DNA; therefore, the possibility that apoptosis might appear as a consequence of the inhibition of translation cannot be ruled out.

6 Toxicity to Cells and Animals

In contrast to the high enzymic activity on ribosomes of RIPs from *Sambucus*, the effects of these proteins on cultured animal cells were found to be much lower than in cell-free systems. All the type 2 RIPs from *Sambucus* show a considerable lower cytotoxicity than ricin and the other toxic type 2 RIPs (Table 2). The first studies carried out for the dimeric type 2 RIPs, nigrin b, and ebulin l, indicated that in contrast to ricin they display very low toxicity to HeLa cells (IC_{50} values of 0.00067, 27.6 and 64.3 nM for ricin, nigrin b, and ebulin l, respectively) (Table 2) and NHC human epithelial cells (Battelli et al. 1997b; Girbes et al. 1993a, b; Citores et al. 1996b). The study was extended to a broad variety of cancer cells in order to determine its potential suitability for the construction of immunotoxins for cancer therapy (Munoz et al. 2001). The studies showed that nigrin b was approximately 10^4–10^5 times less toxic than ricin in all cancer cells studied, with the exception of melanoma cells which are resistant to ricin. The tetrameric RIPs (SNAI, SEA, SSA) display low antiribosomal activities as compared with other RIPs from *Sambucus* and accordingly the proteins only inhibit protein synthesis in intact cells at very high concentrations (Battelli et al. 1997a; Van Damme et al. 1996b; Rojo et al. 1997) (Table 2).

The type 2 RIPs from *Sambucus* were described as nontoxic based on the differential in vivo toxicity with ricin (Girbes et al. 1993a, b). In mice, the LD_{50} of both nigrin b and ebulin l administered by intraperitoneal injection is 12 and 2 mg/kg body weight, respectively (Table 2) while the lethality of ricin is exerted at concentrations in the range of few microgram/kilogram (Battelli et al. 1997b). Intravenous injection of 16 mg/kg of nigrin b killed all the animals within 36 h after injection while ricin did so at 8 μg/kg (Gayoso et al. 2005). A histological study of the organs of mice treated with lethal doses of nigrin b revealed no

apparent signs of tissue damage except in the intestines, where very severe lesions were observed. Under these circumstances the villi and crypt structures disappear and lead to profuse bleeding and death (Gayoso et al. 2005). By contrast, at sublethal doses (5 mg/kg), full recovery of the mice was seen after approximately 9 days. One day after the administration of 5 mg/kg of nigrin b, the crypts were atrophied. Three days after the treatment, lesions in the small intestine began to recover in the crypts and the villi were edematous, with enterocytes displaying different degrees of necrosis and disruption of the covering epithelium (Gayoso et al. 2005). The precise mechanism of the injury is not known, but the result of nigrin b treatment is the apoptosis of cells present in the middle third of intestinal crypts of both the small and large bowel (Gayoso et al. 2005).

7 Interaction with Cells

Seminal work from Lord's group contributed to the clarification of the molecular mechanisms involved in ricin intracellular traffic (Lord et al. 2003, 2005).

Possible explanations for the low toxicity of nigrin b as compared with ricin in HeLa cells have been investigated studying the binding, uptake by cells, and intracellular routing and processing (Battelli et al. 1997b, 2004; Citores et al. 2003). Binding of these RIPs to glycoprotein receptors occurs prior to internalization and intracellular transport and it has been shown that ricin binds to HeLa cells to a greater extent than nigrin b (Battelli et al. 1997b, 2004). On the other hand, studies on the structure of ebulin l indicated that this protein binds to lactose–agarose affinity columns with much less affinity than ricin (Pascal et al. 2001). In addition, X-ray crystallography studies of ebulin l revealed changes in key amino acids of the sugar-binding 2γ subdomain (Pascal et al. 2001). This reduces the affinity of ebulin l for galactosides and therefore for galactose-containing glycoproteins or/and glycolipids present at the surface of the plasma membrane. Nonetheless, this is not the only explanation for its reduced cytotoxicity since volkensin bound to HeLa cells to the same extent as nigrin but was found to be extremely toxic (Battelli et al. 2004).

The high toxicity of ricin for mammalian cells is related to its ability to bind and to be transported to the endoplasmic reticulum via the so-called retrograde transport mechanism (Lord et al. 2005; Spooner et al. 2008). Ricin toxicity is sensitive to brefeldin A and to low temperature (Mayerhofer et al. 2009). In contrast to ricin, nigrin b, and ebulin l follow a pathway that is insensitive to brefeldin A and to temperatures below 37°C indicating that transport from endosomes to the Golgi complex is not required for nigrin and ebulin A-chain translocation (Battelli et al. 1997b; Citores et al. 2003). In fact, nigrin b was found to enter cells like ricin, but was more rapidly and extensively degraded and when excreted by HeLa cells the nigrin b-derived material was completely inactive (Battelli et al. 2004). In an attempt to explain the lack of cellular toxicity of nigrin b as compared with ricin, we formulated the hypothesis that the internalization of ricin and nigrin b might

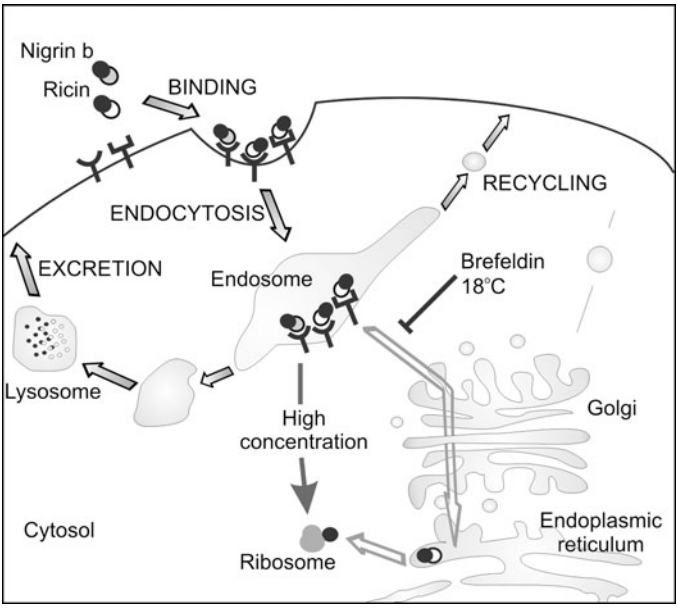

Fig. 3 Intracellular routing of nigrin b and ricin. Ricin binds to glycoproteins of the plasma membrane and internalize into the cell. Some protein molecules are recycled back to the plasma membrane, others undergo degradation in the lysosomes and excretion, and a small number are transported first to the Golgi network and then to the endoplasmic reticulum. In the endoplasmic reticulum, the disulphide bridge is reduced and the A chain translocates to the cytosol. In the cytosol, the A chain inactivates the ribosomes, inhibiting protein synthesis and causing cell death. This pathway is sensitive to low temperature and brefeldin A. Nigrin b can bind to different glycoproteins of the plasma membrane than ricin and internalizes into the cell. All the protein molecules are either recycled back to the plasma membrane or transported to lysosomes for degradation. This pathway is not sensitive to low temperature and brefeldin A

involve different receptors and therefore they could follow different intracellular pathways (Fig. 3). Some of the receptors would carry nigrin b or ricin as a receptor–RIP complex that is either recycled back to the plasma membrane or transported to lysosomes for degradation. This would not be a productive pathway for the internalization of type 2 RIPs. Other receptors would carry ricin and the related highly toxic type 2 RIPs (i.e., viscumin, abrin, modeccin, and volkensin, but not nigrin b) through the endosomal pathway and at some point it diverges to the *trans*-Golgi network. From there, the proteins are retrogradely transported to the endoplasmic reticulum, where temperature-dependent translocation of the A chain to the cytosol occurs (Mayerhofer et al. 2009). The cellular uptake of ricin by a combination of both putative internalization pathways could account for the results reported previously (Battelli et al. 1997b, 2004), namely, high cellular toxicity, and substantial degradation. Therefore, cell protein synthesis inhibition by nigrin b seems to be a consequence of the spontaneous translocation of nigrin b from the endosome when the extracellular concentration of RIP is high.

From the structural data, it has been argued that there is a correlation between the presence of a canonical lipase site and toxicity in type 2 RIPs (Morlon-Guyot et al. 2003). The lipase site is conformed with specific residues belonging to both subunits of the RIPs. The lipase active site of ricin, which has been suggested to have a role in the efficient ricin A-chain translocation and cytotoxicity, is absent in ebulin l. This could also help to explain the lower toxicity of this protein compared with ricin (Morlon-Guyot et al. 2003).

8 Phylogenetic Relationship Among the RIPs and Lectins from *Sambucus*

In order to explore the phylogenetic relationships among RIPs and lectins from *Sambucus*, the full-length sequences of the precursors of 20 of them were aligned using the ClustalW program (Thompson et al. 1994) and the resulting multiple alignment was submitted to Trace Suite II program to display a phylogenetic tree (Innis et al. 2000). The phylogenetic analysis supports a common two-chain gene ancestor for all these proteins (Fig. 4). The proteins from all the three *Sambucus* species tended to be grouped based on their putative structures rather than species relationship; for example, the tetrameric type 2 RIP from *S. nigra* (SNAI), SEA from *S. ebulus,* and SSA from *S. sieboldiana* were clustered together, while distanced from nigrin b, ebulin l, and sieboldin b from the same species in the phylogenetic tree. These facts imply that the ancestral *Sambucus* RIP gene existed as a single gene in the ancestral lineage, and duplications of the type 2 RIP gene occurred prior to the divergence of *S. nigra*, *S. sieboldiana,* and *S. ebulus*. Therefore, these proteins evolved from a small number of ancestral genes that have undergone multiple events of gene duplication and excisions.

The phylogenetic tree shows two major clades (Fig. 4). The upper clade contains both heterodimeric (SNLRPs and SNAI′) and tetrameric type 2 RIPs (SNAI, SNAIf, SEA, and SSA). Mutations in the ancestral gene probably resulted in changes in sugar-binding specificity and lead to proteins of the first clade which are Neu5Ac(α-2,6)Gal/GalNAc-specific lectins with the exception of SNLRPs which contain a B chain that is devoid of carbohydrate-binding activity. The tetrameric type 2 RIPs contain an extra cysteine involved in the intermolecular disulphide bridge of the B chains not found in SNLRPs and SNAI′.

The type 2 RIP ancestral gene gave rise to another clade grouping all the Gal/GalNAc-specific proteins which can be subdivided in two groups. One group contains homodimeric lectins most probably formed by excision of almost the complete A domain and characterized by the presence of an extra cysteine, responsible of the dimerization through a disulphide bridge. This cysteine residue differs from that involved in the dimerization of the B chain of the tetrameric lectins, SNAI, SNAIf, SSA, and SEA (Fig. 1b). The second cluster grouped heterodimeric type 2 RIPs together with monomeric lectins. These monomeric lectins could be

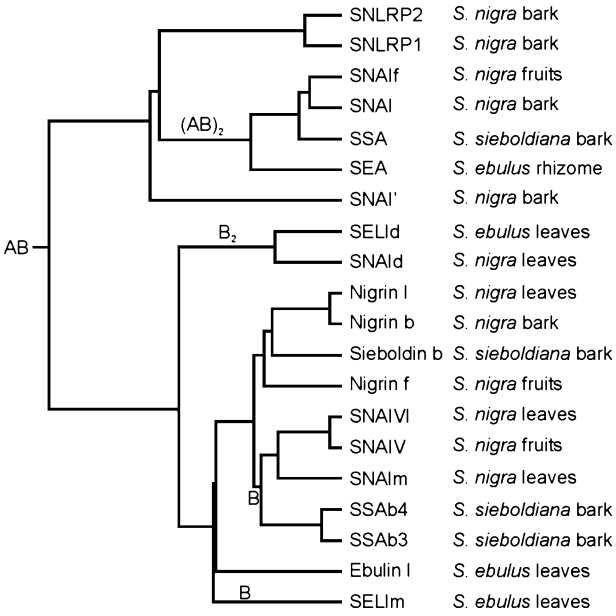

Fig 4 Phylogenetic analysis of the amino acid sequence of the type 2 RIPs and lectins from *Sambucus*. Alignment of the sequences was done using ClustalW (Thompson et al. 1994) and the phylogenetic tree using Trace Suite II (Innis et al. 2000). Names of proteins with protein GenBank accession numbers given in parenthesis are as follows: SNLRP1 (AAC49673); SNLRP2 (AAC49672); SSA (D25317); SNAI (AAC49158); SNAIf (AAC49989); SEA (AM981401); SNAI′ (ABB83821); SNAId (AAN86131); SELld (AAM94880); SSA-b-3 (BAD93342); SSA-b-4 (BAD93343); Nigrin l (AAN86130); Nigrin b (SNAV) (AAB39475, P33183); SNAlm (AAN86132); SNAIV (AAC15885); SNAIV-l (AAL04122); Nigrin f (SNAVf) (AAC15886); Sieboldin b (Rojo et al. 2003a); SELlm (CAM33270); Ebulin l (CAC33178). $(AB)_2$, B_2, and B indicate the branch of the phylogenetic tree where mutations took place to give rise to the tetrameric, dimeric, and monomeric proteins, respectively

encoded by a truncated type 2 RIP gene which lost a substantial part, if not all, coding for the A chain and therefore lost its enzymic activity. Among the heterodimeric type 2 RIPs from *Sambucus*, ebulin l appears the most distant from the other RIPs nigrins b, l and f, and sieboldin.

To date, there are no sequence data available on type 1 RIPs from *S. ebulus* (ebulitins α, β, and γ) because the N-terminals of the proteins studied are blocked (de Benito et al. 1995). Amino acid composition of these proteins indicates that ebulitins β and γ are quite similar and relatively different from ebulitin α (de Benito et al. 1995). Nonetheless, the known N-terminal sequences of nigritins, the type 1 RIPs from *S. nigra* fruits, reveal no sequence homology with other known RIPs nor other proteins (de Benito et al. 1998). Regarding the RIPs from other families, all the RIPs have a common origin, regardless of whether they are type 1 or type 2 RIPs (Girbes et al. 2004). In fact, the A chains of type 2 RIPs from *Sambucaceae* are more related to the type 1 RIPs from *Cucurbitaceae* than to the type 2 RIPs from

Euphorbiaceae, *Fabaceae,* and *Viscaceae*. This suggests that the type 1 RIPs may be formed from type 2 RIPs by a deletion of their B chain.

9 Uses of the RIPs and Lectins From *Sambucus*

Interest in RIPs has increased in recent years because of their use as the toxic moieties of conjugates and immunotoxins for the target therapy of important diseases. Ricin has been the most used RIP for the construction of conjugates and immunotoxins targeting cancer cells (Kreitman 2006; Kreitman and Pastan 2006; Pastan et al. 2007). Immunotoxins with the ricin holoenzyme, its A chain, and the ricin holoenzyme with the sugar-binding domains blocked have been constructed (Girbes et al. 2003; Lambert et al. 1991). Perhaps, the most undesirable feature of ricin is its unspecific toxicity. Nonetheless, since blocked ricin is less toxic than the intact ricin, holoenzyme immunotoxins bearing blocked ricin have been assayed in several clinical trials (Grossbard et al. 1998, 1999; Szatrowski et al. 2003). Nigrin b and ebulin l are 10^3–10^5 times less toxic in cultured cells and mice than ricin. The lack of toxicity of type 2 RIPs from *Sambucus* resemble those of blocked ricin and make them excellent candidates as toxic moieties in the construction of immunotoxins and conjugates directed against specific targets.

Nigrin b and ebulin l have been used in the construction of conjugates containing transferrin to target TfR-overexpressing cancer cells, which were shown to be highly active to HeLa cells (Citores et al. 2002). A useful approach for antitumor therapy is to target tumor neovasculature which nourishes tumor cells using antibodies to the endothelial receptor CD105 (endoglin). Nigrin b and ebulin l have been used to construct immunotoxins containing antiCD105 which are very active on CD105+ cells (Munoz et al. 2001, 2007; Benitez et al. 2005). Furthermore, conjugates containing nigrin b and the dimeric lectin SELld exerted strong cytotoxicity on COLO and Hela cells (Benitez et al. 2004).

Some type 2 RIPs purified from *S. nigra* (SNAIf, SNAI′) have been shown to increase resistance against virus and other parasites in transgenic tobacco plants. The type 2 RIP SNAI′ (Chen et al. 2002a, b), but not SNAIf (Chen et al. 2002c), exhibits in planta antiviral activity in transgenic tobacco. On the other hand, the Neu5Ac(α-2,6)Gal/GalNac-specific type 2 RIPs SNAI and SNAI′ expressed in transgenic tobacco plants have been reported to promote toxic effects when ingested by insects (Shahidi-Noghabi et al. 2008, 2009). Carbohydrate-binding activity of SNAI is necessary for its insecticidal activity (Shahidi-Noghabi et al. 2008).

Interest in lectins has increased as they are highly valuable reagents for the investigation of cell surface sugars (Sharon 2007). On the basis of their Neu5Ac (α-2,6)Gal/GalNac-specificity, the type 2 RIPs SNAI and SSA have been used to detect different expression of glycoproteins carrying α-(2-6)-galactose binding sites (Murayama et al. 1997; Tsokos et al. 2002; Brooks et al. 2001). Besides, the lectin SNAII that preferentially recognizes Gal and GalNAc has also been found to

recognize Gal-related saccharides on the carcinoma Tn epitope (Ser-O-GalNAc) that is a specific human tumor-associated determinant antigen (Maveyraud et al. 2009).

Elderberry plants have been used for food and as herbal medicine for centuries. Almost all the *Sambucus* tissues contain RIPs and lectins that could resist proteolysis and survive passage through the alimentary tract in an active form. In insects, some lectins display toxicity upon ingestion and processing (Shahidi-Noghabi et al. 2008, 2009). In mice, orally ingested lectins can interact with the glycosylated gut surface and trigger complex signals to the gut wall cells, ranging from hyperplasia of the small bowel epithelium, interfering with nutrient absorption (Pusztai et al. 1990), to growth inhibition (Pusztai et al. 2008). The monomeric lectin SNAII has been shown to be a powerful growth factor in the rat small intestine, while SNAI induces changes in receptor expression in the gut (Pusztai et al. 1990).

Since the intestinal intravenous injection of sublethal doses of nigrin b exerted a specific damage on the small intestine, both to crypts and villi (Gayoso et al. 2005), we propose nigrin b sublethal effects as a model for gut derangement and recovery, which would allow studies on epithelial-mediated processes, such as tumor appearance and the absorption of drugs and nutrients. This model might offer a complementary alternative to chemically induced mouse models that use aggressive chemicals to promote intestinal inflammation and derangement (Wirtz et al. 2007).

Acknowledgments This work was supported by grants from the Junta de Castilla y León (VA0150A7, GR106 and Consejería de Sanidad) and UVA-GIR funding to T.G and FISPI04/1279 to J.M.F. We thank Judy Callaghan for correcting the manuscript.

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Ribosome-Inactivating Proteins from *Abrus pulchellus*

Ana Paula Ulian Araújo, Priscila Vasques Castilho, and Leandro Seiji Goto

Abstract Pulchellins are highly toxic type 2 ribosome-inactivating proteins (RIPs) expressed in the seeds of *Abrus pulchellus tenuiflorus*. Four pulchellin isoforms have been characterized, allowing their classification into two subgroups based on the toxicity levels and sugar-binding specificity. The residues involved in the catalytic mechanism are all conserved amongst the pulchellins, suggesting that the differential toxicity is related to variations in their B-chain behavior. In this chapter, insights into the successful production of active A- and B-chains via heterologous production in *Escherichia coli* and their assembly in vitro to produce an active heterodimer are discussed in some detail. Additionally, some features of subcellular sorting of native pulchellins are presented. Pulchellin comparative studies have contributed to the knowledge of the molecular bases of biochemical processes, such as sugar binding and toxicity, observed among RIPs.

1 Introduction

The ribosome-inactivating proteins (RIPs) belong to a class of enzymes (EC 3.2.2.22) widely distributed among plants, fungi, algae, and bacteria (Girbés et al. 2004). Members of this group play a role in the history of clinical medicine and biomedical research (reviewed in Olsnes (2004)). These proteins exhibit rRNA *N*-glycosidase activity, which leads to the excision of a specific adenine residue from a conserved loop of the large rRNA (Endo and Tsurugi 1987). The latter effect is irreversible and prevents the association of the elongation factors with the 60S ribosomal subunit, resulting in the inhibition of protein synthesis and subsequent cell death (Hartley et al. 1991). Moreover, it has been reported that some RIPs

A.P.U. Araújo (✉), P.V. Castilho, and L.S. Goto
Instituto de Física de São Carlos, Universidade de São Paulo, P.O. Box 369, 13560-970, São Carlos SP, Brazil
e-mail: anapaula@ifsc.usp.br

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_7,

possess additional enzymatic activities. These include phosphatase activity on lipids, as well as chitinase, DNase, and superoxide dismutase activities (Li et al. 1996; Shih et al. 1997; Helmy et al. 1999; Wang and Tumer 2000).

The vast majority of RIPs are divided into two groups, which can be distinguished according to the absence (type 1 RIPs) or presence (type 2 RIPs) of a lectin chain (the B-chain), which is linked to the toxic chain (A-chain) by a disulfide bond (VanDamme et al. 1998; Peumans et al. 2001). The A-chain exhibits the toxic rRNA *N*-glycosidase activity (Olsnes and Pihl 1973a; Endo and Tsurugi 1987). The lectin activity of the B-chain is targeted toward specific carbohydrate moieties on the mammalian cell surface (Peumans and van Damme 1995; VanDamme et al. 1998) and mediates the entry of the A-chain (Olsnes and Pihl 1973b; Endo and Tsurugi 1987). The absence of the lectin chain significantly limits the access of type 1 RIPs into cells, resulting in lower cytotoxicity levels. However, the presence of the B-chain alone is not enough to guarantee high levels of cytotoxicity to type 2 RIPs (Stirpe and Battelli 2006).

Most plant RIPs have been found in a small number of families, namely Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, Phytolaccaceae, and Poaceae (reviewed in Girbés et al. (2004)). Within the Leguminosae-Papilionoideae family, in particular, type 2 RIPs have been found and characterized in seeds of only two species, both of the genus *Abrus*: *Abrus precatorius* and *Abrus pulchellus tenuiflorus*. The *Abrus* genus is composed of about 15 species, being mostly found in tropical and subtropical areas in Asia, Africa, and South America (Parrotta 2001). Contrasting with the well-known seeds of *A. precatorius*, which exhibit a glossy red coloration with a black spot, *A. pulchellus tenuiflorus* seeds are thinner and brownish (Fig. 1).

Pulchellins are extracted from the seeds of *A. pulchellus tenuiflorus* and exhibit specificity for galactose and galactose-containing structures. This lectin property causes the agglutination of human and rabbit erythrocytes. Additionally, their toxic property kills mice when injected intraperitoneally (Ramos et al. 1998; Silva et al. 2005). In keeping with other type 2 RIPs, pulchellins are heterodimeric proteins composed of a toxic A-chain (29 kDa) in conjunction with a lectin B-chain (31.5 kDa), and are also highly toxic proteins (Ramos et al. 1998; Silva et al. 2005; Castilho et al. 2008).

Seeds of *Abrus pulchellus* present several pulchellin isoforms and four of them have already been isolated and few aspects of their sugar-binding properties have been investigated in detail (Castilho et al. 2008). All pulchellins are synthesized as precursor forms including an N-terminal presequence and a short intervening linker peptide joining the A- and B-chains, both of which are removed during protein maturation. The presequence, as observed in other type 2 RIPs, contains an endoplasmic reticulum targeting signal peptide that directs the proteins to the secretory pathway (Castilho et al. 2008). As observed by Jolliffe et al. (2003) for proricin, propulchellins possess similar intervening linker peptides (Castilho et al. 2008) which possibly contain a vacuolar targeting signal (Nielsen and Boston 2001).

Regarding cell entry during the intoxication process, subcellular sorting in mammalian cells reveals that the endosomal internalization pathway, as well as

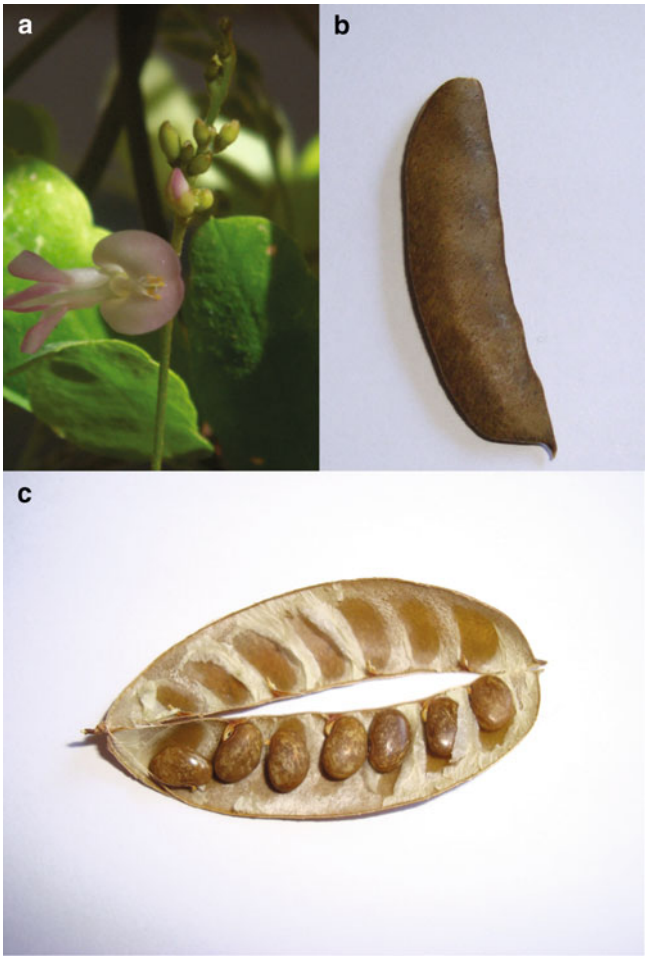

Fig. 1 *Abrus pulchellus tenuiflorus*. Plant details showing: (**a**) a close-up view of the flower; (**b**) a mature fruit; and (**c**) an open fruit showing the seeds. (Photos **a**, **b**, and **c** are not to scale)

retrograde transport through the Golgi apparatus, might be used by both native protein and single pulchellin B-chains.

In order to obtain an alternative source of these holotoxins, other than their direct extraction from seeds, pulchellin synthesis from callus culture has been investigated (Silva et al. 2003) which represents a convenient system of production for biotechnological applications. Callus culture from immature seeds of *A. pulchellus* is able to express pulchellins and their levels of expression are similar to those described for other RIPs (D'Silva et al. 1993; Silva et al. 2003). Although the isolation of pulchellins from callus culture is possible, extraction from seed cotyledons is more efficient, probably because of the higher percentages of protein present in the latter.

The heterologous expression of type 2 RIPs in *Escherichia coli* is an attractive strategy. However, pulchellin is synthesized as a single polypeptide precursor that must be cleaved in order to yield the independent, mature protein chains. Thus, the use of a prokaryotic expression system, such as bacteria, suffers from the shortcoming of having to produce the A- and B-chains separately (although in studies which focus on either one of the chains in isolation, this could be advantageous). Another major difficulty is the empirical refolding process typically required for B-chains.

2 Pulchellin Isoforms

Plants usually produce several RIP isoforms that show variation in their physical and therefore biological properties. RIP isoforms may be present in one or more plant tissues (Stirpe and Battelli 2006) and/or in a season-dependent fashion (Stirpe et al. 1992); however, the reason for such wide heterogeneity remains unclear. Several isoforms were found in the seeds of *A. pulchellus* and four of them, named P I, P II, P III, and P IV, have been isolated and studied in some detail (Castilho et al. 2008).

The pulchellins exhibit isoelectric point between 5.2 and 5.8 and possess subtle differences in their migration pattern on SDS-PAGE. Recently, biochemical characterization has been performed to investigate both their toxic and lectin-like properties. The addition of pulchellin isoforms to cultures of HeLa cells resulted in a high inhibition of protein synthesis. The half-maximal inhibitory concentration (IC_{50}) values showed that P I and P II have similar cytotoxicity (21.7 ng/ml (0.375 nM), and 22.7 ng/ml (0.391 nM), respectively) and are approximately fivefold more potent then P III and P IV (101.9 ng/ml (1.76 nM) and 98.4 ng/ml (1.7 nM), respectively). In agreement, median lethal doses (LD_{50}) in mice pointed at P II as the most toxic isoform (15 μg/kg), followed by P I (25 μg/kg), P IV (60 μg/kg), and P III (70 μg/kg). According to the toxicity values, one can conclude that although the pulchellin isoforms are less toxic than the two well-known RIPs, ricin (IC_{50} = 0.001 nM and LD_{50} = 2.6 μg/kg) (Olsnes and Pihl 1973a) and abrin (IC_{50} 0.0037 = nM and LD_{50} 0.56 = μg/kg) (Olsnes and Pihl 1973b), they are still highly toxic proteins.

The difference in the cytotoxicity values (IC_{50}) shown by pulchellin isoforms and those by abrin and ricin is still controversial because the protein samples used in the pulchellin assays were lyophilized. When the analyses were carried out with fresh samples, higher cytotoxicity values were obtained (unpublished data). This suggests that lyophilization of pulchellins should be avoided as the resuspension of dry protein probably leads to heterogeneity in the structure and activity of the protein samples.

Regarding the lectin properties of pulchellins, even though all isoforms are capable of binding to galactosides, the mechanisms of sugar interaction may vary. Haemagglutination inhibition assays on proteins, preincubated with several serially diluted sugars, showed that whereas agglutination was inhibited by

galactose and its derivatives (such as *N*-acetylgalactosamine (GalNAc), methyl-α-D-galactopyranoside), it was evident that at doses up to 100 mM, glucose, mannose, α-methylmannoside, fucose, maltose, xylose, and saccharose did not inhibit agglutination at all. The failure to bind to these sugars implies that an axial hydroxyl group at C4 is crucial and that a reversed configuration at this position might prevent sugar recognition. Besides, the haemagglutination inhibition caused by methyl-α-D-galactopyranoside suggests that the OH group on C2, C3, and C4, which displays the same configuration as those in galactose and lactose, is responsible for the strong interaction between this sugar and the four isoforms. Interestingly, P II is the only isoform with affinity for rhamnose, lacking the galactose and/or *N*-acetylgalactosamine specificity that is a characteristic feature of the archetypal type 2 RIP, for which there are few exceptions.

The most relevant difference regarding pulchellin–sugar interaction is related to the ability to bind GalNAc. For example, cytotoxicity assays, in which the four pulchellins were preincubated or not with free GalNAc, revealed that for P I and P II increased levels of cellular protein synthesis were seen as the concentration of premixed GalNAc was increased (Fig. 6). However, in contrast with the rescue of protein synthesis observed for P I and P II, GalNAc protection against P III and P IV was only marginal, even when toxin was pretreated with 100 mM GalNAc. That is, pulchellin isoforms P I and P II exhibit a remarkable difference in lectin-like properties compared with P III and P IV because in the presence of this sugar, both haemagglutination and cytotoxicity are differently inhibited. Moreover, these data suggest that the binding and uptake of P III and P IV into cells might not be dependent on receptors containing GalNAc.

On the basis of their toxicity and sugar-binding specificity, therefore, the four pulchellins can be roughly divided into two subgroups: P I and P II, which are more toxic and capable of binding to GalNAc; and P III and P IV, which are less toxic and incapable (or much less capable) of binding to GalNAc. The amino acid sequence alignment of the four isoforms (deduced from cDNA cloning and confirmed by mass spectrometry analysis) shows that the residues of the catalytic site are all conserved. In addition, the highest similarity within each subgroup is in the second domain of the B-chain (see alignment in Castilho et al. (2008)). These findings suggested that what dictates the different levels of toxicity for the two subgroups is the variation seen in the B-chain. That is, the two subgroups might interact with different binding sites on the cell surface, which might at least partially explain the differences in toxicity, given that the active site on the A-chain is conserved in both subgroups.

3 The Heterologous Expression of Pulchellins

Expression in *E. coli* of type 2 RIPs is an attractive strategy, since it may represent an unlimited source of protein (some RIPs are present in a seasonal dependent manner). Additionally, problems regarding differences on the expression patterns – which often occurs and may influence the reproducibility/reliability of the studies – may be overcome.

3.1 The Pulchellin A-Chain

In the 1990s, cloning of several type 2 RIPs, such as ricin and abrin, had already been obtained in several research laboratories (Wood et al. 1991; Roberts et al. 1992; Hung et al. 1994). Analysis of these genes showed that these RIPs belong to multigene families and had no introns. The latter information, in conjunction to the high sequence identity between abrin and ricin allowed the genomic cloning of prepulchellin A-chain (Silva et al. 2005).

In contrast to the recombinant production of the pulchellin B-chain (discussed latter), the A-chain is soluble when overexpressed as a GST-fusion protein in *E. coli* (Silva et al. 2005). The recombinant protein is able to depurinate rRNA in vitro releasing the Endo's fragment (defined below) after acid aniline treatment, as does native, reduced pulchellin, confirming that it was enzymatically active (Silva et al. 2005). It should be recalled that the evaluation of RIPs' enzymatic activity is based on RIP-mediated depurination of the large ribosomal subunit, which results in a susceptibility of the RNA to hydrolysis at the depurination site (Endo et al. 1987). This action leads to the release of a small fragment of nucleotides from the 3′-end of the rRNA (Endo's fragment).

It is known that the recombinant A-chain lacks the lectin-like activity. An effective method to check its toxic activity in cells or animals is via a reassembly the holotoxin by association of the single chains in vitro. Obviously, the protocols for the heterologous production of the B-chain or for its isolation from natural sources must already have been determined. The reconstruction of recombinant heterodimer of pulchellin has been shown to be feasible, and its resultant toxicity is comparable to that of the native molecule when injected into mice (Fig. 2, Silva et al. 2005). Without doubt, the toxic activity itself is accounted for solely by the A-chain. However, the full pulchellin intoxication pathway is clearly dependent on B-chain shuttling into mammalian cells, since neither isolated recombinant A- nor B-chains showed lethality in mice after intraperitoneal injection (Silva et al. 2005). Analogous reassociation of the A- and B-chains was also performed by Eck et al. (1999) for mistletoe lectin. The recombinant A-chain produced by this heterologous system is active and available for the preparation of toxic moieties of conjugates and immunotoxins with great potential as therapeutic agents.

3.2 The Pulchellin B-Chain

Only few studies involving isolated type 2 RIPs B-chains from recombinant expression sources have been reported (Tonevitsky et al. 1994; Pevzner et al. 2005; Chambery et al. 2007). This might be due to the difficulty in overexpressing RIP B-chain genes in *E. coli*. Pulchellin B-chain, which shares 81% sequential identity within the abrin-a isoform and up to 58% identity to ricin (including all key residues for proper folding and function), comes from a refolding process from

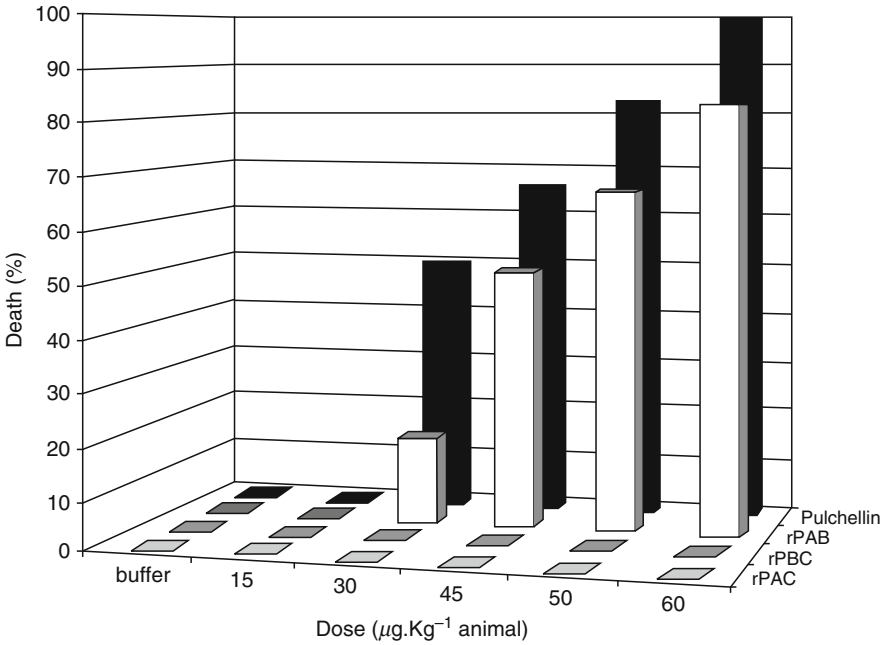

Fig. 2 Lethal activity determined by intraperitoneal injection in mice. Recombinant pulchellin A-chain (rPAC), recombinant pulchellin B-chain (rPBC), recombinant holotoxin pulchellin (rPAB), and native pulchellin were injected at the doses indicated. The buffers of each protein were used as negative control. Groups of six animals were used per dose of each protein preparation. The toxic effects were determined after 48 h. (Figure from Silva et al. 2005)

insoluble inclusion bodies (Goto et al. 2003) which may be considered a relatively difficult process. Although one might imagine that the refolding of the pulchellin B-chain may serve as a guideline for how to refold any similar type 2 RIP B-chain, the process of refolding may be affected by several factors (Maachupalli-Reddy et al. 1997; Tsumoto et al. 2003), many of which are empirical. Consequently, the pulchellin refolding protocol might not be generally applicable to every type 2 RIP B-chain. For the best results, it should be adjusted for each particular case. The problem of protein refolding is beyond the scope of the work discussed here, and there are plenty of refolding protocols available (Zardeneta and Horowitz 1994; Hashimoto et al. 1998; St John et al. 1999; Rariy and Klibanov 1999; Gu et al. 2001; Clark 2001; Tsumoto et al. 2003). What is expected to be true for most (if not all) type 2 RIP B-chains is that they are produced as insoluble inclusion bodies if overexpressed in a cytosolic *E. coli* system. This is due to the intrinsic nature of type 2 RIP B-chain structure which contains multiple disulfide bonds (Lilie et al. 1998; Kadokura et al. 2003). Attempts to obtain active recombinant type 2 RIP B-chains should focus on refolding protocols or changing the host expression system (Wales et al. 1991; Frankel et al. 1994; Sehnke et al. 1994; Sphyris et al. 1995; Frigerio et al. 1998; Chamberlain et al. 2008).

The refolding of recombinant pulchellin B-chain has been shown to be feasible and some characterization has been performed. The recombinant lectin chain was isolated as a monomeric polypeptide (free of a carrier peptide). Circular dichroism measurements were in agreement with the expected β-sheet-rich structure. Haemagglutination activity and inhibition of haemagglutination by the addition of D-galactose corroborated the expected lectin properties (Goto et al. 2003). The recombinant pulchellin heterodimer, reconstituted from individual recombinant expression of both chains, showed a very close median lethal dose (LD_{50}) in mice (45 μg/kg) to that found for a native toxin extract (30 μg/kg) (Silva et al. 2005).

One interesting point coming from the in vitro assembly of the holotoxin was the verification that the heterodimer retained characteristics very similar to those of the native proteins. Reconstituted pulchellin showed very similar toxicity, molecular masses, and CD spectra to that of the native protein (Figs. 2 and 3) (Silva et al. 2005). It is noted that the pulchellin B-chain, responsible for the holotoxin cell uptake, is harmless (at least for the test systems used so far) when administrated to living subjects. One potential advantage of the recombinant pulchellin B-chain over the native B-chain is that the former was free of glycans which are known to affect the applicability of other native RIPs for therapeutic purposes (Blakey and Thorpe 1988;

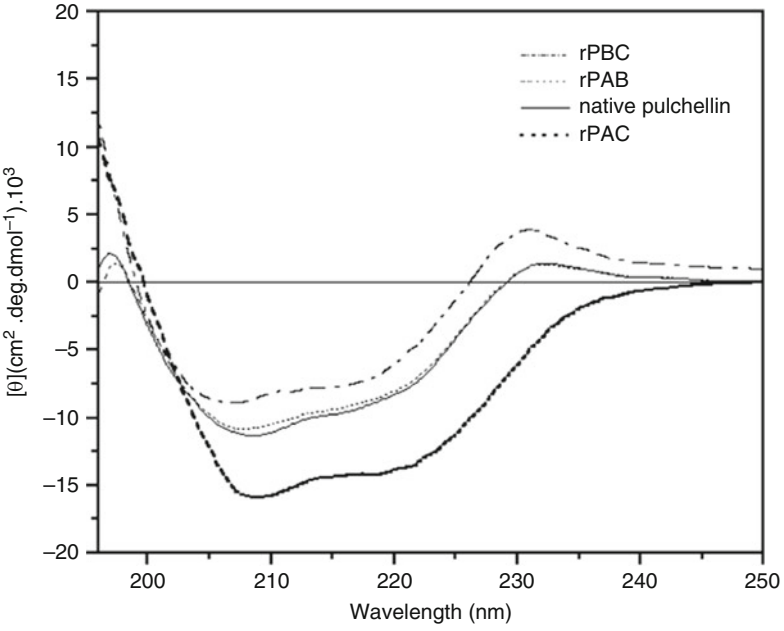

Fig. 3 CD spectra of recombinant pulchellin A-chain (rPAC), recombinant pulchellin B-chain (rPBC), recombinant pulchellin (rPAB), and native pulchellin. The spectra were obtained from each protein at a concentration of 0.3 mg/ml in 20 mM Tris–HCl, pH 8.0. The measurements were performed using quartz cuvettes of 1 mm path length and recorded from 195 to 250 nm as the average of 16 scans at 25°C. (Figure from Silva et al. 2005)

Jansen et al. 1992). The method used for coupling the recombinant chains results from the recombinant B-chain retaining a useful free cysteine residue (most likely the one nearest to the N-terminus) which may be available for coupling to other type of molecules.

4 Pulchellin Endocytosis in Mammalian Cells

The pulchellin B-chain could be used as a targeting molecule mediating cell uptake. This is based on the cell specificity displayed by recombinant pulchellin B-chain, a result of its lectin-like properties which guide it preferentially to given cell types via specific recognition of their carbohydrate composition. Indeed, recombinant pulchellin B-chain showed preferential cell type discrimination, leading to adhesion of MDA-MB-231 (human breast cancer) and K-562 (human bone marrow leukemia cancer) cell lines. This effect was not observed for mice fibroblasts (ATCC CCL-1.3) and human cervix adenocarcinoma (ATCC CCL-2) cells (Goto et al. 2007). On the other hand, native pulchellin, which includes a mixture of isoforms with different properties (Castilho et al. 2008), is able to interact with HeLa cells (ATCC CCL-2), in contrast to the recombinant refolded pulchellin B-chain, as the latter represent only one isoform that is unable to recognize external carbohydrate structure of HeLa cells. A series of chromatographic and surface plasmon resonance experiments has shown that native pulchellins have, besides β-D-galactose, remarkable affinity also for lactose, *N*-acetylgalactosamine and lacto-*N*-biose structures (Ramos et al. 2001).

Endocytosis of pulchellin B-chain should follow a very similar route to that described for other RIPs (Hartley and Lord 2004) as, in fact, this has been shown by confocal laser scanning microscopy subcellular localization experiments (Goto et al. 2007). Recombinant pulchellin B-chain (and also native pulchellin) were both found to accumulate in the region of the Golgi apparatus (Fig. 4), as would be expected if the endocytosis-ERAD retrograde transport mechanism is being used as has been claimed for all type 2 RIPs endocytosis/intoxication pathways (Hartley and Lord 2004; Roberts and Smith 2004).

Despite their similarity, a point of interest comes from the great variation in toxicity found within RIPs, which cannot be accounted for by structural variations or enzymatic activities of the A-chains (Barbieri et al. 2004). This is probably due to differences in B-chains, which may be more or less effective in the intoxication process (Svinth et al. 1998; Barbieri et al. 2004). In this respect, it should be pointed out that the LD_{50} in mice of around 45 μg/kg indicates that the recombinant pulchellin B-chain is a very effective RIP lectin chain, even without its natively attached carbohydrate which could account for an additional form of interaction important for cell entry (Simmons et al. 1986, Magnusson et al. 1993). The reasons for this come from structural features particular to this RIP B-chain and are subjects of current investigation.

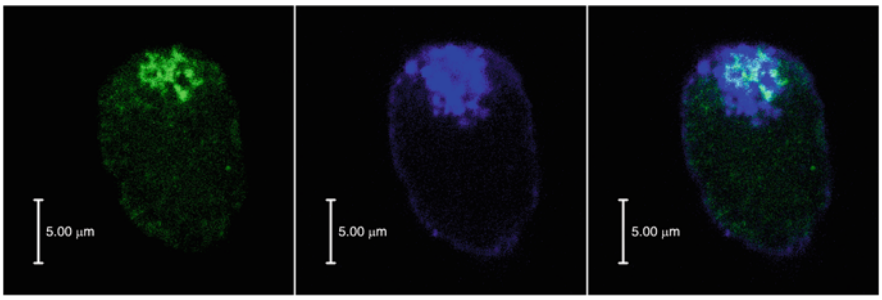

Fig. 4 Subcellular localization of pulchellin by confocal laser scanning microscopy. (**a**) K-562 cell fluorescently immunostained in *green* for golgin, a *trans*-Golgi protein. (**b**) Endocytosed fluorescently labeled pulchellin localization (*in blue*). (**c**) Overlaid images showing that pulchellin accumulated in the Golgi apparatus region (modified from Goto et al. (2007))

5 Structure of Pulchellin

The structure of pulchellin isoform P II (see below) was solved by X-ray crystallography (Fig. 5 (Navarro et al. unpublished)). Overall, the pulchellin fold resembles the structures of the well-known type 2 RIPs abrin, ricin, and mistletoe RIP, as indicated by the root-mean-square deviation calculated for the structurally equivalent Cα atoms (0.718, 1.131, and 1.031 Å for abrin, ricin, and mistletoe I, respectively). As expected, the structure is divided into two chains (the catalytic A-chain and the lectin B-chain) connected by a covalent disulfide bond between the Cys residue at the C-terminus of the A-chain and the Cys residue at the N-terminus of the B-chain (Cys240 and Cys263 respectively; numbering as Castilho et al. (2008)).

The catalytic A-chain (composed of 248 residues) possesses a globular fold and the *N*-glycosidase active site is located at the interface of three defined domains: the A1 domain, formed by the first 107 residues and mainly consisting of a six-stranded β-sheet and two α-helices; the A2 domain, formed by 86 residues organized into five α-helices, being the most conserved region of the A-chain (Bagaria et al. 2006); and the A3 domain, formed by 55 residues at the C-terminus organized as two α-helices, two antiparallel β-sheet strands and an unstructured coil (Fig. 5). All residues (Asn72, Tyr74, Tyr113, Arg124, Gln160, Glu164, Arg167, Glu195, Asn196, Trp198) involved in the catalytic mechanism of type 2 RIPs (Tahirov et al. 1995) are conserved in pulchellin.

The B-chains of type 2 RIPs fold into two globular domains, each one with at least one carbohydrate-binding site, which result in the agglutinating properties of the lectin chain (Rutenber et al. 1987; Sphyris et al. 1995). The B-chain of all the type 2 RIPs is thought to have emerged from a series of gene duplications, as each of the two globular domains seem to be built up from three similar ancestral peptides, named α, β, and γ (Rutenber et al. 1987; Rutenber and Robertus 1991).

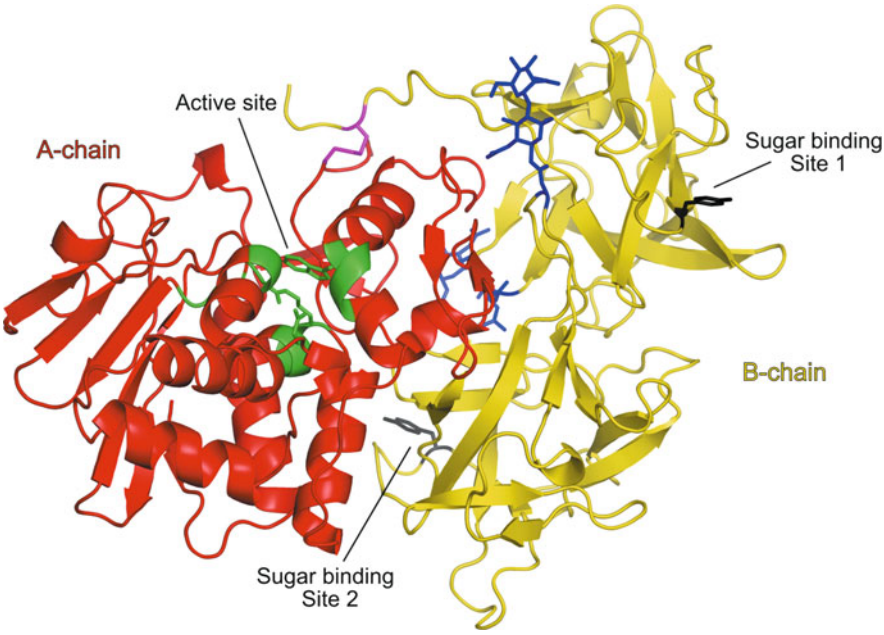

Fig. 5 Crystal structure of Pulchellin (isoform P II). Cartoon representation of the A-chain (*red*) and B-chain (*yellow*). The active site region is highlighted in *green* and the two conserved aromatic residues, which provide the binding platform for the sugar recognition, in *black*. The two glycosylations found in the B-chain are in *blue* and the disulfide bond connecting the catalytic and lectin chains is in *magenta*

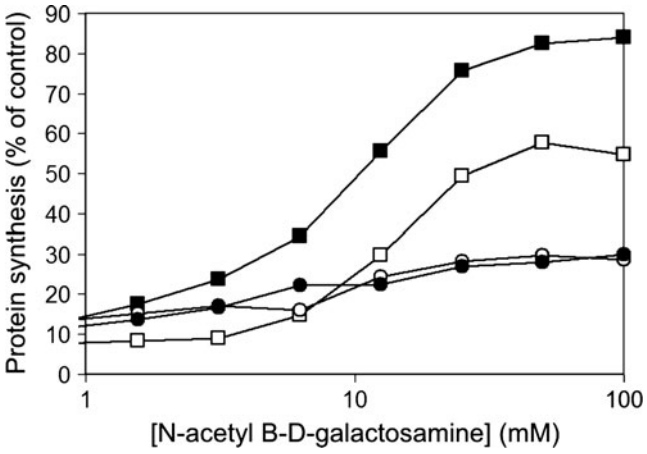

Fig. 6 Competition of pulchellin entry by *N*-acetyl-D-galactosamine. A single dose of toxin (200 ng/ml P I and P II or 800 ng/ml P III and P IV), previously shown capable of inhibiting 90% protein synthesis within 4 h, was used in all preincubations. Each toxin was mixed with increasing concentrations of GalNAc in DMEM/FCS for 30 min at 37°C. The mixtures were added to cells for 4 h and remaining protein synthesis determined as detailed in the Castilho et al. 2008. (Figure extracted from Castilho et al. 2008) (*open square*) P I; (*filled square*) P II; (*open circle*) P III; (*filled circle*) P IV

The pulchellin lectin chain, like other B subunits of type 2 RIPs, contains 256 amino acids arranged into two subdomains well conserved amongst this protein family, exclusively constituted of β-sheet regions interconnected by coiled structural elements. These subdomains contain the two galactose-binding sites. All the residues constituting the abrin B-chain carbohydrate-binding site 1 are conserved in the pulchellin B-chain: Asp274, Ile287, Tyr289, Asn298, and Gln299. Similarly, the residues of the abrin B-chain galactose-binding site 2 are conserved in pulchellin: Asp486, Ile498, Tyr500, Asn508, and Gln509. In both sites, the only one exception is the replacement of the aromatic residue that serves as the platform for sugar-binding (a Trp in abrin is replaced by Tyr in pulchellin). As in all type 2 RIPs, pulchellin also possesses *N*-glycans, and the crystal structure revealed that two (B-chain Asn347 and Asn387) out of the three predicted sites (Castilho et al. 2008) are indeed glycosylated.

6 Conclusion

Pulchellins have been described only recently. They are members of the highly toxic type 2 RIP family. Seeds of *A. pulchellus* present several different isoforms and four of them (PI, PII, PIII, and PIV) have already been isolated and studied in some detail. The toxicity values exhibited by the isoforms showed that they are less toxic than ricin and abrin, but nevertheless they are still highly toxic proteins. P I and P II are the most toxic isoforms and they are able to bind GalNAc; P III and P IV are about five times less toxic and not capable of binding to GalNAc. Taken together, an analysis of the sequence alignments and experimental results suggests that minor changes to the sugar-binding site domains could account for the differences in the isoform toxicities, since their catalytic sites of the A-chain and their ability to depurinate rRNA is conserved in both subgroups.

Additionally, the PII A-chain has been expressed in a heterologous system allowing for the production of a soluble and active toxic chain. This protein is available for the preparation of toxic moieties of conjugates and immunotoxins with potential for use as a therapeutic agent. The B-chain has also been overexpressed in a prokaryotic system and its refolding is feasible. Thus, it is possible to reconstruct the holotoxin from the recombinant A- and B-chains resembling the native protein in terms of toxicity and structure. The recombinant pulchellin B-chain has also the potential to be used as a cell targeting molecule and mediator of cellular uptake.

The crystal structure of at least one isoform from each subgroup would provide a deeper and more detailed understanding of the sugar-binding site. Moreover, the knowledge of the structure–function relationship of the residues interacting with galactosides would help outline the distinct structural features that are relevant for galactose binding and thereby the modulation of toxicity, which could help make them more appropriate for therapeutic use. Further studies comparing the different pulchellin isoforms may contribute to the understanding of how sugar binding is related to cell toxicity in type 2 RIPs.

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Ribosome-Inactivating Proteins in Cereals

Carlotta Balconi, Chiara Lanzanova, and Mario Motto

Abstract Plants constitutively accumulate proteins that are either toxic or inhibitory against pathogens, including ribosome-inactivating proteins (RIPs) and *N*-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs. Cereal RIPs share a high similarity with all the other RIPs; however, they retain characteristic features forming a distinct class which diversified significantly during evolution**.** They appear involved in several different physiological roles, such as defense against pathogens and/or involved in regulatory and developmental processes. RIPs from cereals generally have low activity against plant ribosomes. In this chapter are reported recent advances in research related to cereal RIPs, with particular emphasis to the maize RIP (b-32) expressed in transgenic plants as an antifungal protein and reliable tool in crop disease management programs.

1 Introduction

Ribosome-inactivating proteins (RIPs) are a widely distributed family of toxic plant proteins that catalytically inactivate eukaryotic ribosomes (Barbieri et al. 1993; Metha and Boston 1998). RIPs function as *N*-glycosidases to depurinate the universally conserved α-sarcin loop of large rRNAs, selectively cleaving an adenine residue at a conserved site of the 28S rRNA (26S rRNA in yeast), such as the adenine$_{4324}$ of rat liver 28S rRNA (Endo and Tsurugi 1988). This depurination inactivates the ribosome, thereby blocking its further participation in protein synthesis; in particular, this irreversible modification blocks elongation factor EF-1- and EF-2-dependent GTPases activities and renders the ribosome unable to bind EF-2 with consequent arrest of protein synthesis (Barbieri et al. 1993; Metha

C. Balconi (✉), C. Lanzanova, and M. Motto
Consiglio per la Ricerca e Sperimentazione in Agricoltura, CRA-MAC – Unità di Ricerca per la Maiscoltura, Via Stezzano, 24, 24126 Bergamo, Italy
e-mail: carlotta.balconi@entecra.it

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_8,

and Boston 1998; Endo and Tsurugi 1988; Nielsen and Boston 2001; Peumans et al. 2001; van Damme et al. 2001).

Although, RIPs were first identified more than 25 years ago, their biological function(s) still remains open to speculation. In addition, to their known activity of depurinating ribosomes at the sarcin/ricin (S/R) loop in vitro, there is no unequivocal answer to the question of why plants synthesize and accumulate RIPs. To summarize and rationalize their biological function in plants, progress on RIPs and a working model have been recently presented and discussed (Park et al. 2004).

It was originally believed that RIPs do not occur universally among plants, pointed out by the failure to identify RIPs in model plant systems such as *Arabidopsis thaliana* or *Nicotiana tabacum*. However, Sharma et al. (2004) have isolated and characterized from *N. tabacum*, a 26-kDa RIP-like protein termed TRIP, possessing *N*-glycosidase activity. In this context, it was found that TRIP is expressed in the leaf at very low levels (≈0.01% fresh weight), a finding that suggests the difficulty in identifying an RIP from tobacco. This discovery raised the possibility that other plant species also possess one or more *N*-glycosidases. Accordingly, it has been shown that plants possess multiple RIPs and their activity has been found in different organs (seed, root, leaf) in concentrations ranging from few micrograms to several hundred milligrams per 100 g of tissue (Stirpe et al. 1992; Hartley and Lord 1993).

2 Classification of RIPs

On the basis of their physical properties, RIPs are classified into three classes (Metha and Boston 1998; Mundy et al. 1994). Type 1 RIPs such as Pokeweed Antiviral Protein (PAP), saporin (from soap-wort, *Saponaria officinalis* L.), trichosanthin, gelonin, and barley seed RIP (RIP30) have basic isoelectric point, and are monomeric enzymes of approximately 30 kDa with a single polypeptide chain that contains the ribosome-inactivating activity (Irvin 1975; Stirpe et al. 1980; Asano et al. 1984; Maraganore et al. 1987; Yeung et al. 1988). Type 1 RIPs are only weakly toxic to intact cells, although their enzymatic activity appears to be at least greater than that of type-2 RIPs (Barbieri et al. 1993).

Type 2 RIPs, such as ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of an approximate MW of 30 kDa; a polypeptide chain (A-chain) that contains the ribosome-inactivating activity is linked by a disulphide bridge to a second chain galactose-binding lectin (B-chain) that promotes uptake by the cell (Olsnes and Pihl 1973a; Olsnes and Pihl 1973b; Stirpe et al. 1978). The B-lectin chain can bind to galactosyl moieties of glycoproteins and/or glycolipids that are found on the surface of eukaryotic cells and mediates retrograde transport of the A-chain through the secretory pathways into the cytosol (Beaumelle et al. 1993; Sandvig and van Deurs 1994). Therefore, when it reaches the cytosol, the A-chain of the RIP has access to the translational machinery and inactivates ribosomes interrupting protein

synthesis. Type 2 RIPs were valuable for investigating endocytosis and intracellular transport in mammalian cells (Lord and Roberts 1996; Sandvig and van Deurs 1999; Hazes and Read 1997). Only some type 2 RIPs, namely ricin, abrin, modeccin, volkensin, and viscumin, are highly toxic to cells and animals; on the contrary, others type 2 RIPs, namely ebulin, nigrin, cinnamomin and iris lectin are not toxic, the reason(s) for the difference being still unknown.

Type 3 RIPs, such as maize b-32 and barley JI60 (Walsh et al. 1991; Chaudhry et al. 1994), are synthesized as single-chain proenzymes, inactive precursors (pro-RIPs) that require proteolytic processing events to produce two noncovalently linked chains equivalent to a type 1 RIP. Recently, b-32 has been described as a holo-RIP, two-chain type-1 RIP, whereas JIP60 as a chimero-RIP, true type-3 RIP (van Damme et al. 2001). These RIPs are less abundant than type 1 or type 2 RIPs. The function of the extra domains in the type 3 RIP is not known: once they are removed, the processed active protein is similar in charge and enzymatic activity to type 1 RIPs (Hey et al. 1995; Krawetz and Boston 2000; Walsh et al. 1991). For maize, the extra domains are unlikely protective features to prevent self-inactivation of maize ribosomes because ribosomes from seed and other plant parts are resistant to maize proRIP and active RIP (Hey et al. 1995; Krawetz and Boston 2000; Bass et al. 1992). For barley, however, induction of JIP60 was reported to coincide with a decrease in protein synthesis followed by a decrease in polysome size (Reinbothe et al. 1994b). Thus, the possibility of JIP60 accumulating as a proenzyme to protect barley ribosomes cannot be ruled out. The mode of uptake of types 1 and 3 RIPs by cells is still unknown.

3 Applied Research on RIPs

Because of their peculiar biological activities towards animals and human cells as cell-killing agents, RIPs have received remarkable attention in biological and biomedical applied research. In fact, due to their selective toxicity, RIPs have been primary candidates for the toxic moiety of immunotherapeutics (Spooner and Lord 1990; Pastan and Fitzgerald 1991; Olsnes and Pihl 1982). Therefore, a great deal of the literature reflects attempts to isolate and characterize RIPs from new plant sources and to exploit these RIPs as anticancer agents (Barbieri et al. 1993; Gasperi-Campani et al. 1985; Stirpe et al. 1992). Studies were also focused on enzymology, uptake of lectin-associated RIPs into target cells, and subsequent transport to ribosomal targets in the cytosol (Lord and Roberts 1998; Olsnes and Sandvig 1988; Sandvig and van Deurs 1999). This research has provided a broad knowledge base for understanding biochemical and therapeutic properties of RIPs. However, less frequent are studies into the biological function of RIPs in plants, even if in recent years, investigations of RIP activities have increased, especially as tools for gene isolation and transgenic expression became available.

RIPs are widely distributed throughout the plant kingdom and are active against ribosomes from different species, though the level of activity depends on the source

of RIP and of the ribosome (Bass et al. 1992; Battelli et al. 1984; Harley and Beevers 1982; Stirpe et al. 1992).

Although, the enzymatic activities of RIPs have been shown in vitro, their role in plant defense is less clearly defined. Historically, RIPs have been linked to plant protection, because crude extracts of pokeweed (*Phytolacca americana*) leaves were first shown to have inhibitory activity against viral infections in plants (Irvin 1975). Work performed to assay extracts from over 50 plant species pointed out that most had translational inhibitory activity in vitro (Barbieri et al. 1993; Gasperi-Campani et al. 1985; Stirpe et al. 1992), while purification of the inhibitory proteins have led to their identification as RIPs. Collectively, this information has promoted several biotechnological approaches to generate transgenic plants to exploit the antimicrobial activity of RIPs. For example, in tobacco, increased virus resistance was achieved with the expression of trichosanthin (Lam et al. 1996), PAP (Lodge et al. 1993), PAP II (Wang et al. 1998), virus-induced dianthin (Hong et al. 1996) and C-terminally deleted, inactive PAP (Tumer et al. 1997); altogether these data indicate that the resistance may not necessarily be linked to *N*-glycosidase activity on "self" ribosomes. Increased fungal resistance against *Rhizoctonia solani* was obtained with PAP II (Wang et al. 1998), and a truncated PAP version (Zoubenko et al. 1997).

4 Properties of Cereal RIPs

RIPs from cereals share a high similarity to all other RIPs, retaining, however, characteristic features which group them into a distinct class which diversified significantly during evolution (Jensen et al. 1999). These proteins appear to be involved in quite different physiological roles, such as defense against pathogens and/or in regulatory and developmental processes (Motto and Lupotto 2004). While some RIPs, such as PAP, are very active against both animal and plant ribosomes, on the other side, RIPs from cereals generally have low activity against plant ribosomes (Madin et al. 2000); an exception to this rule is the RIP of wheat leaves, which can modify plant ribosomes at concentrations where the seed RIP does not (Massiah and Hartley 1995).

4.1 Rice RIPs

A genome-wide identification of the RIP family in rice, based on the complete genome sequence analysis, was recently reported by Jiang et al. (2008), who identified at least 31 members of the RIP family all belonging to the type 1 RIP genes. It was also found that some members of this family were expressed in various tissues with differentiated expression abundances, whereas several members showed no expression under normal growth conditions or various environmental stresses.

On the other hand, the expression of many RIP members appears regulated by various abiotic and biotic stresses. Therefore, the previous authors suggested that specific members of the RIP family in rice might play important roles in biotic and abiotic stress-related biological processes and function as regulators of various environmental cues and hormone signaling. Consequently, they can potentially be useful in improving plant tolerance to various abiotic and biotic stresses by over-expressing or suppressing their genes.

4.2 Wheat RIPs

Tritin, a single-chain RIP in wheat seeds, was first identified as a component of a wheat-germ protein-synthesizing system that caused inhibition of protein synthesis in an ascites cell-free system (Stewart et al. 1977); this protein represents approximately 2% of the total soluble protein in mature wheat seeds (Coleman and Roberts 1982). Furthermore, two distinct forms of RIPs were purified from wheat germ and leaves, termed tritin-S and tritin-L, respectively; these forms differ in size and charge and are antigenically unrelated (Massiah and Hartley 1995). Tritin-S and tritin-L differ in both their ribosome substrate specificities and cofactor requirements: tritin-S shows only barely detectable activity on ribosomes from the endosperm, whereas tritin-L is highly active on leaf ribosomes; tritin-S, unlike tritin-L shows a marked requirement for ATP in its action (Massiah and Hartley 1995). The finding that tritin-S is inactive on wheat-germ ribosomes is consistent with the observation that the genomic sequence does not encode an *N*-terminal signal sequence (Habuka et al. 1993); from this it can be inferred that the RIP accumulates in the cytosol in contact with ribosomes.

Sawasaki et al. (2008) recently reported that: (1) tritin RIP activity could be one of the key steps in the development of senescence in wheat coleoptiles (2) transgenic tobacco plants expressing glucocorticoid-induced tritin developed senescence-like phenotype. The above mentioned data confirm previous indications about the induction of RIP activity in stressed leaves, including senescent leaves, of several plant species (Stirpe et al. 1996; Rippmann et al. 1997).

4.3 Barley RIPs

In the barley endosperm, three similar type 1 RIP isoforms – I, II (RIP30), and III – have been identified and described. The RIP30 isozyme fraction showed 50% inhibition of RNA translation (reticulocyte lysate) at concentration of 3–30 nM (Asano et al. 1986; Leah et al. 1991). Barley RIP30 inactivates rat liver ribosomes in the same manner as ricin A-chain by hydrolysing a single *N*-glycoside bond at A_{4324} of 28S rRNA to release adenine (Endo and Tsurugi 1988). Additionally, this barley toxin has been shown to be especially active on isolated fungal ribosomes of

Neurospora crassa (Roberts and Selitrennikoff 1986). RIP30 is a cytosolic protein lacking a signal peptide extension and it is probably only weakly active or completely inactive on ribosomes of the producing cells (Leah et al. 1991). The starchy endosperm-specific deposition of RIP30 suggests that it may also function as an albumin in storage polypeptide. Starchy endosperm cells differentiate terminally during development and are metabolically senescent at maturity. Therefore, it is likely that RIP30, despite its inhibitory specificity towards "foreign" ribosomes (Stirpe and Hughes 1989), is only mildly cyototoxic to barley cells. If this is the case, starchy endosperm cells apparently form one of the tissues where high levels of ribosome-inactivating proteins accumulate in cereal plants. These proteins are likely potential determinants of the terminally differentiated fate of this cell type. However, addition of barley RIP30 was inhibitory to in vitro translation and to fungal growth on solid media when tested against *Trichoderma reesei* (Leah et al. 1991), suggesting a protective role. On the other hand, the same authors reported that inhibition of growth in liquid media was minimal with barley RIP alone, but increased dramatically when β-1,3-glucanase or a chitinase, or both were included (Leah et al. 1991).

Studies with the type 3 barley jasmonate-induced RIP, termed JIP60, suggested that ribosome susceptibility to RIPs is a dynamic process than an innate property. Incubation of polysomes with JIP60 resulted in a shift to monosomes only if the polysomes had been prepared from stressed leaf tissue that had undergone 36 h of desiccation or 24 h of methyl jasmonate treatment. On the other hand, water-treated controls had no change in polysome size (Reinbothe et al. 1994a,b). However, these indications should be carefully interpreted, because ribosomes were not assayed for depurination at the α-sarcin loop; moreover, it was reported that JIP60 is not competent for translational inhibition unless proteolytic processing with at least two cleavage events has occurred (Chaudhry et al. 1994).

4.4 Maize RIPs

In maize, proRIP – classified as type 3 RIPs (Nielsen and Boston 2001) or, as two-chain type 1 RIPs (van Damme et al. 2001), are present in at least two forms encoded by nonallelic genes, one expressed in the endosperm (Walsh et al. 1991; Di Fonzo et al. 1986, 1988) and the other in leaf tissues (Bass et al. 1995). The maize endosperm RIP (b-32) has been widely investigated (Motto and Lupotto 2004).

This protein is a cytosolic albumin with a molecular weight of 32 kDa synthesized in temporal and quantitative coordination with the deposition of storage proteins (Soave et al. 1981a; Barbieri et al. 1997); it is present in the endosperm as inactive zymogen (proRIP), representing up to 1% of the total seed proteins (Soave et al. 1981b).

N-terminal, C-terminal and internal domains can be enzymatically removed from proRIP to yield two chains α–β that interact noncovalently to form a much more active enzyme (Walsh et al. 1991; Bass et al. 1992). The process involves

removal of a 16 amino acid residue of 1763 D from the N-terminus (residues 1–16), a 25 amino acid residue of 2708 D from the acidic central region of polypeptide (residues 162–186), and 14 amino acids of 1336 D from the C-terminus (residues 289–301) (Hey et al. 1995). The two final peptides of 16.5 and 8.5 kDa generated, tightly linked in a noncovalent manner, represent the activated form of RIP, termed αβ-RIP (Walsh et al. 1991). The activated form inhibits translation in a cell-free rabbit reticulocyte system with an IC_{50} (concentration causing 50% inhibition) of 28–66 pM, at least 10,000 times more active than the proRIP (Walsh et al. 1991). Further support for a proteolytic activation of proRIP was found in the demonstration of increases in RIP activity in coincidence with the onset of protease synthesis and protein degradation during germination (Bass et al. 1992; Hay et al. 1991). The proteolytic cleavage that occurs in vivo during germination, can also be performed in vitro by a variety of nonspecific proteases such as papain and subtilisin Carlsberg (Chaudhry et al. 1994), thus demonstrating that the RIP activation is due to a proteolytic processing of central acidic domain. Bass et al. (1992) have identified a second maize RIP that appears to require both N-terminal and internal processing events for enhancing its enzymatic activity. This second RIP shows no enhanced expression in the kernel, but it appears expressed in all maize tissues.

The synthesis of inactive precursor forms of enzymes, the zymogens, appears to be a specific way to regulate their activity by suppressing the enzymatic capacity until conversion of the zymogens to the active form, when needed, occurs by proteolytic cleavage (Neurath and Walsh 1976; Neurath 1989). There is no evidence that maize endosperm RIP b-32 is housed in intracellular organelles or secreted (Walsh et al. 1991; Di Fonzo et al. 1986); the maize proRIP is, in all cases described, a cytosolic protein not secreted via the endoplasmic reticulum (ER). This feature distinguishes the maize RIP from many other plant RIPs such as ricin (Lamb et al. 1985), trichosanthin (Chow et al. 1990) or momorcharin (Ho et al. 1991).

Gene expression studies have demonstrated that the *b-32* gene, as well as genes encoding the 22 kDa zeins, are coordinately controlled by the endosperm regulatory locus *Opaque-2* (*O2*) (Soave et al. 1981a, b). O2 protein belongs to the b-ZIP family of transcriptional regulators (Hartings et al. 1989), and affects expression of the major seed storage protein genes, in particular those encoding the 22 kDa α-zeins (Hartings et al. 1989; Schmidt et al. 1990). Levels of b-32 and 22 kDa zeins are greatly decreased in *o2* mutants. The role of b-32 in defense against pathogens is therefore suggested by an increased susceptibility of *o2* mutant kernels, where the level of b-32 is greatly decreased, to fungal attack (Loesch et al. 1976; Warren 1978) and insect feeding (Gupta et al. 1970). Moreover, the results obtained by testing a set of pure inbred lines, and their isogenic *o2*-mutants, in field experiments with Silk Channel Inoculation Assay (SCIA) on adult plants, showed that the *o2* mutants resulted significantly more susceptible to the *Fusarium verticillioides* attack than the normal version (Balconi et al. 2005). The increased susceptibility in the absence of the proRIP b-32 is consistent with a defense function, although the experimental results cannot be attributed to the maize proRIP b-32 a priori because O2 regulates transcription of a large number of genes that may contribute to

a complex mutant phenotype (Hartings et al. 2009). These observations suggest that it would be interesting to verify if the expression of b-32 in an *o2* mutant could increase the tolerance to fungal pathogen attack in the kernel.

The toxicity of maize RIP (RIP1) toward fungi has been tested by Nielsen et al. (2001). These authors developed a microculture assay useful to monitor the cellular growth and morphology of the fungi upon addition of purified RIP. In this study, it has been found that the activated maize RIP altered the growth and morphology of *Aspergillus flavus*, a corn fungal pathogen, and *Aspergillus nidulans*, a nonpathogen. Specifically, data reported by Nielsen et al. (2001) from the enzymatically inactive MOD1 mutant treatment argue that the effect of RIP on *A. flavus* and *A. nidulans* requires the catalytic ribosome-inactivating activity of the protein. In the above mentioned study, proRIP did not show any antifungal activity against tested fungi; these data suggest that the protein must be activated to have antifungal activity but do not rule out activation occurring in a number of ways in vivo. For example, *A. flavus* has been shown to lyse and degrade cells at the fungal invasion front (Smart et al. 1990), presumably by the action of proteases and other degradative enzymes secreted by the fungus. Proteases stored intracellularly might also be released from plant cells damaged by the invading fungus. The inhibitory activity of activated maize RIP against normal fungal growth is consistent with a biological function to protect the seed from fungal invasion.

5 Transgenic Plants Expressing RIPs

The ectopic expression of antimicrobial proteins in plants or plant tissues has the potential to limit pathogen infection or growth. In this perspective, a successful strategy would be to deploy an antifungal protein (normally expressed in the kernel) in a nonseed tissue that is a site of infection (e.g., silk, husk, leaves). Ectopic expression of RIPs in transgenic plants may solve this infection problem by allowing the exposure of the pest or pathogen to the RIP only during interactions with the plant. In several studies, transgenic plants expressing cereal RIPs were used to test defense properties attributed to this group of proteins (Hartley et al. 1996; Punja 2001). The type 1 barley RIP expressed under a 35S-CaMV promoter or a wound-inducible promoter in tobacco conferred a reduction of disease symptoms caused by the fungus *R. solani*; addition of a signal sequence to target the RIP to endomembrane system improved resistance in transgenic plants producing detectable levels of RIP (Logemann et al. 1992; Jach et al. 1995). In contrast, expression of type 1 barley RIP30, expressed under the control of a strong constitutive promoter *35S-CaMV*, had little effect against infection by the fungal pathogen *Erysiphe graminis* in transgenic wheat (Bieri et al. 2000). In the last cited study, the RIP30 was targeted through the ER to the apoplastic space in order to ensure the presence of RIP at the place where initial interactions with the fungus occur; RIP30 was effectively localized to the intracellular space, and the intercellular wash fluids (IWF) of expressing transgenic wheat lines, strongly inhibited a rabbit reticulocyte

lysate transcription/translation system, but the antifungal effects of RIP30, as assayed by infection of detached leaves with *E. graminis*, were small (Bieri et al. 2000). Likewise, RIP30 driven by the strong maize constitutive *ubiquitin-1* promoter was introduced in wheat and transgenic plants engineered with three single antifungal genes – *RIP30*, *Ag-AFP* and *chitinase-II* – then challenged for response to powdery mildew and rust; the results obtained showed a significant reduction of infection in Ag-AFP and Chi-II expressing plants, but not in RIP30-expressing wheat lines compared with the control (Oldach et al. 2001).

Further studies have demonstrated that combined expression of chitinase and RIP in transgenic tobacco had a more inhibitory effect on *R. solani* development than the individual proteins (Jach et al. 1995). Therefore, dissolution of the fungal cell wall by hydrolytic enzymes should enhance the efficacy of antifungal proteins and peptides in transgenic plants.

Transgenic rice plants expressing the maize RIP b-32 have been produced by Kim et al. (1999). These workers noted that the level of the b-32 expression was approximately 0.5–1% of total soluble protein in leaf tissues, a value comparable to the expression of barley RIP detected in fungus-resistant transgenic tobacco plants (Logemann et al. 1992). The data reported in this research indicated that the b-32 was proteolytically processed in germinating rice seeds and young leaves of transgenic plants, in a similar manner to that found in germinating maize kernels; however, no protein processing was detected in mature leaf tissues (Kim et al. 1999). The authors also reported that disease severity caused by infection with the fungal pathogens *R. solani* and *Magnaporthe grisea*, was not significantly reduced in the transgenic plants expressing the RIP b-32 as compared to control plants, suggesting that processing of b-32 protein may be required to exhibit antifungal activity *in planta*. Whether transgenic plant fungal resistance requires proteolytic processing of the maize proRIP (b-32) is not clearly defined. In fact, a research developed in our laboratory showed that transgenic tobacco plants, expressing the maize RIP b-32 gene driven by the *wun 1* promoter, had increased protection against infection of the soil-borne fungal pathogen *R. solani* (Maddaloni et al. 1997).

Similarly, further research carried out in our laboratory with wheat transgenic lines, indicated that maize RIP b-32 protein was effective, as an antifungal protein, in reducing Fusarium head blight (FHB) symptoms (Balconi et al. 2007). Transgenic approaches to combat FHB in wheat and barley were recently reviewed by Dahleen et al. (2001). They pointed out that various degrees of protection against FHB may be achieved by introducing, *in planta*, heterologous genes encoding for proteins with anti*Fusarium* activity.

A variety of antifungal genes have been isolated: some of their products have been shown to inhibit *Fusarium* growth in vitro and *in planta* (McKeehen et al. 1999). Our experiments to evaluate the action of the protein RIP b-32 in transgenic wheat plants, confirmed an increase of FHB resistance. Transgenic wheat plants were obtained via biolistic transformation, in which the *b-32* gene is driven by the *35S-CaMV* promoter in association with the *bar* gene as a selectable marker. The six homozygous transgenic lines used for the investigation were all phenotypically

normal when compared to the parental nontransgenic cv. Veery, except for a slightly smaller size, were fully fertile and set seeds, confirming that expression of the exogenous RIP did not interfere with normal plant development. B-32 expression was maintained at comparable levels during various developmental stages of the transgenic wheat plants which include the seedling stage, tillering, and up to 10 days after anthesis. It was also interesting to note that no endogenous b-32 expression was observed in control wheat plants, while the typical pattern (a double banding) was observed in the transgenic line at the three stages of development at comparable levels, and in the control maize endosperm W64A, as expected (Balconi et al. 2007).

A similar pattern of b-32 expression was also detected in immature spikelets and rachis. Collectively, these results confirmed a stable expression level of b-32 in green tissues of transgenic lines throughout their development. The comparison of b-32 amounts in protein leaf extracts of transgenic lines at the heading stage allowed the identification of lines with high, intermediate, and low b-32 content in leaves. This finding is a useful range of expression for pathogenicity experiments, in order to evaluate an eventual differential response to fungal pathogens attack. Resistance to FHB was evaluated by the "single floret injection inoculation method" on immature spikes of the b-32 transgenic wheat lines in comparison to the parental cv. Veery, with spores of *Fusarium culmorum*. The plants were analyzed for FBH by visual inspection and the data suggest that protection due to the presence of b-32 was not dependent on increasing levels of the RIP protein in the tissues, but also that the lowest level of b-32 was effective.

In this study, it was also observed, by applying another index associated with tolerance, such as the percentage of "tombstones" (shriveled, light weight, dull grayish or pinkish in color kernels)/total seeds, recorded at maturity, that independently from the differential b-32 content of the transgenic lines, the percentage of "tombstones" was equally reduced in all cases, in comparison with control cv. Veery plants (Balconi et al. 2007). Therefore, disease control by b-32 protein was observed as a reduction of visible FHB symptoms, early after inoculation, reflected in reduced fungal colonization after artificial single spikelet inoculation and also at maturity, as a reduction in damaged seeds percentage.

Collectively, these results indicate that RIP b-32 was effective as an in vivo antifungal protein in wheat, which normally does not produce this protein. In fact, one of the most devastating fungal diseases of wheat was therefore controlled at significant level.

To further explore the antifungal activity of the maize RIP b-32, Lanzanova et al. (2009) developed transgenic maize plants containing the *b-32* coding sequence driven by a constitutive *35S-CaMV* promoter were obtained through genetic transformation. In this study four homozygous independent maize transgenic lines, with differential ectopic expression of b-32 (SM 3.4; SM 16.1; SM 19.4; SM 20.2), were evaluated, in comparison with plants expressing b-32 only in the endosperm (SM 20.4), for response to *F. verticillioides* colonization by leaf tissue bioassays. All transgenic plants were phenotypically normal, when compared to the negative control and fertile, confirming that the ectopic expression of the b-32 RIP did not

interfere with normal plant development, as previously underlined (Maddaloni et al. 1997; Balconi et al. 2007).

The evaluation, in the B73 inbred line and in the negative control (SM 20.4), of b-32 expression at the protein level, in endosperm and leaf tissues, confirmed the endosperm-tissue specificity of this gene (Fig. 1, lanes 1 and 3); it is also evident in the same figure that those materials did not exhibit any expression of cross-reacting proteins in leaf tissues (lanes 2 and 4, respectively). On the other hand, SM 20.2 transgenic progeny showed detectable b-32 cross-reacting bands both in leaf and endosperm tissues (Fig. 1, lanes 5 and 6).

Additionally, proteomic analyzes on leaf extracts showed that the presence of both the b-32 and herbicide resistance enzyme, were the only significant variations detected between the transgenic and the Basta-sensitive progeny protein profiles. The identification of progeny with a differential b-32 expression in the leaves was useful for setting up pathogenicity experiments. These were aimed at evaluating a possible differential response to a *Fusarium* attack in leaf tissue colonization bioassays.

A comparison of b-32 protein amounts in leaf extracts at flowering stage, performed by immunoblot image scanning, manifested a differential b-32 expression among the various progeny. Progeny SM 3.4, SM 16.1 and SM 20.2 showed b-32 contents significantly higher than observed in progeny SM 19.4; as expected, SM 20.4, i.e. negative control, showed nondetectable b-32 content (n.d.) in leaf tissues.

The transgenic progenies expressing b-32 in leaf tissues were less susceptible than the negative control, when evaluated for response to the *F. verticillioides* attack, showing significantly reduced colony diameter around the inoculated leaves (Table 1). Similar results were reported concerning blast inoculation assays conducted on detached leaves of transgenic rice plants expressing the antifungal AFP protein (Coca et al. 2004). A good correlation between the b-32 content in the leaves and the level of resistance to *Fusarium* attack was observed (Table 1). In particular, data in this table indicated that the suppression of *Fusarium* leaf colonization (growth inhibition,% relative to the control) in the SM 19.4 progeny was significantly lower than that observed in the other three transgenic progenies, at all detection times. The expression of herbicide resistance protein (the enzyme, phosphinothricin acetyltransferase) apparently does not interfere with mechanism(s)

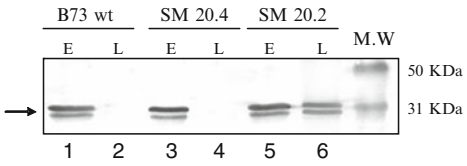

Fig. 1 Western-blot analysis of b-32 expression at protein level. Endosperm and leaf tissue respectively of inbred line B73 (*lanes 1 and 2*), negative control line SM 20.4 (*lanes 3 and 4*), and transgenic line SM 20.2 (*lanes 5 and 6*), were tested for the presence of b-32 protein (modified from Lanzanova et al. 2009)

Table 1 Severity of *Fusarium* leaf tissue colonization and suppression of *Fusarium* leaf colonization in the b-32-expressing transgenic progenies (modified from Lanzanova et al. 2009)

Progenies	Days after inoculation (DAI)			
	3 DAI		7 DAI	
	Colony diameter (mm)[a]	Growth inhibition (%)[b]	Colony diameter (mm)[a]	Growth inhibition (%)[b]
SM 20.4	7.6 ± 0.5	–	25.0 ± 0.0	–
SM 3.4	2.7 ± 0.9	64.0 ± 11.3	12.3 ± 0.9	50.8 ± 3.5
SM 16.1	2.9 ± 0.6	61.9 ± 7.7	12.8 ± 1.3	47.3 ± 3.7
SM 19.4	5.3 ± 0.7	30.6 ± 7.8	18.9 ± 0.9	23.0 ± 3.2
SM 20.2	2.7 ± 0.5	64.2 ± 7.9	12.6 ± 0.5	49.8 ± 2.0
LSD (0.05)	0.8	12.7	0.9	3.3

[a]Triplicate samples measured as mycelial radial growth 3–7 days after *F. verticillioides* leaf tissues inoculation (10^6 spores/ml) in the negative control (SM 20.4 progeny) and in the transgenic progenies. All values were analyzed by MSTAT-C-Program (Michigan State University, Version 1991)

[b]Percent of radial growth inhibition compared to negative control, SM 20.4 progeny, calculated individually for each replicate before statistical analysis

involved in *F. verticillioides* maize leaf colonization; the increased resistance to *Fusarium* colonization, observed in the transgenic progenies is attributable to the expression of b-32, excluding potential additional transgenic effects (Lanzanova et al. 2009).

Even though the two low molecular weight peptides, reported to be the activated form of RIP (Walsh et al. 1991), were not detected in the transgenic leaf tissues, the expression of b-32 protein (reported to be the 32 kDa proenzyme, proRIP) supported the increase of *Fusarium* leaf colonization resistance. This result is in accordance with previous studies on tobacco, showing that transformed plants were more tolerant to *R. solani* infection than the negative control even if no low molecular weight immunoreactants were detectable with b-32 antiserum (Maddaloni et al. 1997).

In all tested transgenic progenies and in the negative control, 100% of inoculated leaf squares showed *Fusarium* colonization (data not shown) suggesting, as expected, that b-32 protein does not prevent the *Fusarium* attack, but rather promotes the reduction of mycelial growth on the colonized tissue. As previously reported for FHB, b-32 crop protection may be due to the disease being limited in its spread in all directions from the point of inoculation (PI) (Balconi et al. 2007).

An important issue in view of fungal protection against maize fusariosis is to verify the influence of the engineered antifungal b-32 protein in the containment of mycotoxins (fumonisins, FB) in the plants infected by *F. verticillioides* (Duvick 2001). Since most mycotoxin problems develop in the field, strategies are needed to prevent infection of growing plants by toxigenic fungi (Munkvold 2003). The expression of antifungal proteins in plants or plant tissues, in which they are not normally expressed, is very appropriate to reduce pathogen colonization and growth; in this perspective, a reduction of *F. verticillioides* infection in maize leaves and stalk, could be very useful to limit the fungal infection from spreading

to the exposed silks and consequently to reduce the grain fumonisin contamination (Lauren and Di Menna 1999). In this respect, various benchmarks for the success of this approach to FB reduction will all determine the extent of disease tolerance in a transgene. These include: (1) protein localization in the plant tissue in relation to fungal substrate accessibility; (2) kinetic parameters of the enzyme(s) in the context of its interaction with the plant substrate (substrate Km, pH optimum, substrate range, potential inhibitors); (3) stability and activity of the enzyme during plant growth and development conductive to fungal growth. Equally important will be experiments that verify the nutritional properties of the transgenic grain under various environmental conditions.

6 Conclusions

In spite of RIPs having been thoroughly investigated as potentially useful toxins, their role and function in plants and their distribution in nature remain enigmatic. Use of the recently identified RIP from *N. tabacum* (Sharma et al. 2004), in conjunction with genomic and biotechnological approaches, such as mutagenesis and gene silencing, appear as an effective strategy to bridge the existing gaps in our knowledge. In the future, it is likely that progress in this field will accelerate, leading to elucidation of the biological function of RIPs and establishing their fundamental role in the plant cell and their potential significance in several therapeutic applications, including the preparation of immunotoxins targeting tumors and hematological malignancies.

The maize b-32 RIP is one of the most thoroughly investigated cereal RIPs because of its peculiar presence, action, processing, and effects in the maize kernel physiology. In addition, its presence in connection to regulatory processes involved in the zein storage protein deposition, as well as direct evidence of a protective role of the seed, render the maize RIP an interesting matter of investigation as a multifunctional system of the plant biology. Despite of the body of work performed, several topics correlated to RIP b-32 role in the maize kernel, remain to be clarified. RIP b-32 is expressed under the control of the seed-specific transcriptional activator *O2* and is synthesized as zymogen that increases its catalytic activity after proteolytic activation. B-32 accumulates in the kernel as a proenzyme (proRIP) accounting for 1–3% of the soluble protein; therefore its role is also consistent with a storage albumin function. This RIP clearly shows unusual regulatory properties at both the gene and protein level. The differences in maize RIP and other RIPs might also be useful in understanding the mechanisms involved in physiology and biochemistry of endosperm development and reserve mobilization.

Attempts to use RIPs as biological pesticides or cell-killing agents can benefit from information about the mechanism of RIP uptake by pathogens and about their protective role in transgenic plants. The effectiveness of an antifungal protein *in planta* may be predicted, in part, by its expression levels in the crucial host tissues and by the timing of expression because suitable levels should accumulate

before the host becomes most vulnerable to infection. In this context, the maize endosperm albumin b-32, as a RIP has been the subject of extensive studies aimed at investigating and at exploiting its action as a defense protein against fungi.

Recent results from our laboratory (Lanzanova et al. 2009), in agreement with other findings reported for tobacco and wheat (Maddaloni et al. 1997; Balconi et al. 2007), confirm that the incorporation of maize *b-32* gene and the ectopical expression of b-32 RIP protein, may represent an effective and reliable tool in crop disease management programs.

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Ribosome Inactivating Proteins and Apoptosis

Deepa Sikriwal and Janendra K. Batra

Abstract Ribosome inactivating proteins (RIPs) are RNA *N*-glycosidases which potently inhibit translation by inactivating ribosomes. RIPs have also been shown to possess the ability to induce apoptosis. A number of RIPs from different sources have been used to study the mechanism of apoptosis induction. However, it is being observed that these toxins trigger apoptosis in different cell types via different mechanisms; although in most cases mitochondria have been involved, no single common pathway that is followed by the RIPs for apoptosis induction has emerged. There appears to be a consensus that the protein synthesis inhibition and induction of apoptosis by RIPs are independent of each other. In this chapter, we bring together the available studies on apoptosis induction by RIPs.

1 Introduction

Ribosome-inactivating proteins (RIPs) inactivate ribosomes which results in potent inhibition of protein synthesis. They are widely distributed in nature and are almost ubiquitously present in plants. RIPs are not only found in many exotic plants but also in some edible crop plants including wheat, maize, barley, tomato, spinach, etc. (Prestle et al. 1992; Ishizaki et al. 2002; Barbieri et al. 2006). In addition to plants, RIPs are also found in bacteria, fungi, and alga (Liu et al. 2002), and have been shown to be phylogenetically related (Girbes et al. 2004). RIPs have been shown to possess RNA *N*-glycosidase activity which is responsible for their RNA depurination ability (Endo et al. 1987). They manifest toxicity by irreversibly damaging the ribosome. The interest in RIPs gained a new momentum with the growing

D. Sikriwal and J.K. Batra (✉)
Immunochemistry Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India
e-mail: janendra@nii.res.in

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_9,

evidence of their action on nonribosomal substrates (Barbieri et al. 1997, 2000; Hudak et al. 2000).

RIPs are classically categorized into type 1 and type 2. The type 1 RIPs, like saporin and pokeweed antiviral protein (PAP), include a number of basic monomeric enzymes of approximately 30 kDa. Type 1 RIPs share a number of highly conserved active site cleft residues and secondary structure within the active site region; however, they are distinctly different in overall sequence and posttranslational modifications (Monzingo and Robertus 1992; Mlsna et al. 1993; Barbieri et al. 1993; Husain et al. 1994). There are some smaller type 1 RIPs, with a molecular mass below 30 kDa which are characterized by an N-terminal sequence abundant in arginine and glutamate residues (Ng and Parkash 2002; Ng et al. 2002, 2003; Parkash et al. 2002a, b). Besides the classical type 1 RIPs, there are few type 1 RIPs, like maize b 32, which are synthesized as inactive precursors termed proRIPs. These RIPs are much less prevalent than classical type 1 RIPs and have been characterized only from maize and barley (Bass et al. 1992; Reinbothe et al. 1994; Walsh et al. 1991). Type 1 RIPs play a defensive role in plants and inhibit both plant and animal viruses (Robertus 1996). They penetrate virus-infected cells, inactivate ribosomes and kill the infected cells, thus, terminating viral proliferation (Rappuoli 1997).

Type 2 RIPs, like ricin, abrin and modeccin are heterodimeric proteins consisting of two chains, an A-chain of approximately 30 kDa with enzymic activity, and a B-chain of approximately 35 kDa with lectin properties. The A-chain is linked to the B-chain through a disulfide bond (Olsnes and Pihl 1973, 1981; Stirpe et al. 1977). The B-chain can bind to galactosyl moieties of glycoproteins and/or glycolipids found on the surface of eukaryotic cells (Sandvig et al. 1976; Olsnes and Sandvig 1988; Swimmer et al. 1992; Lehar et al. 1994; Steeves et al. 1999) and mediate retrograde transport of the A-chain to the cytosol where it has access to the translational machinery (Olsnes and Pihl 1981; van Deurs et al. 1986; Beaumelle et al. 1993; Sandvig and van Deurs 1996). The type 2 RIPs vary in their toxicity by about three orders of magnitude (Battelli 2004; Stirpe 2004). On the basis of the considerable differences in their cytotoxicity, and consequently in their toxicity to animals, the type 2 RIPs have been broadly divided into two groups, toxic and nontoxic. The reasons for the difference in toxicities are not completely understood, however binding and entry of the toxin into the cells and/or their degradation and exocytosis appear to be the major contributors (Stirpe and Battelli 2006).

2 Mechanism of Action of RIPs

RIPs exhibit RNA *N*-glycosidase activity, which was first demonstrated by Endo et al. (1987). They discovered that the RIPs catalyze removal of a single adenine residue, A4324 in rat liver rRNA, from a GAGA sequence in a universally conserved loop at the top of a stem in 28S ribosomal RNA, which was subsequently termed sarcin/ricin domain or loop (Endo et al. 1987; Endo and Tsurugi 1988).

The catalytic depurination disrupts the binding of elongation factors to the ribosomes, thus arresting protein synthesis at the translocation step (Endo et al. 1987). Later, when the study was extended to more RIPs and they were all found to have similar activity, RIPs were classified as rRNA *N*-glycosidases (EC 3.2.2.22). Although the catalytic mechanism of all RIPs is identical, their activity on ribosomes from sources other than eukaryotes is markedly different (Barbieri et al. 1993; Stirpe et al. 1988). The differences in the toxicity of RIPs toward various cell lines, requirements for different cofactors and variations in the minimal structure of the adenine-containing loop that they can attack, point to their substantial diversity (Carnicelli et al. 1992; Marchant and Hartley 1995). Differential inhibition pattern by molecules that bind and inactivate RIPs has also suggested that local sequence and structure variability exists among RIPs (Brigotti et al. 2000).

Besides having the functional *N*-glycosidase activity there are evidences of RIPs showing activities on nonribosomal substrates (Barbieri et al. 1997, 2000; Hudak et al. 2000). Most of the novel enzymatic activities are related to a presumed RNase or DNase activity (Li et al. 1991; Mock et al. 1996; Nicolas et al. 1997, 1998, 2000; Roncuzzi and Gasperi-Campani 1996). Other enzymatic activities reported for individual RIPs include phospholipase, chitinase and superoxide dismutase activity (Li et al. 1997; Helmy et al. 1999; Sharma et al. 2004; Xu et al. 2008). PAP has been shown to cleave the double-stranded supercoiled DNA using the same active site required to depurinate rRNA, whereas momordin has been shown to have intrinsic RNase activity (Wang and Tumer 1999; Fong et al. 2000).

3 Apoptosis

Apoptosis or programmed cell death is a well orchestrated collapse of a cell whereby the specific signaling is activated which ultimately leads to controlled cellular death. The term apoptosis was first used to describe a morphologically distinct form of cell death (Kerr et al. 1972). The mechanism of apoptosis is highly complex and involves a cascade of energy dependent molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. In addition, there is now evidence that the two pathways are linked and molecules in one pathway can influence the other (Igney and Krammer 2002).

The extrinsic pathway is initiated by the interaction of the transmembrane receptor with a ligand. FasL/FasR and TNF-α/TNFR1 are the best models that characterize the sequence of events that define the extrinsic pathway of apoptosis. Briefly, the receptors cluster when they bind with the homologous trimeric ligand. Consequently, upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD (Fas-Associated protein with Death Domain), and the binding of TNF to TNF receptor results in the binding of the adapter protein TRADD (TNFR1-associated

death domain protein) with recruitment of FADD (Hsu et al. 1995; Kelliher et al. 1998; Wajant 2002). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the autocatalytic activation of procaspase-8 (Kischkel et al. 1995). Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP (FLICE inhibitory protein) which binds to FADD and caspase-8, rendering them ineffective (Kataoka et al. 1998; Scaffidi et al. 1999).

The intrinsic pathway of apoptosis, as its name suggests, is initiated from within the cell. This pathway involves a diverse array of nonreceptor-mediated stimuli that produce intracellular signals which act directly on targets within the cell and are mostly mitochondrial-initiated events. The stimulus that initiates the intrinsic pathway may either be a positive or a negative factor. In other words, there has to be a balance between the pro- and anti-apoptotic factors for a continued cell growth. The presence of negative signals, which could be through the absence of growth factors, hormones and cytokines, can lead to failure of suppression of death programs, thereby triggering apoptosis. The positive signals could be due to a specific factor(s) like radiation, toxins, hypoxia, hyperthermia, viral infections, free radicals, etc. Any of these stimuli can cause changes in the inner mitochondrial membrane that results in opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of proapoptotic proteins from the intermembrane space into the cytosol (Saelens et al. 2004). The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi, which activate the caspase dependent mitochondrial pathway (Du et al. 2000; van Loo et al. 2002a; Garrido et al. 2006). Cytochrome c binds and activates Apaf-1 (apoptotic protease activating factor-1) as well as procaspase-9, forming an "apoptosome" (Chinnaiyan 1999; Hill et al. 2004). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting the activity of IAP (inhibitor of apoptosis proteins) (Schimmer 2004; van Loo et al. 2002b). The second group of proapoptotic proteins, AIF (apoptosis-activating factor), endonuclease G and CAD (caspase-activated DNase), are released from the mitochondria during apoptosis, but this is a late event and occurs after the cell has committed to die.

Both, the extrinsic and intrinsic pathways converge at the point of the execution phase which is the final stage of apoptosis. The activation of the effector caspases is the most important step that begins the execution phase of apoptosis. The activated execution caspases in turn activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases, which cleave various substrates (Slee et al. 2001). Caspase-3 is the most important of the executioner caspases and is activated by a number of the initiator caspases like caspase-8, caspase-9, or caspase-10. Caspase-3 specifically activates the endonuclease, CAD (Caspase-Activated DNase). In proliferating cells, CAD is present with its inhibitor, ICAD (inhibitor of caspase-activated DNase) but in the apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira et al. 1998).

CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3.

4 Ribosome Inactivating Proteins and Apoptosis

Initially, the cytotoxicity of RIPs was ascribed solely to the inhibition of protein synthesis; however Griffiths et al. (1987) observed for the first time that the morphology of the cells treated with ricin and abrin was similar to that of the cells undergoing apoptosis. They observed a large number of apoptotic bodies in paraaortic lymph nodes, Peyer's patches and ileal crypts of rats intramuscularly injected with ricin and abrin. Abrin, compared with ricin was found to cause more pronounced changes in these tissues. Later, abrin and ricin treatment of bovine pulmonary endothelial cells was also shown to produce apoptotic morphology, in addition to heterochromatin condensation and DNA laddering (Hughes et al. 1996). Soon after the first report on ricin and abrin induced apoptosis, many other plant and bacterial toxins were also found to induce apoptosis in mammalian cells (Chang et al. 1989; Morimoto and Bonavida 1992; Kochi and Collier 1993; Allam et al. 1997; Brinkmann et al. 1997; Narayanan et al. 2004).

As more and more studies were conducted on RIPs, it was clearly established that RIPs induced apoptosis since the toxin treated cells demonstrated the morphological and biochemical events associated with apoptosis. Ricin was observed to induce apoptosis in macrophages independent of the inhibition of protein synthesis (Khan and Waring 1993). Also, it was observed that the ricin-induced apoptosis did not involve the activation of Ca^{2+} dependent endonuclease(s) as there was no immediate increase in Ca^{2+} concentration when macrophages were treated with ricin (Khan and Waring 1993). The cell death induced by ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in MDCK cells was found to be strongly inhibited by 1,9-Dideoxyforskolin (DDF) suggesting these protein toxins to invoke a DDF-sensitive common cell death pathway (Oda et al. 1997). However, despite the strong inhibitory effect, DDF did not block toxin-induced DNA fragmentation which suggested that apoptosis and cell death may be triggered through separate pathways by these toxins (Oda et al. 1997).

RIPs have been explored to be developed as therapeutic proteins by coupling with antibodies recognizing cell surface proteins. The conjugates containing RIPs and antibodies, termed immunotoxins have also been studied for their apoptosis inducing properties. Saporin and a saporin containing immunotoxin were found to induce apoptosis in human peripheral blood B lymphocytes and neutrophils, in the B-cell line, Daudi, and in the haemopoietic cell lines, HL-60 and TF-1 (Bergamaschi et al. 1996). The saporin containing immunotoxin was 2–3 logs more effective than the native saporin in inducing apoptosis (Bergamaschi et al. 1996). Momordin, pokeweed antiviral protein from seeds (PAP-S) and saporin, and

their immunotoxins with Ber-H2, a monoclonal antibody directed against the CD30 antigen of human lymphocytes induced apoptosis in the CD30+ L540 cell line (Bolognesi et al. 1996). The immunotoxins made with RIPs were much more potent in inducing apoptosis compared to their free toxin counterparts because of better cell binding and internalization (Bergamaschi et al. 1996, Bolognesi et al. 1996). A replication-defective adenovirus enhanced the apoptotic and cytotoxic activity of a basic fibroblast growth factor-saporin fusion protein by more than ten fold, and caused *in vivo* tumor cell killing at nontoxic concentrations due to enhanced internalization of the ligand–receptor complex and release of the active toxin from the endosomes (Satyamoorthy et al. 1997).

Despite a large number of studies on RIP-induced apoptosis, the exact mechanism by which these toxins induce apoptosis is not very clear. Several reports on various RIPs like abrin, ricin, saporin, gelonin, mistletoe lectins (MLs), Shiga toxins (Stx), etc. describe the induction of apoptosis involving different apoptotic pathways and so far no single general mechanism has emerged for the induction of apoptosis by RIPs. We will address below in this chapter the various mechanisms put forth for the induction of apoptosis by RIPs.

4.1 Activation of Intrinsic Pathway of Apoptosis by General Stress

Mitochondria play a key role in stress induced cell death. Damage to mitochondria leads to loss of mitochondrial membrane potential (MMP) and has been shown to be the key point when the cell commits to die. A cell on exposure to different stress signals, which include toxins, heat, infection by viruses, loss of ATP, etc. responds either to overcome stress by activating various stress genes or can decide to undergo apoptosis. Most studies relating to induction of apoptosis by RIPs suggest that apoptosis is caused by the intrinsic pathway where the MMP changes, followed by rapid release of cytochrome c and activation of caspase-9.

One of the early reports depicted the direct role of mitochondria in RIP-induced apoptosis (Shih et al. 2001). The study showed that abrin could induce apoptosis by directly interacting and activating a thiol-specific 30-kDa antioxidant protein-1 (AOP-1), which resulted in an increase in the levels of intracellular reactive oxygen species (ROS) and release of cytochrome c from the mitochondria to the cytosol, and subsequently activation of caspase-9 and caspase-3 (Shih et al. 2001). Furthermore, ROS scavengers, *N*-acetylcysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl delayed the onset of apoptosis indicating ROS to be an important mediator of abrin-induced apoptosis (Shih et al. 2001).

Saporin-6, a type I RIP expressed in the seeds of *Saponaria officinalis* plant, induced caspase-dependent apoptosis in human histiocytic lymphoma cell line U937 via the mitochondrial or intrinsic pathway (Sikriwal et al. 2008). Saporin-6, unlike many other RIPs, did not require *N*-glycosidase activity for apoptosis induction, and the apoptosis onset occurred before any significant inhibition of protein synthesis ensued (Bagga et al. 2003; Sikriwal et al. 2008). In another study,

His-tagged saporin was found to be more cytotoxic to U937 cells when combined with *Gypsophila* saponins (Weng et al. 2008). The cytotoxicity was a result of induction of apoptosis triggered by the internalization of saporin facilitated by *Gypsophila* saponins (Weng et al. 2008).

The release of mitochondrial cytochrome c, and the sequential caspase-9 and caspase-3 activations have been shown to be important events in the signal transduction pathway of abrin-induced apoptotic cell death in the HeLa cell line (Qu and Qing 2004). Generation of ROS in response to toxins may cause oxidative stress to cells which might be one of the key factors in inducing apoptosis through the mitochondrial pathway. Abrin was shown to induce apoptosis in Jurkat cells following the intrinsic mitochondrial pathway that involved MMP damage and production of ROS (Narayanan et al. 2004).

In a similar way trichosanthin (TCS), a type-I ribosome-inactivating protein was observed to induce apoptosis in human choriocarcinoma cells, JAR due to generation of ROS (Zhang et al. 2000, 2001). The ROS formation, which preceded the activation of caspase-3, was shown to be dependent on the presence of extracellular Ca^{2+}. Furthermore, the antioxidant α-tocopherol prevented TCS-induced ROS formation and thereby rescued the cells from death (Zhang et al. 2000). There are many established roles of calcium in mitochondria induced apoptosis (Hajnoczky et al. 2003). The study by Zhang et al. (2000) indicated the possible role of Ca^{2+} signaling in RIP-induced apoptosis. Though TCS has been shown to induce calcium dependent ROS generation in JAR cells, there is a reported exception in human chronic myeloid leukemia cell line K562 (Li et al. 2007a). In this study, TCS treatment induced a transient elevation in the intracellular calcium concentration followed by a slow increase in ROS production. Calcium chelators and antioxidants did not affect the TCS-induced apoptosis, suggesting that calcium changes and ROS may not be involved in TCS-mediated apoptosis in K562 cells (Li et al. 2007a). TCS was also able to induce effective apoptosis in HIV-1 infected cells which was suggested to account, in part, for its antiviral activity (Wang et al. 2005).

Korean ML treatment resulted in a significant increase in ROS and loss of MMP in human hepatocarcinoma cells (Kim et al. 2004). Furthermore, treatment with the antioxidant *N*-acetyl-L-cysteine reduced ROS induction by ML, preventing apoptosis in Hep3B cells, indicating that oxidative stress is involved in ML-mediated cell death (Kim et al. 2004).

Ricin was shown to induce cell death in human cervical cancer cell line, HeLa which was mediated by the generation of ROS and subsequent activation of caspase-3 cascade followed by downstream events leading to apoptosis (Rao et al. 2005).

Viscum album agglutinin-I (VAA-I) was shown to induce apoptosis by ROS-independent mechanism as treatment with catalase, known to degrade H_2O_2, failed to reverse VAA-I-induced apoptosis (Lavastre et al. 2002).

Though there are reports on the activation of apoptosis exclusively by the mitochondrial pathway, in some instances apoptosis is induced by RIPs involving caspase-8 through the receptor-independent mitochondria-controlled apoptotic pathway as well. One such example is ML, ML-1 induced apoptosis in leukemic T- and B-cell lines where activation of caspase-8 has been observed along with

caspase-9 and -3 (Bantel et al. 1999). Since caspase-8 is implicated as a regulator of apoptosis mediated by death receptors, it is concluded that apoptosis induced by ML-1 is a receptor-independent mitochondria-controlled apoptotic pathway (Bantel et al. 1999).

Shiga toxin-1 (Stx1) has been shown to induce apoptosis in HeLa cells along with the activation of caspase-8, -6, and -3, loss of MMP, increased release of cytochrome c from mitochondria at 3 to 4 h post-treatment and DNA fragmentation (Fujii et al. 2003). It was concluded that the primary pathway of Stx1-induced apoptosis and DNA fragmentation in HeLa cells was unique and included caspases 8, 6, and 3 but was independent of events in the mitochondrial pathway (Fujii et al. 2003). Similarly, in macrophage-like cells, THP-1, Stx1 activated a broad array of caspases, disrupted the MMP and released cytochrome c into the cytoplasm (Lee et al. 2007). Earlier, it was shown that in THP-1 cells Stx1 and Stx2 activated caspase-3, and the apoptotic signals increased after Stx had reached the Golgi apparatus (Kojio et al. 2000).

Stress to organelles other than mitochondria can also induce apoptosis. Treatment of HL-60 cells with TCS demonstrated the involvement of mitochondrial pathway as there was reduction of MMP and release of cytochrome c and Smac besides the activation of caspase-9 (Li et al. 2007b). Furthermore, TCS treatment induced upregulation of endoplasmic reticulum chaperone BiP and transcription factor CHOP (CCAAT/enhancer-binding protein (C/EBP)-homologous protein), and also activated caspase-4, which for the first time strongly supported the involvement of the endoplasmic reticulum stress pathway in TCS-induced apoptosis (Li et al. 2007b). Subsequently, Stx1 was also shown to induce apoptosis through endoplasmic reticulum stress response in myelogenous leukemia cell line, THP-1 (Lee et al. 2008). Treatment of THP-1 cells with Stx 1 resulted in the increased activation of the ER stress sensors IRE1, PERK and ATF6, and increased expression of the transcriptional regulator CHOP and the death domain-containing receptor DR5 (Lee et al. 2008).

4.2 Activation of the Extrinsic Pathway of Apoptosis

There are few reports demonstrating the involvement of death receptors in the induction of apoptosis by RIPs. One such study demonstrated that apart from direct induction of apoptosis in response to inhibition of protein synthesis by the enzymic action of ML- A chain, it could also indirectly induce apoptosis in Fas+ tumor cells through activated FasL+ lymphocytes (Büssing et al. 1999).

Korean ML induced apoptosis in a human colon cancer cell line, COLO, and an antagonizing antibody against tumor necrosis factor receptor 1 was able to decrease activation of caspases, particularly caspase-8, in COLO cells treated with ML suggesting the possibility of the extrinsic pathway of apoptosis to be involved (Khil et al. 2007). Shiga toxin- 2 (Stx2) has been shown to induce apoptosis by activation of both the intrinsic and extrinsic pathways of apoptosis (Fujii et al.

2008). Similarly, Polito et al. (2009) provided evidence for the involvement of more than one pathway in the apoptosis induced by ricin and saporin. However, it was suggested that the activation of the extrinsic pathway may not be essential in apoptosis induced by these RIPs. There are few other reports mentioning the activation of caspase-8 by RIPs, however they have ruled out the involvement of the receptor pathways in the cell death (Bantel et al. 1999; Kiyokawa et al. 2001).

4.3 Impaired Balance Between and Pro- and Anti-Apoptotic Factors

In the normal cell there exists a delicate balance between the pro- and anti-apoptotic factors. The antiapoptotic factors, Bcl-XL and Bcl-2 are located in the outer mitochondrial membrane and promote cell survival, whereas the proapoptotic factors, Bax, Bid, Bak and Bad are in the cytosol where they act as sensors of cellular damage or stress. Some RIPs have been shown to alter the balance of pro- and anti-apoptotic factors by either increasing the expression of proapoptotic or decreasing the expression of antiapoptotic proteins.

ML was observed to induce apoptosis by down-regulation of Bcl-2 and up-regulation of Bax, thereby activating caspases in p53-positive, SK-Hep-1 and p53-negative, Hep 3B cell lines (Lyu et al. 2002). Induction of apoptosis by the *N*-acetyl-galactosamine-specific toxic lectin from *V. album*, ML-III in human lymphocytes has been shown to be associated with a decrease of nuclear p53 and Bcl-2 proteins and induction of telomeric associations (Bussing et al. 1998).

Apoptosis induced by Stxs (Stx1 and Stx2) in epithelial cell line HEp-2 was observed to be mediated through the enhanced expression of the proapoptotic protein Bax which could be blocked by the over expression of Bcl-2 by transient transfection (Jones et al. 2000). Subsequently, it was found that Bid, a proapoptotic member of the Bcl-2 family was also induced upon Stx1 treatment of HEp-2 cells followed by the activation of various caspases (Ching et al. 2002). Stx also induced cell death in human renal proximal tubular epithelial cells, HK-2 by stimulating the expression of proapoptotic protein Bak, and silencing of Bak gene gave partial protection against Stx-mediated apoptosis (Wilson et al. 2005). In another study, Stx-1 and Stx-2-induced death in endothelial cells was found to be accompanied by a dose dependent decrease in the expression of Mcl-1, an antiapoptotic Bcl-2 family member, with no change in the expression of Bcl-2 and Bcl-xl (Erwert et al. 2003). Mcl-1 is structurally similar to Bcl-2 except that it harbors two PEST sequences that target the protein for degradation by proteasome. Using proteasome specific inhibitors, the degradation of the Mcl-1 could be prevented which rescued the cells from Stx-induced apoptosis suggesting a role for Mcl-1 in protecting endothelial cells against Stx-1-induced apoptosis (Erwert et al. 2003).

TCS-induced apoptosis in HeLa cells was accompanied by a decreased expression of Bcl-2 and phosphorylation of cyclic AMP response element-binding protein (CREB), which regulates the expression of Bcl-2 (Wang et al. 2007). The study thus

suggested the possibility of CREB playing a critical role in the regulation of Bcl-2 expression in TCS-induced HeLa cell death (Wang et al. 2007).

Agrostin, a type 1 RIP isolated from the seeds of *Agrostemma githago* showed down-regulation of the intracellular level of Bcl-2 protein (Chiu et al. 2001). Ricin-induced apoptosis in hepatoma cells, BEL7404, was accompanied by increased expression of Bak and decreased levels of Bcl-xl (Hu et al. 2001). In a similar way, abrin-derived peptide (ABP) was also observed to induce apoptosis in Dalton's lymphoma which was marked by a reduction in the ratio of Bcl-2 and Bax protein expression, and consequently activation of caspase-3 (Bhutia et al. 2009).

Protein phosphorylation–dephosphorylation is one of the major signaling mechanisms for modulating the functional properties of proteins involved in gene expression, cell adhesion, cell cycle, cell proliferation, and differentiation. It has been shown that phosphorylation of Bcl-2 proteins regulates their ability to inhibit apoptosis (Adams and Cory 2001). Phosphorylated Bcl-2, Bad, and Bax have an antiapoptotic function and their dephosphorylation is required for proapoptotic activity (Verma et al. 2001). Khwaja et al. (2008) highlighted the potential phosphorylation and glycosylation sites on evolutionarily conserved residues of Bad, Bax and Bcl-2 proteins *in silico*, and suggested that ML-I may induce downstream signaling events that include alternative phosphorylation and O-GlcNAc modification of Bcl-2, Bax, and Bad for tumor cell apoptosis through binding to the cell surface receptors.

4.4 Induction of Apoptosis in Response to Ribotoxic Stress

The term ribotoxic stress was first used to describe the cellular response to toxicants that perturb the functioning of the 3′-end of the large 28S ribosomal RNA (Iordanov et al. 1997). During translation, the 3′-end of the large 28S rRNA functions in aminoacyl-tRNA binding, peptidyltransferase activity, and ribosomal translocation (Uptain et al. 1997). Toxin induced disruption of this activity results in the activation of various kinase pathways like JNK and p38 MAP kinase and/or alterations in ERK1/2 signaling (Iordanov et al. 2002, 1997; Iordanov and Magun 1998). In most cases, active ribosomes appear to be required as mediators of this signaling response and many of the inducers of the ribotoxic stress response at least partially inhibit protein synthesis. However, not all inhibitors of protein synthesis were able to elicit the ribotoxic stress response. Thus, it was proposed that ribotoxic stress response is specific for inhibitors that, either, induce damage to the α-sarcin/ricin loop of 28S rRNA or ADP-ribosylate the EF-2/EF-G and arrest translation at the translocation step (Iordanov et al. 1997).

The first evidence which highlighted the role of kinases in apoptosis came from a study with two different protein synthesis inhibitors, ricin and cycloheximide (Geier et al. 1996). Treatment of MDA-231 cells with ricin and cycloheximide induced apoptosis, and the results indicated the possibility of the involvement

of several distinctive pathways with protein kinase C also playing a role (Geier et al. 1996). Later, Iordanov et al. (1997) observed that ricin, α-sarcin and anisomycin were able to activate SAPK or JNK1 in Rat-1 cells. This study also suggested that activation of SAPK/JNK1 was not only due to protein synthesis inhibition, but also due to signaling from 28S rRNA triggered by the toxins. Thus, damage to 28S rRNA by RIPs resulted in ribotoxic stress response. Subsequently, ML was shown to induce apoptosis in cancer cells which was mediated by activation of JNK/SAPK (Kim et al. 2000). Furthermore, three distinct components of mistletoe, including β-galactoside- and *N*-acetyl-D-galactosamine-specific lectin II, polysaccharides, and viscotoxin were found to induce apoptotic cell death in U937 cells (Park et al. 2000). The mistletoe extracts markedly increased the phosphotransferase activity of JNK1/SAPK in these cells. Lectin II was the most potent in inducing apoptosis as well as JNK1 activation in U937 cells (Park et al. 2000). The ML-II-induced apoptosis in U937 cells was preceded by the activation of ERK1/2, p38 MAPK and SAPK/JNK (Pae et al. 2001). The apoptosis was significantly enhanced when ERK1/2 activation was selectively inhibited by PD098059, a MAP kinase inhibitor and was markedly reduced when an activator of ERK, 12-O-tetradecanoylphorbol-13-acetate, was used in U937 cells. Inhibition of p38 MAPK activity with p38-specific inhibitor, SB203580, partially inhibited lectin-II-induced DNA fragmentation. These results suggested that ERK1/2 and p38 MAPK may have opposite effects on cell survival in response to cytotoxic ML-II (Pae et al. 2001).

Two protein kinases, protein kinase A (PKA) and C (PKC), were shown to play a crucial role in apoptosis induced in cancer cells by Korean ML-II (Pae et al. 2000). The study demonstrated that exposure of human leukemia cells, HL-60 to ML-II induced apoptosis but the treatment of these cells with a PKA or PKC activator suppressed apoptosis. PKA and PKC inhibitors reversed the suppression of apoptosis by the activators, suggesting the involvement of PKA or PKC in the ML-II-induced apoptosis in HL-60 cells (Pae et al. 2000). The ML-II has also been shown to induce apoptotic cell death through Akt signaling pathway along with the inhibition of telomerase activity and the activation of caspase-3 (Choi et al. 2004). *Viscum album coloratum* agglutinin (VCA), isolated from Korean mistletoe induced apoptotic killing in hepatocarcinoma Hep3B cells which was preceded by a significant increase in ROS and loss of MMP (Kim et al. 2004). Treatment of Hep3B cells with VCA resulted in JNK phosphorylation which was abolished with the pretreatment of cells with a JNK inhibitor suggesting the necessary role of the phosphorylation in VCA-induced apoptosis. Furthermore, Hep3B cells overexpressing JNK1 or stress-activated protein kinase (SEK1) were more susceptible to cell death induced by VCA- (Kim et al. 2004).

A contradictory report on the role of PKC came to light using a specific inhibitor, participation of calcium-dependent proteases, or when PKC was excluded, in the apoptotic process induced by ricin (Hu et al. 2001).

Ricin- induced apoptosis was preceded by the release of TNF-α in a dose dependent manner in mouse macrophage cell line RAW 264.7 (Higuchi et al. 2003). However, galactose-specific ricin B-chain alone did not cause release of

TNF-α and apoptosis suggesting that receptor-binding of ricin through the B-chain is not enough. Inhibition of the release of TNF-α by pretreatment of the RAW 264.7 cells with a specific p38 MAP kinase inhibitor resulted in significant inhibition of ricin-induced apoptosis indicating that a specific attack on 28S rRNA by ricin resulting in ribotoxic stress and the activation of p38 MAP kinase are contributors to ricin-induced apoptosis (Higuchi et al. 2003). In case of ML, only the hololectin was able to induce apoptosis and isolated A- and B-chains were not cytotoxic (Vervecken et al. 2000).

Exposure of primary macrophages to ricin *in vitro* also led to the activation of SAP kinases, increased expression of proinflammatory mRNA transcripts and subsequently increase in the synthesis and secretion of TNF-α, and apoptotic cell death (Korcheva et al. 2007).

Tamura et al. (2003) demonstrated that in Vero cells the apoptosis signaling pathways, triggered by ricin were sensitized in butyric acid-treated cells, while the pathways leading to protein synthesis inhibition by the toxin were relatively unchanged.

Stx1 has also been shown to induce the ribotoxic stress response (Smith et al. 2003). Treatment of intestinal epithelial cell line, HCT-8 with Stx1 induced expression of c-jun and c-fos, and activated JNK and p38 within 1 h which persisted for 24 h. However, using a catalytically defective mutant toxin, in which the active site glutamate was replaced with aspartate, could not activate JNK and p38 indicating that RNA *N*-glycosidase activity is required for the induction of apoptosis. Moreover, blocking Stx1-induced p38 and JNK activation with the inhibitor SB202190 prevented cell death and was able to rescue cells from Stx- induced apoptosis (Smith et al. 2003). Treatment of macrophage-like cells, THP-1 *in vitro* with Stx1 resulted in the simultaneous induction of apoptotic and survival signaling pathways in these cells; and a limited apoptosis and prolonged JNK and p38 MAPK activation was observed (Lee et al. 2007). JNK is known to be involved in stress-induced apoptosis triggered via the mitochondria (Tournier et al. 2000). The absence of JNK causes a defect in the mitochondrial death signaling pathway, including the failure to release cytochrome c, thus indicating that mitochondria are influenced by proapoptotic signal transduction through the JNK pathway (Tournier et al. 2000).

Verotoxins (VT1 and VT2) stimulated a weak, transient increase in JNK activity and a strong activation of both p38 MAP kinase and ERK activity in human monocytes, which was sustained in the case of p38 MAP kinase 3 (Cameron et al. 2003). 293T cells expressing PAP did not show inhibition of translation even when approximately 15% of the ribosomal RNA was depurinated (Chan Tung et al. 2008). PAP expression induced the activation of JNK, and the enzymatically inactive mutant PAPx did not affect kinase activity. However, JNK activation did not result in apoptosis as there was an absence of caspase-3 and poly (ADP-ribose) polymerase cleavage. Thus, unlike other RIPs discussed above, the stress response triggered by PAP expression did not result in cell death (Chan Tung et al. 2008).

4.5 The Intrinsic Nuclease Activity of Toxins

As mentioned previously, RIPs possess many different types of activities and among them is their nonspecific DNase activity. RIPs like dianthin 30, saporin-6 and gelonin were identified to exert a specific nuclease activity on supercoiled DNA (Roncuzzi and Gasperi-Campani 1996). In the plasmid, pBR322 four specific sites of cleavage by dianthin 30 and saporin-6, and two specific sites of cleavage by gelonin were identified and mapped (Roncuzzi and Gasperi-Campani 1996). TCS has been shown to cleave the supercoiled double-stranded DNA and relaxed circular DNA to produce linear DNA (Li et al. 1991). In addition, TCS was observed to contain one calcium ion per protein molecule, suggesting a role for calcium in its endonucleolytic activity (Li et al. 1991). Stx1 was also shown to damage the single-stranded DNA by depurination (Brigotti et al. 2001). Ricin and Stx have been suggested to damage nuclear DNA in whole cells by means that are not secondary to ribosome inactivation (Brigotti et al. 2002). The non-specific degradation of DNA by RIPs can, in turn, induce apoptosis. It has been previously shown that most of the saporin-6 was found to be present in the nucleus before the onset of apoptosis (Bagga et al. 2003). Recently, an immunotoxin, StxA1-GM-CSF comprising of the catalytic domain of Stx, as the killing moiety, and GM-CSF as the cell targeting moiety showed the ability to induce apoptosis and DNA double strand breaks in different cell lines (Roudkenar et al. 2008).

4.6 Alternate Pathways

Though generally RIPs have been found to induce apoptosis by following the known classical pathways of apoptosis, they have also been shown to induce apoptosis through alternative pathways. In the subsequent section we highlight some studies providing evidences for alternate pathways being activated by RIPs to induce apoptosis.

4.6.1 PARP Activation Resulting in NAD^+ Depletion

PARP (poly(ADP-ribose)polymerase) is an abundant nuclear protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death. PARP, in response to DNA damage, undergoes auto-modification by forming poly (ADP-ribose) polymers using NAD^+ (Lindahl et al. 1995). A prolonged PARP activation leads to an excessive consumption of NAD^+ resulting in the depletion of ATP pool (Sims et al. 1983), which has been proposed as a mechanism for DNA damage-induced cell death in many cell types (Cherney et al. 1987). It has been conclusively shown that the depletion of NAD^+ levels as a result of PARP-1 hyperactivation induces mitochondrial damage and apoptosis (Chiarugi

and Moskowitz 2002; Yu et al. 2002). The first evidence for the involvement of PARP activation and NAD depletion came to light in the case of ricin-induced apoptosis (Komatsu et al. 2000). It was observed that U937 cells exposed to ricin showed an increase in PARP activity and depletion of intracellular NAD^+ and ATP. A PARP inhibitor, 3-aminobenzamide (3-ABA), prevented the depletion in NAD^+ and ATP levels and concomitantly protected U937 cells from the lysis that followed the ricin treatment (Komatsu et al. 2000).

Later, some RIPs, including ricin, saporin-L2, saporin-S6, gelonin and momordin, were observed to depurinate the automodified enzyme poly(ADP-ribosylated) poly(ADP-ribose) polymerase, thereby releasing adenine from the ADP-ribosyl group (Barbieri et al. 2003). It was suggested that depurination of auto-modified PARP could result in the inhibition of DNA repair pathway as well as the availability of PARP for further ADP-ribosylation, leading to depletion of intracellular levels of NAD^+ thus inducing apoptosis (Barbieri et al. 2003).

4.6.2 Down-Regulation of Telomerase

Telomerase is a cellular reverse transcriptase which adds DNA sequence repeats, TTAGGG to the 3′ end of DNA strands in the telomere regions in all vertebrates, thus providing stability to the chromosomes. The enzyme is usually not active in normal somatic cells and is specifically activated in many malignant cells. Several protooncogenes and tumor suppressor genes either directly or indirectly have been implicated in the regulation of telomerase activity (Liu 1999). Telomerase dysfunction has been found to be a key determinant in governing the sensitivity to anticancer agents (Lee et al. 2001).

Korean ML was shown to induce apoptosis in hepatocarcinoma cells by inhibiting the telomerase activity (Lyu et al. 2002). ML induced apoptosis in both p53-positive, SK-Hep-1 and p53-negative, Hep 3B cells through down-regulation of telomerase activity. Telomerase activity in p53 positive cells was greatly reduced after 24 h of treatment with ML, whereas the telomerase activity decreased gradually in p53 negative cells (Lyu et al. 2002). Subsequently, it was observed that the inhibition of telomerase activity and induction of apoptosis resulted from decreased phosphorylation of Akt survival signaling pathways (Choi et al. 2004).

4.6.3 Inhibition of Histone Deacetylase

Histone deacetylases (HDACs) catalyze the removal of acetyl groups from N-terminus of histones, leading to chromatin condensation and transcriptional repression. Recently, a 30-kDa type I RIP, MCP 30 isolated from bitter melon, *Momordica charantia* seeds has been shown to induce apoptosis as a result of inhibition of HDACs (Xiong et al. 2009). Furthermore, it was found that MCP 30 could also promote acetylation of histone-3 and -4 proteins (Xiong et al. 2009).

4.6.4 Degradation of Cytoskeleton Proteins

Cytoskeleton proteins, e.g., actin, lamin and tubulin, provide mechanical support to the cells and hardwire the cytoplasm with the surroundings to support signal transduction. *V. album* agglutinin-I (VAA-I) induced apoptosis in eosinophilic AML14.3D10 (3D10) cells was found to be associated with the degradation of lamin B1 and activation of caspase-1, -2, -3, -4, -7, -8, -9, and -10. VAA-I induced gelsolin degradation was reversed by the pan-caspase inhibitor *N*-benzyloxycarbonyl-V-A-D-O-methylfluoromethyl ketone (z-VAD). Also, paxillin, vimentin and lamin B1 were cleaved by caspases in VAA-I-induced 3D10 cells (Lavastre et al. 2005). Moreover, treatment of purified human eosinophils with VAA-I was found to induce apoptosis, degradation of gelsolin and lamin B1, but unlike 3D10 cells, cleavage of lamin B1 and cell apoptosis was not reversed by z-VAD in eosinophils (Lavastre et al. 2005).

4.6.5 Nitric Oxide-Mediated Apoptosis Pathway

TCS was found to induce apoptosis by increasing the expression of inducible nitric oxide synthase (iNOS)mRNA expression and protein levels and this phenomenon was significantly inhibited when L-NIL, a specific inhibitor of iNOS, was added to the cells treated with TCS (Li et al. 2005).

5 Conclusion

It is now clearly evident that most RIPs induce apoptosis. Generally, the apoptosis induced by RIPs involves the caspase dependent mitochondrial pathway, and is independent of protein synthesis inhibition. The triggers include ROS, ribotoxic stress, activation of kinases and in some instances consequences of the direct enzymatic activities of RIPs. RIPs have now been acknowledged as multifunctional proteins which may account for the absence of a single common pathway for the induction of apoptosis by them.

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The Synthesis of *Ricinus communis* Lectins

Lorenzo Frigerio and Lynne M. Roberts

Abstract *Ricinus communis* agglutinin II (ricin) and *R. communis* agglutinin I are cytotoxic seed lectins whose study has contributed to our understanding of precursor synthesis, membrane translocation, ribosome inactivation, intracellular trafficking, vacuolar targeting, protein assembly and quality control in plant cells. This chapter will focus largely on the targeting, quality control and protein assembly of these two closely related sugar binding, ribotoxic proteins.

1 Introduction

The two lectins from the seeds of *Ricinus communis* (castor oil plant) are carbohydrate-binding proteins, each possessing at least two sugar-binding sites. As such they can agglutinate cells, as was first observed over a century ago by Stillmark (1889) who was studying the toxic effects of castor bean extracts on blood. The toxic agglutinating factor he discovered was a protein he termed ricin. Much later, it was shown that the toxic and agglutinating properties reside in two distinct but closely related proteins: ricin (known alternatively as *R. communis* agglutinin II; RCA II), which is a relatively weak haemagglutinin but a very potent cytotoxin, and *R. communis* agglutinin I (RCA I), which is a strong haemagglutinin showing weak cytotoxicity (Nicolson et al. 1974; Olsnes et al. 1974). Since these proteins were derived from plants, they came to be known as phytohaemagglutinins, but were later classified as 7S globulins, vicilin-like lectins, and as type II ribosome-inactivating proteins (RIPs).

Besides providing the blueprint for the biosynthesis and cell biology of type II RIPs, the study of the synthesis of ricin and RCA has shed light on several

L. Frigerio (✉) and L.M. Roberts
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
e-mail: Lorenzo.Frigerio@warwick.ac.uk

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_10,

important cellular processes such as translocation into the endoplasmic reticulum (ER), ER quality control and protein sorting to storage vacuoles. In particular, the study of ricin A chain provided the first indication for the presence of an operational ER-associated degradation (ERAD) machinery in plant cells (Di Cola et al. 2001). The study of vacuolar sorting of proricin challenged the "multivacuole hypothesis" and contributed to a revision of the current model for sorting to such organelles (Frigerio et al. 2008).

The history, cell biology, toxicity and potential uses of ricin have been reviewed extensively (Lord et al. 1994; Frigerio and Roberts 1998; Olsnes 2004; Audi et al. 2005). In this chapter, we focus on recent advances in the understanding of the synthesis and intracellular fate of ricin, RCA and their individual sub-units.

2 Ricin

2.1 Synthesis and Quality Control of Proricin

Ricin is the best known of the castor bean lectins, primarily because it is the archetypal member of the A–B family of plant and bacterial proteins that are potently toxic to the protein synthesis machinery of eukaryotic cells. Over the years, this has led to its use in cell ablation, particularly as the toxic component of immunotoxins targeted to malignant cells (see Kawakami et al. (2006) for a recent review). Recent terrorist attacks have further increased research efforts into the mode of entry and action of ricin in animal cells, and have intensified the search for a protective vaccine or antidote (Audi et al. 2005; Marsden et al. 2005).

The castor bean genome encodes approximately eight isoforms of ricin including ricin D and ricin E, although at least three of these are non-functional (Tregear and Roberts 1992). All ricin isoforms are heterodimeric lectins. Ricin holotoxin itself is composed of a 32-kDa catalytic, ribosome-inactivating A chain (RTA) disulfide-linked to a 34 kDa B chain (RTB). The latter is a bilobal polypeptide in which each globular domain contains a sugar binding pocket that can interact with one molecule of either galactose or *N*-acetyl galactosamine (Nicolson et al. 1974). A detailed description of the enzymatic activity and biological properties of ricin is provided in other chapters of this book.

Expression of the ricin genes is both developmentally regulated and tissue specific, with synthesis occurring exclusively within the endosperm cells of maturing seeds (Peumans et al. 2001). Here it becomes stored in protein storage vacuoles (PSV) in common with the other major storage proteins until, at seed dessication, it accounts for around 5% of the total particulate protein (Tully and Beevers 1976; Youle and Huang 1976). The most obvious biological role of ricin in castor bean seeds is that of a storage protein. Following imbibition, programmed cell death permits mobilisation of storage reserves to the cotyledons (Schmid et al. 1999, 2001; Gietl and Schmid 2001) although during early germination the 7S lectins are

hydrolysed more slowly than the 11S (legumin-like) crystalloid proteins and the 2S albumins (Gifford et al. 1982). Alternatively, the conserved activity of ricin isoforms and their relatedness to other cytotoxic RIPs has prompted suggestions of a defensive function (Hartley et al. 1996).

2.1.1 Synthesis and ER Translocation

Ricin is synthesised during the endosperm maturation stage in which the testa is clearly evident (Roberts and Lord 1981). The primary translation product is a 576 residue precursor (preproricin) comprising at the N-terminus of a 26-amino acid signal peptide to permit ER import and a 9-residue propeptide that is later cleaved (Ferrini et al. 1995; Jolliffe et al. 2006). This is followed by RTA linked to RTB via a 12 amino acyl residue linker propeptide (Harley and Lord 1985; Lamb et al. 1985). Preproricin is co-translationally *N*-glycosylated (Lord 1985a) and multiply disulphide bonded within RTB with an additional disulphide between the mature RTA and RTB sequences (Lord 1985b). In this precursor form that now lacks the 26 residue signal peptide, but retains the two propeptides, the single chain proricin molecule is catalytically inactive. This is because the sugar binding RTB sterically hinders the catalytic site of RTA (Richardson et al. 1989). Indeed, this masking persists in the mature heterodimer (Montfort et al. 1987) but, during the entry into mammalian cells, the active site of RTA becomes exposed once RTA and RTB are reductively cleaved – an event that appears to occur in the ER of intoxicated cells (Spooner et al. 2004).

The fact that *Ricinus* cells are manufactured as inactive precursors perhaps explains why they are able to transport and store such large amounts of ricin even though endogenous ribosomes are susceptible to its action, albeit much less so than animal ribosomes (Cawley et al. 1977; Harley and Beevers 1982; Taylor et al. 1994). In this way, should any precursor be inefficiently or incompletely imported, the hindered active site would help prevent ribosome inactivation.

2.1.2 Anterograde Trafficking

ER import is mediated by a 26-residue signal peptide. The subsequent 9-residue propeptide that becomes exposed at the N-terminus after signal peptide cleavage and that precedes the proricin sequence acts as a spacer that influences the efficiencies of both co-translational import and glycosylation (Jolliffe et al. 2006). Import of the precursor is accompanied not only by signal peptide cleavage and *N*-glycosylation but also by disulphide bond formation. Proricin is then transported to the PSV through the secretory pathway. Early radiolabelling experiments have shown that proricin travels through the Golgi complex where the glycans on both chains become processed in a Golgi-specific manner (Lord 1985a). Indeed, one of the two RTA glycans (but neither of the two B chain glycans) contains an α-1,3 fucose linked to the proximal *N*-acetylglucosamine residue (Lord and Harley 1985)

by Golgi-located fucosyl transferase (Zhang and Staehelin 1992). The presence of this fucose renders the oligosaccharide resistant to peptide:*N*-glycanase F (Di Cola et al. 2001).

Ricin is processed to its mature disulfide-linked heterodimeric form by removal of both the N-terminal 9-residue propeptide and the 12-amino acid internal "linker" propeptide (Harley and Lord 1985). The enzyme responsible is likely to be a cysteine proteinase known as vacuolar processing enzyme (VPE) (Hara-Nishimura et al. 1993) that cleaves on the C-terminal side of asparagine residues. Both proricin propeptides terminate in asparagine. This type of proteolysis is typical in the maturation of seed storage proteins (Hara-Nishimura et al. 1991, 1995; Hiraiwa et al. 1997; Shimada et al. 2003; Wang et al. 2009), although induced VPE has also been identified as a caspase involved in vacuole-mediated cell death in vegetative tissues (Hatsugai et al. 2004; Hara-Nishimura et al. 2005). In the seeds, VPE is transported to vacuoles in latent form and is only active in the vacuolar matrix following its activation (Hara-Nishimura et al. 1993). Despite the generation of mature ricin throughout seed maturation process the cells show no signs of ribosome damage. This strongly indicates that reduction of the interchain disulphide bond and translocation of the active RTA to the cytosol does not occur from PSV. Thus, the generation of active holotoxin only when sheltered in vacuoles is yet another safeguard that permits *Ricinus* seeds to store large quantities of a highly potent toxin without putting its own survival at risk.

The biosynthetic events in castor bean endosperm have been recapitulated in tobacco protoplasts using transient expression and metabolic labelling (Frigerio et al. 1998b). These experiments provided a first indication that the linker propeptide may contain the vacuolar sorting signal. When constructs encoding preproRTA and preRTB were co-expressed, RTA and RTB assembled into disulfide-linked heterodimers which were completely secreted, rather than targeted to the vacuole (Frigerio et al. 1998b). The linker peptide was sufficient to restore vacuolar sorting of the RTA–RTB heterodimers when appended to the C-terminus of RTA or to the N-terminus of RTB (Frigerio et al. 2001a). The linker peptide was also sufficient to target two reporter proteins, secreted phaseolin (Frigerio et al. 1998a) and GFP/RFP, to the vacuole (Frigerio et al. 2001a; Hunter et al. 2007). The 12 residue intervening linker peptide contains the amino acid motif LLIRP, which is reminiscent of the "sequence-specific" vacuolar sorting signal (ssVSS) NPIRL, found in proteins such as sweet potato sporamin (Matsuoka and Nakamura 1991; Koide et al. 1997) and barley aleurain (Holwerda et al. 1992). The bulky hydrophobic side chain of isoleucine has been identified as the crucial residue for sorting within this motif (Matsuoka and Nakamura 1999). Indeed, mutagenesis of Ile to Gly in the ricin linker resulted in complete secretion of proricin (Frigerio et al. 2001a) or fluorescent proteins (Hunter et al. 2007), indicating that the linker is a bona fide, ssVSS. The linker was also the first internal propeptide to be identified that carries vacuolar sorting information (Vitale and Raikhel 1999). It was subsequently shown that the position of the ricin linker within a reporter protein was important, with the linker functioning in an isoleucine-dependent manner when positioned at the N-terminus but not at the C-terminus, thus reinforcing the idea that this was a "canonical"

ssVSS (Jolliffe et al. 2003). The description of the internal ssVSS of proricin was followed by the discovery that propeptide II of castor bean pro2S albumin also contains a ssVSS (Brown et al. 2003). This finding also indicated that leucine can perform the same function of isoleucine within the VSS. More recently, the analysis of the *Arabidopsis* vacuolar proteome has revealed that the consensus sequence for ssVSS is indeed less strict than initially anticipated on the basis of a handful of model proteins containing NPIR and allows for the presence of leucine as well as isoleucine (Carter et al. 2004).

For the last 15 years, the working model for vacuolar sorting hypothesised the existence of separate vacuoles within plant cells: a vegetative, lytic vacuole and a PSV (Paris et al. 1996). Accordingly, at least two vacuolar sorting pathways were identified (Matsuoka et al. 1995): one mediated by ssVSS and directed to the lytic vacuole, and one mediated by C-terminal, hydrophobic VSS and directed to the PSV (reviewed in (Matsuoka and Neuhaus 1999; Vitale and Raikhel 1999; Vitale and Hinz 2005). Therefore the model predicted that all proteins carrying a ssVSS would be recognised by a trans-Golgi located vacuolar sorting receptor (first named BP-80, than renamed VSR; (Paris and Neuhaus 2002)) and delivered to the lytic vacuole. As the VSR was first identified in clathrin-coated vesicle (CCV)-enriched preparations (Kirsch et al. 1994; Ahmed et al. 1997), the hypothesis was that VSS-carrying proteins would be packaged into CCV and delivered to the lytic vacuole. Proteins directed to the PSV were hypothesised to follow a distinct route, possibly involving aggregation (Castelli and Vitale 2005) and exit from early Golgi stacks (Hillmer et al. 2001), but no interaction with VSR (Robinson et al. 2005; Vitale and Hinz 2005). Therefore the discovery that two castor bean proteins, proricin and pro2SA, carry ssVSS and yet are targeted to PSV challenged this model and raised the question as to whether these proteins would interact with a VSR-like receptor. Affinity chromatography experiments using immobilised ricin linker peptides identified castor bean proteins cross-reacting with *Arabidopsis* VSR antibodies in an exquisitely sequence-specific manner, thus strongly suggesting that two storage proteins are indeed VSR ligands in maturing castor bean endosperm (Jolliffe et al. 2004). Sub-cellular fractionation also revealed that proricin co-fractionated with VSR and clathrin heavy chain (Jolliffe et al. 2004). More recently, a secretory reporter (red fluorescent protein) carrying the ricin linker VSS (Hunter et al. 2007) was found to be missorted to the apoplast in mutant *Arabidopsis* seeds lacking the most abundant VSR isoform, VSR1 (Craddock et al. 2008). Taken together, these observations indicate that ricin is a bona fide VSR substrate. The wider implication of these findings is that VSR may be central to the sorting of the majority of vacuolar proteins, including storage proteins. Therefore the distinction between vacuolar sorting signals and indeed sorting routes may be less well-defined than originally predicted. In addition, the fact that the existence of multiple vacuoles within plant cells seems to be the exception rather than the rule (Frigerio et al. 2008) and the recent finding that proteins carrying different types of VSS have been observed to converge at the prevacuolar compartment (Miao et al. 2008) seems to indicate that multiple transport routes may serve the same vacuolar destination.

2.2 *Ricin A Chain: ER Synthesis and Turnover in the Cytosol*

As tobacco ribosomes are less sensitive to RTA than their mammalian counterparts (Taylor et al. 1994), tobacco protoplasts have provided a useful system in which to understand the behaviour of single ricin sub-units in vivo. Transient expression of preproRTA in tobacco protoplasts revealed two striking phenotypes: RTA was turned over very rapidly but was also toxic to the cells (Frigerio et al. 1998b). Toxicity was measured by the co-expression of a translational reporter (phaseolin) in the ricin-transfected cells. Synthesis of phaseolin was unaffected by the expression of preproricin, as expected given the sequestration of this catalytically inactive protein within the endomembrane system. Expression of RTA alone however caused a significant (~40%) reduction in phaseolin synthesis. This toxicity of RTA could be abolished by co-expression of RTB. Under these conditions, the two sub-units formed disulfide-linked heterodimers within the ER lumen that were subsequently secreted (Frigerio et al. 1998b). These findings indicated that RTA was being efficiently translocated into the ER lumen, as testified by the fact that it acquired N-linked glycans, but then was subsequently able to reach the cytosol and inactivate ribosomes. Moreover, the permanence of RTA in the ER was short-lived. It was shown that RTA degradation and toxicity were insensitive to treatment with the fungal metabolite brefeldin A (BFA) (Robinson et al. 2008). This provided a general indication that degradation and exit from the ER were independent of trafficking through the Golgi and hinted that the degradation of RTA was occurring within – or in close proximity of – the ER (Pedrazzini et al. 1997).

The discovery of ERAD (Sommer and Jentsch 1993; Jensen et al. 1995; Ward et al. 1995; Wiertz et al. 1996) provided a timely framework for the study of RTA degradation in plant cells. In this branch of protein quality control, which is now particularly well characterised in mammalian and yeast cells (see Vembar and Brodsky 2008) for a review, and elsewhere in this book), newly synthesised proteins within the ER are scrutinised by an elaborate array of chaperones and enzymes that serve as the primary mediators of ER quality control. This scrutiny helps ensure that only those proteins that fold and assemble correctly are allowed to exit this organelle for onward transport to other destinations. However, mutations, transcription/translation errors, inefficient folding, unbalanced synthesis of oligomeric protein sub-units, various stresses, and protein ageing can each contribute to the presence of misfolded or unassembled polypeptides in the ER. Such proteins would be damaging if allowed to accumulate so, in ways that are not yet fully understood, they are singled out from proteins on the correct folding pathway and retained. If acknowledged as terminally misfolded they are then retrotranslocated to the cytosol via some kind of channel, recognised on the cytosolic plane of the membrane by the ubiquitylation machinery, extracted by the cytosolic CDC48/p97 complex, deglycosylated (if glycosylated) by cytosolic peptide:*N*-glycanase and ultimately degraded by 26S proteasomes, as described elsewhere in this book. The individual factors that are required for each ERAD substrate may depend on the

location of the recognised lesion (Vashist and Ng 2004; Carvalho et al. 2006; Denic et al. 2006), although for some substrates, export to the cytosol is metabolically controlled (DeBose-Boyd 2008).

For free RTA, a protein that can be readily expressed in functional form in a variety of systems, it is assumed that transport back to the cytosol in plant cells will involve some degree of "assisted" or "spontaneous" unfolding or exposure of a normally hidden hydrophobic surface in the ER environment. This is based on evidence from mammalian cells where ricin containing a constrained RTA showed impaired cytotoxicity (Argent et al. 1994; Beaumelle et al. 1997). How such unfolding occurs is not known with certainty but it may involve lipid interactions accompanied by structural change (Day et al. 2002; Mayerhofer et al. 2009) However, since the trigger for expulsion of RTA to the cytosol in plant cells remains unclear, just how strong is the evidence to support such an event? Pulse-chase analyses in transfected tobacco cells have shown that following its retrotranslocation into a soluble fraction away from the ER marker BiP, RTA degradation could be largely prevented with proteasomal inhibitors such as clasto-lactacystin-β-lactone and is preceded by deglycosylation (Di Cola et al. 2001), an event known to involve cytosolic peptide:*N*-glycanase (Suzuki et al. 1998, 2000; Park et al. 2001) These two findings, together with the insensitivity of the degradation process to anterograde trafficking inhibitors, indicate that orphan RTA is handled by an ERAD-like process in plant cells (Di Cola et al. 2001).

Although RTA was the first example of a protein utilising an ERAD-like pathway in plant cells, others have since been reported (Brandizzi et al. 2003; Muller et al. 2005; Hong et al. 2008). Nevertheless we remain almost completely ignorant, not only of the full range of plant proteins that undergo ERAD in plant cells, but of the endogenous proteins devoted to its operation. In *Arabidopsis*, over 1,300 genes (5% of the proteome) are devoted to the selective breakdown of proteins by the ubiquitin/26S proteasome pathway (Smalle and Vierstra 2004) although virtually nothing is known about these components in relation to ERAD, nor of the overall impact of this pathway relative to alternative disposal in vacuoles (Carter et al. 2004; Contento et al. 2004). Homologues of ERAD proteins certainly exist in all *Arabidopsis* tissues (Schmid et al. 2005) and in other plants (Kirst et al. 2005) suggesting that they are also likely to be expressed in tobacco and *R. communis* as well.

More recently, the ERAD-like pathway of RTA in tobacco cells has been further characterised. This toxin subunit contains only two lysine residues. Since lysine is the primary target for ubiquitylation – a common step in mammalian/yeast ERAD – this paucity helps explain why RTA can partially escape degradation in animal cells (Hazes and Read 1997). When expressed in protoplasts, a mutant RTA completely devoid of lysine (RTA0K) was considerably more stable in the cytosol and significantly more toxic to protoplasts than native RTA (Di Cola et al. 2005). Furthermore, a deglycosylated form of this protein could be observed in cytosolic fractions even in the absence of proteasomal inhibitors, supporting the notion that peptide:*N*-glycanase acts prior to proteasome delivery and not on cleaved peptides following

delivery (Di Cola et al. 2005). Conversely, a more rapid degradation and a concomitant reduction in toxicity were observed after engineering additional lysines into RTA (RTA6K). This observation mirrors findings in animal cells (Deeks et al. 2002). However the increased potency of RTA0K (which was not observed in animal cells) strongly indicates that in plant cells, the two naturally occurring lysine residues are canonical targets for ubiquitylation. Again, this suggests a safeguard to the plant should any mistargeting or premature termination of translation occur in the ricin producing cells. Interestingly, modulating lysine content had no effect on the rate of dislocation of RTA from the ER. Rather, it affected only stability and therefore toxicity once in the cytosol (Di Cola et al. 2005). This is rather different from the tight coupling between ER dislocation, ubiquitylation and degradation typically observed in animal cells (de Virgilio et al. 1998). It has also been demonstrated that dislocation of RTA requires the AAA ATPase complex, CDC48 (Lord et al. 2002). Co-expression of a dominant-negative variant of CDC48 together with RTA prevented retrotranslocation of the latter with a corresponding reduction in ribotoxicity (Marshall et al. 2008).

Attempts have been made to visualise the retrotranslocation process by generating green fluorescent protein (GFP) fusions to RTA. However, expression of both N- or C-terminal GFP-RTA fusions by agro-infiltration in tobacco protoplasts (Sparkes et al. 2006) resulted in the illumination of ER and Golgi bodies (Fig. 1), suggesting that the fusions were being transported to vacuoles where GFP is no longer fluorescent (Tamura et al. 2003). Such data indicates that fusion to a tightly folded protein such as GFP is probably sufficient to rescue RTA from ERAD, making direct visualisation of the ERAD process problematic. The altered localisation of RTA serves as a note of caution when using GFP fusions in plant cells (Moore and Murphy 2009).

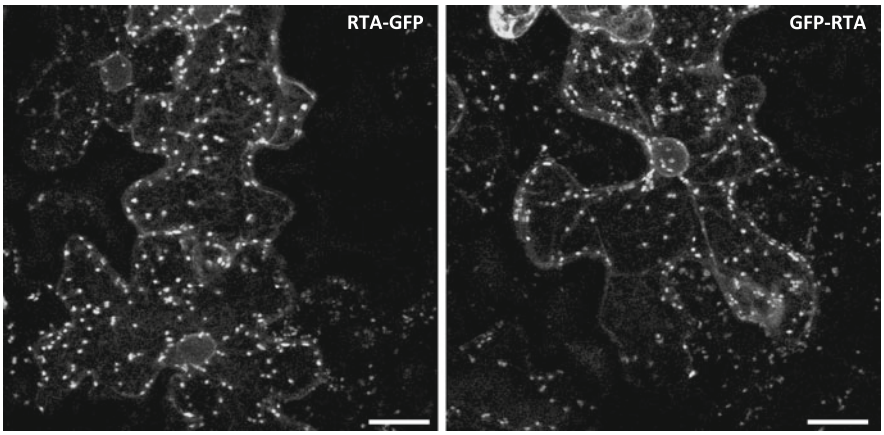

Fig. 1 Fusions between RTA and GFP localise to the ER and the Golgi complex. Tobacco leaves were infiltrated with agrobacteria harbouring the indicated constructs and visualised by confocal microscopy after 72 h. *Bars*, 20 μm

2.3 Ricin B Chain: Synthesis and Quality Control

When expressed in transfected or transgenic tobacco protoplasts in isolation of RTA, only a proportion (~25%) of newly made RTB adopts a conformation able to bind galactose that permits its secretion via the Golgi in an endoglycosidase H (endoH)-resistant form (Frigerio et al. 1998b; Chamberlain et al. 2008). Pulse-chase analysis revealed that most RTB is retained as an endoH-sensitive species, much of it in association with BiP, with a significant amount (~40%) then disappearing over a period of time. However, unlike RTA, RTB degradation could not be inhibited by proteasome inhibitors or by a mutant CDC48 in which the conserved glutamate residues of the Walker B motifs (E308 and E581) of the two ATPase domains had been replaced by glutamine (Chamberlain et al. 2008). These data suggest that if degradation is occurring in the cytosol then it involves a novel CDC48- and proteasome-independent pathway. Vacuolar degradation was ruled out in this study since the turnover was independent of vacuole-dependent processing of an appended propeptide and was insensitive to BFA treatment and to the inhibition of COPII-dependent ER export (Chamberlain et al. 2008) via overexpression of Sec12 (Phillipson et al. 2001). Transport to the vacuole on a route that bypasses the Golgi complex (Frigerio et al. 2001b) was also excluded, and deliberate signal peptide-mediated anchoring in the ER as an endoH-sensitive RTB did not prevent its disappearance either. Collectively, these data support the degradation of RTB within the early secretory pathway itself (Chamberlain et al. 2008). Although the proteases responsible have not yet been isolated, if proven to exist in the ER/Golgi compartment this would represent a novel location for protein degradation in plant cells that adds to the ERAD pathway exceptions, reported in other systems (Schmitz and Herzog 2004).

3 RCA 1

RCA 1 (hereon RCA) is a tetramer composed of two ricin-like heterodimers. The A chain of RCA is 93% identical to RTA, while the two B chains are 84% identical (Roberts et al. 1985). While RTB has two glycans, RCA B chain possesses three, with the additional glycan containing a fucose residue (Lord and Harley 1985). This additional oligosaccharide accounts for the higher apparent molecular mass of RCA B chain (~36 kDa) compared with RTB (34 kDa). Two of the 18 differences between the respective A chains involve the substitution of cysteine residues in RCA, one of which (Cys 156) is reported to form a disulphide bond with an adjacent molecule to generate a mature ~128 kDa lectin with the subunit arrangement B–A–A–B (Cawley and Houston 1979; Sweeney et al. 1997). In contrast to ricin, RCA interacts solely with galactose and not with *N*-acetylgalactosamine, a feature that permits convenient separation of these closely related lectins from affinity columns during protein purification (Nicolson et al. 1974).

3.1 RCA Synthesis and Assembly

The signal peptide and two propeptides in the RCA precursor are identical to the equivalent sequences in preproricin (Roberts et al. 1985), and events in transport are essentially the same for both lectins (Butterworth and Lord 1983). However, the assembly of RCA into a disulphide bonded tetramer has not been studied until recently. It appears that in the castor oil seed, single chain proRCA precursors are folded and glycosylated in the ER such that they are competent for transport without assembly into precursor dimers. Consequently, they are delivered to vacuoles along a BFA-sensitive pathway that involves trafficking through the Golgi and fucosylation of one of the RCA B chain glycans (Lord and Harley 1985). Upon arrival, proRCA molecules are proteolytically processed to remove the propeptides. Surprisingly, the assembly into B–A–A–B tetramers takes place within PSV rather gradually leading eventually to a mixed population of B–A and B–A–A–B forms of RCA in mature endosperm. This suggests that the ER may not be the only site for disulphide bond formation in plant cells (Marshall et al. 2010).

4 Concluding Remarks

Well characterised plant lectins are present in the Leguminosae, Euphorbiacae, Gramineae, and Solanaceae. The synthesis of most shares features with plant storage proteins. The abundance of the castor bean lectins suggests that they too are storage proteins, although their RIP activity and resultant cytotoxicity has prompted suggestions that they may also have a defensive role. Studies of ricin in particular have led to a better understanding, not only of the roles of individual segments of this fascinating toxin and its precursor, but also of a number of important cell biological processes in plant cells. It seems likely that the castor bean RIPs will continue to be used as reporters in investigations of protein assembly and turnover, and in the fine dissection of membrane translocation and trafficking to vacuoles.

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How Ricin Reaches its Target in the Cytosol of Mammalian Cells

Robert A. Spooner, Jonathan P. Cook, Shuyu Li, Paula Pietroni, and J. Michael Lord

Abstract The cytotoxic plant protein ricin comprises a lectin B chain that binds promiscuously to glycolipids and glycoproteins at the surface of mammalian cells, disulphide-coupled to a toxic A chain which depurinates target ribosomes. To find these cytosolic targets, the A chain has to cross a biological membrane, which is not a simple task for a folded protein. The secretory pathway of eukaryotic cells is reversible and ricin can take advantage of this to move from the plasma membrane, via the Golgi, to the ER whose membrane is crossed to gain access to the cytosol. Since membrane traversal is preceded by an unfolding step, there is a clear requirement for cytosolic re-folding of ricin to gain a catalytic conformation. This final step for ricin is accomplished after triage by cytosolic chaperones, underlining the central role of these in cellular protein folding.

1 Introduction

The cytotoxic heterodimeric plant protein ricin is laid down in the developing endosperm of the seeds of the castor oil plant, *Ricinus communis*, where it constitutes up to 5% of the dry mass of the seed protein (Harley and Beevers 1986). Its B chain (RTB) is a lectin, interacting with terminal non-reducing galactose residues (exposed β1 → 4 linked galactosyls) (Olsnes et al. 1974); thus RTB binds promiscuously to many glycolipids and glycoproteins at the surface of mammalian cells. RTB acts as a delivery agent for the catalytic toxic A chain (RTA), which depurinates target ribosomes (Endo et al. 1987). To find

R.A. Spooner (✉), J.P. Cook, S. Li, P. Pietroni, and J.M. Lord
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
e-mail: R.A.Spooner@warwick.ac.uk; J.P.Cook@warwick.ac.uk; Shuyu.Li@warwick.ac.uk; P.Pietroni@warwick.ac.uk; and Mike.Lord@warwick.ac.uk

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_11,

these cytosolic targets, RTA has to cross a biological membrane, which is not a trivial task for a folded protein.

2 Cytotoxicity Model

The model for intoxication of target mammalian cells by ricin is deceptively simple (Fig. 1a). Ricin holotoxin binds cell-surface receptors and is then internalised and trafficked in a retrograde manner, via early/sorting endosomes and the Golgi apparatus, to the endoplasmic reticulum (ER), where RTB and RTA are separated. Free RTA is then recognised as an unstable or misfolded protein and is exported (retrotranslocated/dislocated) to the cytosol for its destruction, using pre-existing cellular mechanisms for clearing misfolded proteins from the ER. However, instead of being destroyed, at least a proportion of cytosolic RTA gains an active catalytic conformation that depurinates the target ribosomes. Some details of this model are known, but many remain obscure.

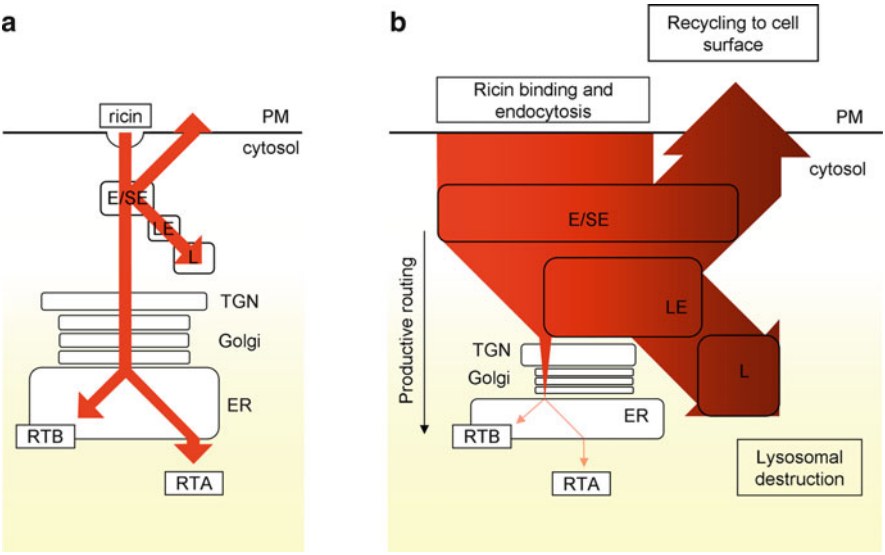

Fig. 1 Ricin trafficking schemes. (**a**) Receptor-bound ricin at the cell surface (plasma membrane, PM), is taken up by endocytosis into early/sorting endosomes (*E*/*SE*). From here proceed multiple routes – a recycling path back to the cell surface, a destructive track via the late endosome (*LE*) – lysosome (*L*) pathway, and a productive cytotoxic route via the *trans*-Golgi network (*TGN*) and the Golgi stack to the endoplasmic reticulum (*ER*). Here the A (*RTA*) and B (*RTB*) chains of ricin are separated reductively, and free RTA dislocates, crossing the ER membrane to enter the cytosol. (**b**) These routes are not equal: when the width of the *arrows* is adjusted to represent the approximate flow of ricin through these pathways, it becomes clear that the majority of ricin traffics endosomally, and the major cytotoxic route implied in *a* is utilised by only a small proportion of the ricin bound initially at the cell surface

3 Cell Entry

3.1 Cell Surface Events Remain Cryptic

Upon cell binding, the bulk of ricin is slowly internalised via clathrin-coated pits, but if coated pit formation at the cell surface is arrested then ricin cytotoxicity is unaltered, even though there is a 50% reduction in overall ricin internalisation (Moya et al. 1985; Sandvig et al. 1988). Thus productive (i.e. cytotoxic) routing of ricin does not appear to depend upon the recruitment of receptors to clathrin-coated pits. Furthermore, interfering with caveolar function makes little difference to ricin toxicity, suggesting that productive routing is caveolae-independent (Simpson et al. 1998). This invokes either a third endocytotic route or suggests that ricin intoxication involves multiple productive receptors that can enter cells via multiple mechanisms, so that the effect of inhibiting individual entry pathways makes little overall difference.

Favouring this latter interpretation is the promiscuous, low affinity, high capacity binding of ricin to cells (Sandvig et al. 1976; Spooner et al. 2004). To date, the identity of any ricin receptors that are required for cytotoxicity is unknown. The majority are likely to be proteinaceous, since manipulating cellular levels of glycosphingolipids does not measurably affect ricin toxicity, suggesting that ricin receptors are not glycolipid in nature (Spilsberg et al. 2003). However, cells which are unable to synthesise complex *N*-glycans owing to loss of *N*-acetylglucosaminyltransferase I bear glycans on their glycoproteins that are trapped at a defined stage prior to galactose modification (Reeves et al. 2002) and are only protected from a ricin challenge by a factor of ~20-fold (Crispin et al. 2009). This suggests that ~5% of productive ricin routing occurs via a non-protein, presumably glycolipid, targeted route.

This lack of knowledge about cell surface events that lead to ricin intoxication contrasts strongly with the knowledge of internalisation of the bacterial Shiga (-like) toxin (STx) and cholera toxin (CTx), both of which cross-link cognate lipid receptors, forcing membrane curvature and invagination (Römer et al. 2007; Windschiegl et al. 2009) and STx further regulates its own entry by stimulating activity of Syk kinase (Lauvrak et al. 2006).

For ricin, uncertainty of events at the cell surface means that trafficking pathways productive for ricin cytotoxicity cannot be studied by examining the behaviour of known receptors.

3.2 Retrograde Trafficking

Despite some confusion about cell surface and very early endocytic events, retrograde trafficking pathways for ricin appear to merge at or near the early endosome. A level of uncertainty also exists for some early endosome events, which may

indicate confounding cell-type specific effects. Thus the role of the small GTPase dynamin acting as a scission agent at the early endosome is not clear, with expression of mutant dynamin having either no effect on ricin toxicity in COS7 cells (Simpson et al. 1998), or else protecting HeLa cells about tenfold from ricin challenge (Llorente et al. 1998). Nevertheless, a number of cellular molecules required for ricin toxicity have been identified.

At least a portion of an endosomal trafficking toxin must avoid destruction by the lysosomes. This process is efficient for STx (Fuchs et al. 2007) but very inefficient for ricin (van Deurs et al. 1988), suggesting that access to a retrograde sorting complex is difficult for ricin (Fig. 1b). A candidate for this difficult step is the access to retromer components (Fig. 2a). Mammalian retromer mediates retrograde transport between the endosome and *trans*-Golgi network (TGN), and is associated with tubular–vesicular structures that spread from early endosomes or from intermediates in the maturation pathway from early to late endosomes (Arighi et al. 2004). These may constitute part of the tubular endosomal network (TEN) that sorts and recycles cargoes. Retromer appears to sequester membrane cargo proteins from vacuolar endosomal membranes into retrograde transport intermediates, thereby preventing default delivery to lysosomes. The retromer coat comprises a dimer of

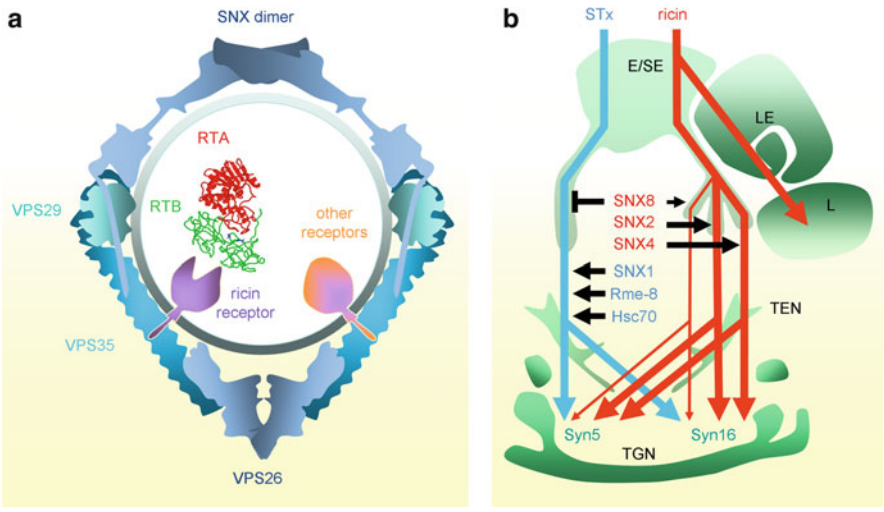

Fig. 2 A model for endosomal sorting of ricin. (**a**) A diagrammatic cross-section through a tubular portion of an early/sorting endosome, showing the arrangement of the retromer coat proteins (SNX, VPS29, VPS35 and VPS26). It is likely that ricin receptors are bound by VPS35, allowing them to enter the tubular portions of the endosome and gain access to the retrograde transporting TEN. (**b**) Sorting events at the early endosome can be complex. STx (*blue arrow*) gains efficient entry to TEN and subsequently the TGN by utilising a retromer complex containing SNX1 and the chaperones Hsc70 and its partner the DnaJ containing protein Rme-8. Ricin (*red arrows*) enters TEN inefficiently, via multiple routes, but the majority is not sorted in the retrograde direction. Two TGN docking mechanisms are currently identified, using SNARE complexes containing either syntaxin 5 or syntaxin 16

different sorting nexin combinations (typically SNX1, SNX2, SNX5 and SNX6) and a Vps26–Vps29–Vps35 trimer (Bonifacino and Hurley 2008). Early endosome trafficking also requires phosphoinositide (PI)-3 kinase activity, that generates the PI(3)P lipid that is bound by the PX domain of sorting nexins. To date, hVps34 is the only identified kinase that phosphorylates PI in the third position to produce PI (3)P. Since access to retromer-controlled retrograde transport intermediates would be an advantage for a protein toxin, it is not too surprising that roles for SNX2, SNX4 and hVps34 have been elucidated in the transport of ricin from early endosomes to the TGN (Skånland et al. 2007). What is surprising is that SNX2 and SNX4 appear to control separate retrograde routes, pointing to some complexity of trafficking events at the early endosome. Presumably, the interaction with retromer is indirect, in that the cargo being sorted is the (ricin-bound) ricin receptor, rather than ricin itself.

Choice of retrograde routes by toxins is not universal (Fig. 2b) – for examples, STx achieves efficient retrograde routing by utilising SNX1 (Bujny et al. 2007; Popoff et al. 2007); CTx has only a small requirement for SNX1 (Bujny et al. 2007), so it may utilise other components; and the subAB toxin that traffics from the plasma membrane to the ER uses neither SNX1 nor SNX2 to access the TGN (Smith et al. 2009), emphasising the complex manner in which eukaryotic cells sort and traffic endogenous components. Sorting nexin 8 has unusual roles – it has a weak promoting effect for ricin transport but inhibits retrograde transport of STx (Dyve et al. 2009). STx transport also requires RME-8, the receptor mediated endocytosis-8 protein, that interacts with SNX1 and the molecular chaperone Hsc70 (Popoff et al. 2009). In contrast, retrograde transport of ricin appears to be independent of Hsc70 function (Spooner et al. 2008a).

From the retrograde endosomal transport intermediates, ricin is transported to the TGN, and early studies, using immuno-gold labelling and electron microscopic techniques, demonstrated that this is inefficient, with only ~5% of cell-surface bound ricin reaching this compartment (van Deurs et al. 1988). Fusion of transport intermediates with target membranes is mediated by SNARE complexes. Ricin transport specifically requires SNARE complexes localised to early endosomes and the TGN. One complex comprises syntaxin 5, Ykt6, GS15 and GS28: the other complex comprises VAMP3 or VAMP4, syntaxin 16, syntaxin 6 and Vti1a (Amessou et al. 2007). This requirement for at least two separate SNARE-controlled docking systems points to multiple transport routes between endosomes and the TGN. A similar argument can be made for roles of multiple sorting nexins in ricin transport (Skånland et al. 2007).

When ricin is reconstituted from a recombinant RTA with a sulphatable tag and native plant RTB and this is used to intoxicate mammalian cells, [^{35}S]-labelled RTA can be immunoprecipitated from the cytosol (Rapak et al. 1997). Thus at least a proportion of ricin traffics from the TGN via the *trans*-Golgi cisternae, where the relevant sulphotransferase resides (Spooner et al. 2008b) and presumably on from there via the Golgi stack to the ER.

The evidence that the Golgi stack is an important conduit for retrograde transport is extensive. For some proteins there is a critical dependence on binding KDEL

receptors which cycle between the TGN and the ER via the Golgi cisternae (Miesenbock and Rothman 1995) in a COP1-dependent manner which typifies retrograde transport in the classic secretory pathway. *Pseudomonas* exotoxin A, which bears a KDEL-like sequence at its C-terminus, can utilise this route (Smith et al. 2006) as can subAB toxin (Smith et al. 2009). However, STx traffics in a COP-1 independent manner, instead requiring the small GTPase Arl1 (Tai et al. 2005) and its effector the Golgi tethering factor golgin-97 (Lu et al. 2004; Tai et al. 2005) and its targeting co-factor ARFRP1 (Shin et al. 2005). It also requires the Golgi tethering factors golgin-245 (Yoshino et al. 2005) and GCC88 (Lieu et al. 2007), the conserved oligomeric Golgi COG complex (Zolov and Lupashin 2005), the Golgi-associated retrograde protein GARP (Perez-Victoria et al. 2008) and the TGN tethering factor GCC185 (Derby et al. 2007). Roles for the Golgi docking and fusion promoter Rab6a′ (Girod et al. 1999; Mallard et al. 1998; White et al. 1999), for its RabGAP Rab6IP2 (Monier et al. 2002) and for Rab11 (Wilcke et al. 2000) have also been established. *Pseudomonas* exotoxin A can also utilise a Rab6-dependent route (Smith et al. 2006), and subAB toxin also utilises this and the COG complex (Smith et al. 2009), remarkable examples of toxins with single cognate receptors taking advantage of multiple retrograde trafficking options through the Golgi stack. Finally, sub-cellular microsurgery to remove the Golgi stack halts retrograde transport of the STx B chain to the ER (McKenzie et al. 2009).

In contrast to this wealth of detail for other toxins, the evidence that ricin proceeds from the *trans*-Golgi cisternae through the Golgi stack is much scarcer. Simultaneous blocking of the COPI-mediated and the Rab6a′-controlled routes through the Golgi do not affect ricin toxicity, suggesting that there is a third, uncharacterised route from the TGN to the ER (Chen et al. 2003). Transport of ricin through the Golgi stack may not even be unidirectional, since ricin intoxication is governed by Rab1, a small GTPase that controls vesicular traffic in the (opposite) anterograde direction, between the ER and the Golgi (Simpson et al. 1995a). Consistent with this, ricin can be co-immunoprecipitated with calreticulin, an ER chaperone that recycles between the ER and the Golgi (Day et al. 2001) that may act as a fortuitous delivery agent to the ER. Overall, then, the manner of ricin's progress through the Golgi stack remains somewhat mysterious.

3.3 Ricin Is Delivered to the ER

Utilising a ricin holotoxin whose A chain is modified by addition of sulphation and glycosylation motifs allows biochemical tracking of ricin to the ER, where the glycosylation motifs become core-*N*-glycosylated (Rapak et al. 1997). Furthermore, a holotoxin with a KDEL retrieval sequence appended to the C-terminus of RTA is more toxic than native holotoxin (Tagge et al. 1996; Wales et al. 1992; Wesche et al. 1999), consistent with increased ER delivery by forcing at least a proportion of Golgi-delivered ricin through the efficient COPI-dependent retrograde route.

3.4 Ricin Is Reduced to its Constituent Chains in the ER

The disulphide bond linking RTA and RTB is occluded, since, in the absence of a denaturant and heat, high concentrations of the small molecule reducing agent DTT are required to separate the two chains of ricin in vitro (Emmanuel et al. 1988; Simpson et al. 1995c). Furthermore, the ER is a relatively oxidising environment that favours the formation, not the scission, of disulphide bonds. These considerations immediately invoke a protein or proteins capable of remodelling the structure of ricin holotoxin to open the interface between the two chains and expose the interchain disulphide bond, allowing reductive cleavage. Protein disulphide isomerase (PDI) possesses these qualities, in that it acts both as a chaperone and as a disulphide exchanger (Ferrari and Söling 1999; Klappa et al. 1997), and also is found predominantly in the ER.

The tiny amounts of ricin that reach the ER (Fig. 1) impose severe experimental difficulties, particularly if a chaperone interaction is suspected, since recombinant tagged RTA versions are not suitable because they may force chaperone interactions. Despite these difficulties, evidence has accumulated that PDI is responsible for the reductive separation of RTA and RTB (Fig. 3). In vitro, on microsomal membranes and in crude cell extracts, PDI can reduce ricin in the presence of thioredoxin, thioredoxin reductase and NADPH (Bellisola et al. 2004). Supporting this, auranofin, an irreversible inactivator of thioredoxin reductase, protects cells against ricin, but not against challenge with pre-reduced ricin, suggesting that activation of PDI by thioredoxin reductase has some physiological significance for ricin intoxication.

Excess RTB at the site of reduction of ricin should act as a dead-end receptor for newly liberated RTA, and therefore protect cells against ricin challenge. When RTB is expressed in the ER of mammalian cells, this is precisely what happens (Spooner et al. 2004). ER-targeted RTB is retained by a thiol anchor in the ER for some time before disposal, and breaking this bond in vivo by treating cells with DTT reverses the protective effect against ricin challenge of RTB expression. This thiol anchor is a mixed disulphide between RTB and PDI, suggesting that PDI can both make and break bonds in ricin confirmed in vitro using glutathione reduced PDI. Free RTB in the ER has two fates – a proportion is an ERAD substrate and is degraded by the cytosolic proteasomes whilst the remainder is secreted (Spooner et al. 2004). The fate of RTA, though, is much more interesting.

3.5 RTA Unfolds in the ER

For CTx, interactions with PDI also result in the unfolding of its A chain (CTxA), making it susceptible to trypsin cleavage (Tsai et al. 2001). A model has been presented where unfolded CTxA is then released from PDI by reduced Ero1p at the ER membrane as a prelude to dislocation (Tsai and Rapoport 2002). For ricin and

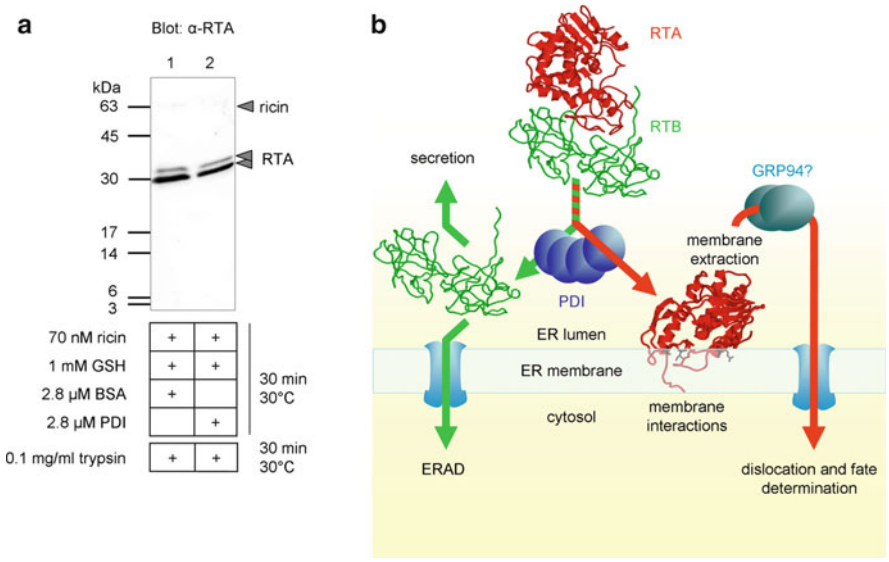

Fig. 3 A model for ER events. (**a**) Treatment of ricin with reduced PDI does not result in increased trypsin sensitivity, suggesting that, unlike CTX, ricin is not substantially unfolded by PDI interactions. *Lane 1*, BSA-treated ricin; *lane 2*, ricin treated with reduced PDI. After treatment, samples were incubated with trypsin and subjected to reducing SDS-PAGE and immunoblotting, probing with anti-RTA antibodies. (**b**) Ricin entering the ER lumen is reductively separated into its constituent RTA (*red*) and RTB (*green*) chains by reduced protein disulphide isomerise (*PDI*). A proportion of RTB is removed from the ER by ERAD and the rest by secretion. The A chain undergoes structural alteration at or near the ER membrane, and partially inserts, with the majority of the inserted region being derived from the hydrophobic stretch of amino acids towards the C-terminus of free RTA. It thus presents to the ER quality control mechanisms as a misfolded membrane-associated protein, stimulating membrane extraction and dislocation to the cytosol as an ERAD substrate. The Hsp90 chaperone GRP94 may play in role in maintaining solubility of RTA during the membrane extraction/dislocation preparation steps

RTA, interactions with PDI do not cause obvious protease sensitivity (Fig. 3a), suggesting that unfolding of RTA occurs by a different mechanism.

A patch of hydrophobic amino acids near the C-terminus of RTA is occluded by RTB until revealed by reduction of the holotoxin in the ER. Adding positively charged residues near this site reduces ricin toxicity (Simpson et al. 1995b), suggesting that this patch might normally interact with membrane lipids. When fused to the N-terminus of other proteins, this region can act as a signal sequence, directing these nascent proteins into the ER (Chaddock et al. 1995), so this region, at least in this context, can enter the lipid bilayer of the ER. After Triton-X114 extraction, RTA (but not ricin) partitions in the detergent phase, suggesting that also in the context of native RTA structure, this region can interact with lipid membranes (Day et al. 2002). Furthermore, in the presence of a negatively charged phospholipid RTA undergoes spontaneous structural alterations, leading to a model where lipid-induced partial unfolding of RTA allows its recognition as an ERAD

substrate (Day et al. 2002). A major caveat of this is that the negatively charged phospholipid that was used is not found physiologically, but this has been addressed recently, using liposomes generated from phosphatidyl serine, a physiologically relevant lipid, to promote identical changes in RTA structure (Mayerhofer et al. 2009).

When RTA is presented to microsomal membranes saturated with a lipophilic quenching agent, sophisticated experiments that use RTA tagged with fluorophores in different positions around the molecule demonstrate that the interaction with ER membranes is not random (Mayerhofer et al. 2009): fluorophores in some positions are quenched, showing membrane insertion, whilst fluorophores in other positions are not quenched. Thus RTA inserts into membranes in a precise, ordered manner (Fig. 3b). While the majority of amino acids from RTA that insert into the membrane are clustered at or near the C-terminal hydrophobic patch, there is a curious exception – a positively charged amino acid (arginine) on a loop near the N-terminus also inserts into the lipid bilayer. When a signal peptide is released from the translocon into the lipid environment of the mammalian ER membrane, its hydrophobic core forms a strong anchor in the membrane and its removal requires signal peptide peptidase to cut the signal peptide into two halves which can be extracted with ease. Perhaps insertion of a hydrophobic RTA-derived structure that includes a positively charged amino acid reduces the strength of the interaction with the ER membrane, facilitating extraction of RTA as a prelude to dislocation.

Insertion into the membrane is also a temperature-dependent process, with membrane binding evident at low temperatures, but with structural changes increasing as the temperature approaches 37°C (Mayerhofer et al. 2009). Purified RTA in vitro aggregates and rapidly becomes insoluble at 45°C, but even at 37°C (the physiological temperature for intoxication of mammalian cells) it is relatively unstable (Spooner et al. 2008a). Thus the driving force for RTA unfolding appears to be thermal instability of RTA released from RTB, coupled with an ordered insertion into the ER membrane.

3.6 Chaperone Interactions in the ER

From its partially buried state in the ER membrane, RTA must be extracted and interact with a dislocation mechanism that can transfer it across the ER membrane into the cytosol. The partial unfolding of RTA to permit membrane entry may also allow recognition by ER molecular chaperones. For the ER-trafficking STx, interactions of the A chain have been noted with the ER Hsp70 chaperone BiP (Falguières and Johannes 2006) and its Hsp40 co-chaperone ERdj3, whose over expression protects cells from toxin challenge (Nakanishi et al. 2004; Yu and Haslam 2005). It is likely that these chaperones promote solubility of the STx A chain, so aiding its recognition as an ERAD substrate, in the same way that ERAD of many misfolded proteins in yeast is aided by DnaJ domain-containing Hsp40 co-chaperones that interact with the ER luminal chaperone Kar2p/BiP (Nishikawa

et al. 2001). To date, though, no clear role for BiP and its co-chaperones has emerged for ricin.

There is a hint of a role, though, for the ER Hsp90 GRP94 chaperone (Fig. 3b), since *N*-ethylcarboxamidoadenosine, a GRP94 inhibitor that does not affect cytosolic Hsp90 (Rosser and Nicchitta 2000) protects cells slightly from ricin (Spooner et al. 2008a). This may suggest a role for GRP94 in preparing RTA for dislocation, consistent with its ability to direct the null Hong Kong variant of α1-anti-trypsin to ERAD (Christianson et al. 2008).

Manipulating expression levels of EDEM, the ER degradation enhancing α-mannosidase I-like protein, alters the sensitivity of cells to ricin: overexpression protects cells from ricin, but knockdown reduces dislocation of RTA and so also protects (Slominska-Wojewodzka et al. 2006). Co-immunoprecipitation of RTA and EDEM suggested a direct role for EDEM, perhaps as a transporter of RTA to the dislocation machinery. Subsequently, the yeast homologue of EDEM, Htm1p, has been shown to retain mannosidase activity, which generates an exposed α1 → 6 mannose residue on glucose-trimmed *N*-glycans of misfolded proteins (Quan et al. 2008). These constitute signals for ERAD recognition, delivering the glycoprotein to the lectin Yos9p and from there to the Hrd3p subunit of an E3 ubiquitin–ligase complex required for dislocation of ERAD substrates with lesions in their luminal domains. Since ricin reconstituted with recombinant RTA contains a non-glycosylated RTA, but is as toxic as the native plant holotoxin (which contains glycosylated RTA), the role of EDEM becomes less clear. It may be that the EDEM target is RTB, which is glycosylated. However, the effects may be caused indirectly. CHO cells up-regulated for ERAD can display a CTx/*Pseudomonas* exotoxin A/ricin-resistant phenotype by increasing the coupling efficiency between toxin dislocation and toxin degradation (Teter et al. 2003). Down-regulation of ERAD can produce the same multitoxin-resistant phenotype, attributed here to decreased retrotranslocation (Teter and Holmes 2002). Thus, manipulating the rate of ERAD steps influences the cytotoxicity of ER-trafficking toxins, but by multiple possible mechanisms. Such indirect effects may explain, in part, the effect of manipulating expression levels of EDEM.

3.7 The Dislocation Process for RTA Remains Mysterious

RTA can be co-immunoprecipitated from cells with Sec61α (Slominska-Wojewodzka et al. 2006; Wesche et al. 1999), a component of the translocon through which nascent proteins enter the ER, and which has been implicated in the dislocation of a number of ERAD substrates in both yeast (Scott and Schekman 2008; Willer et al. 2008) and mammalian systems (Imai et al. 2005; Koopmann et al. 2000; Wiertz et al. 1996). Supporting evidence that the translocon constitutes part of the dislocation system comes from expression of RTA in the yeast ER (Simpson et al. 1999). However, in mammalian cells, a functional requirement remains to be demonstrated. To date, no other molecules required for RTA dislocation have been identified,

although a number of candidates, such as derlins, have been shown to have no obvious functions in this process (Slominska-Wojewodzka et al. 2006). Dislocation of RTA, then, remains a cryptic process.

4 Recovery of Activity in the Cytosol

The conserved Sec61 (mammals, yeast) or SecY (bacterial) heterotrimeric membrane protein complex forms a protein-conducting channel, allowing polypeptides to be transferred across or integrated into membranes. Structural studies of the bacterial SecY translocon show that the translocation pore is narrow, so large structures are unlikely to be extruded (Van den Berg et al. 2004). This leads to the notion that dislocated substrates are to a large extent unfolded, and enter the cytosol in a vulnerable non-native conformation. Thus a dislocated toxin with cytosolic targets might be expected to refold in the cytosol.

In principle, acquisition of a catalytic conformation by dislocated RTA could be accomplished by multiple mechanisms. RTA carefully unfolded by heat to a molten globule state can regain activity when presented to ribosomes in vitro, leading to a model whereby dislocated RTA can undergo substrate (ribosome)-mediated re-folding (Argent et al. 2000). An alternative might be by spontaneous re-folding, proposed for dislocated CTxA chain (Rodighiero et al. 2002). However, in vivo, the Hsc70 inhibitor deoxyspergualin protects cells from ricin, and overexpression of Hsc70 co-chaperones alters the cytotoxic response, demonstrating a vital role for the molecular chaperone Hsc70 in the activation of cytosolic RTA (Spooner et al. 2008a). Since Hsc70 prevents heat-inactivation of RTA, then one role for Hsc70 might be to prevent aggregation of vulnerable, unfolded dislocated RTA in the cytosol.

Dihydrofolate reductase (DHFR) fusions with RTA or with Class I major histocompatibility complex heavy chain still act as ERAD substrates, even when the DHFR domains are stabilised into fully folded structures by addition of a folate analogue (Beaumelle et al. 1997; Tirosh et al. 2003). Thus either the Sec61 channel can be modified to accommodate a folded DHFR domain for dislocation, (but not for translocation into the ER), or a different channel is used for dislocation. Since folded domains can dislocate, dislocated RTA may still have considerable structure. In vitro, RTA shows a degree of thermal instability at 37°C which is reduced in the presence of Hsc70 (Spooner et al. 2008a), so the role of Hsc70 may be to stabilise RTA in the cytosol, perhaps by masking the C-terminal hydrophobic patch implicated in membrane associations.

Furthermore, there is a chaperone triage of RTA in the cytosol (Fig. 4) that includes interactions with the chaperone Hsp90, but only after previous interactions with Hsc70 (Spooner et al. 2008a). RTA possesses only two lysyl residues, a remarkable shortage when compared with the number of lysines found in other non-cytotoxic RIPs. This led to the proposal that ricin traffics to the ER in order to allow the A chain to somehow disguise itself as a substrate for ERAD, the

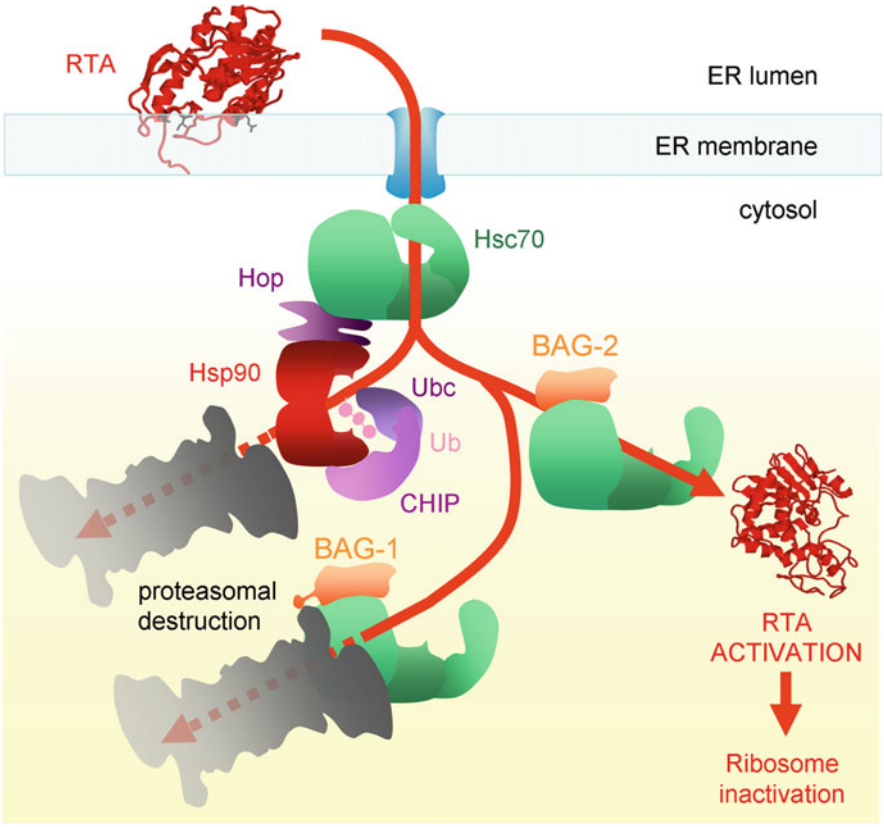

Fig. 4 RTA fate is determined after triage by cytosolic chaperones. RTA enters the mammalian cytosol by a dislocation mechanism that has yet to be elucidated, in a vulnerable non-native conformation. Interactions with the cytosolic chaperone Hsc70 are vital in the recovery of an active conformation. From Hsc70, multiple routes lead to inactivation: these appear to be controlled by ubiquitin (*Ub*) or ubiquitin-like signals that permit engagement with the cytosolic proteasomes. Direct passage to the proteasome occurs after ubiquitylation of RTA by CHIP (C-terminus of Hsc70 interacting protein) and associated ubiquitin conjugating enzymes (*Ubc*) following sequential interactions of RTA with Hsc70 and Hsp90 that are co-ordinated by the dual co-chaperone Hop (Hsc70-Hsp90 organising protein). Indirect routes probably also include "piggy-back" transport to the proteasome through CHIP-mediated ubiquitylation of Hsc70 and via CHIP auto-ubiquitylation (not shown). Uncoupling from the final destructive steps of ERAD requires release of RTA from Hsc70. Release by BAG family (BCL2-associated anathogene protein family) nucleotide exchange factors can lead to either inactivation or activation of RTA. BAG-1 interactions result in release of RTA at the proteasome, which is engaged by the interlaced ubiquitin-like domain of the nucleotide exchange factor. However, RTA release from Hsc70 by BAG-2, which has no ubiquitin-like domain, permits RTA to acquire a catalytically active structure which then inhibits protein synthesis ability by specifically depurinating a large ribosomal RNA

ER-associated protein degradation mechanism that clears misfolded and orphan proteins from the ER, dispatching them to the cytosol for proteasomal elimination (Hazes and Read 1997). Seen in this light, the reduction in lysyl residues may reduce the potential for polyubiquitylation, and so hamper processing by the proteasomal core. Experimental support for this was obtained by replacing some of the arginyl residues of RTA with lysyls, in positions where RTA activity and structure were not compromised. The resulting holotoxins were much reduced in toxicity, but inhibition of proteasomal activity increased the toxicity of lysine-rich ricin variants to close to normal (Deeks et al. 2002). Despite possession of only a low number of lysyl residues that reduce polyubiquitylation and subsequent proteasomal targeting (Deeks et al. 2002; Hazes and Read 1997), inhibition of proteasomal core activities sensitises cells to ricin (Deeks et al. 2002; Slominska-Wojewodzka et al. 2006; Wesche et al. 1999), so at least a proportion of RTA is degraded by proteasomes. Sensitivity to ricin correlates with ubiquitylation or ubiquitin-like signals (Spooner et al. 2008a). Thus overexpression of the Hsc70 nucleotide exchange factor BAG-1 (which has a ubiquitin-like domain that targets the BAG-1: Hsc70: client complex to the proteasome) protects cells from ricin challenge, and overexpression of the Hsc70-Hsp90 associated E3 ubiquitin ligase CHIP also protects cells. Furthermore, over expression of the dual co-chaperone Hop, that transfers RTA from Hsc70 to Hsp90, also protects cells, reflecting the ability of CHIP to interact with RTA and mark it for destruction only in the context of Hsp90 binding. Since replacement of the two lysyls in RTA with arginine residues does not increase the toxicity of ricin (Deeks et al. 2002), it is likely that cytosolic ubiquitylation of RTA is non-canonical, and does not occur on the two remaining lysine residues (Fig. 4).

5 Conclusions

The secretory pathway of eukaryotic cells is fully reversible and some plant protein toxins such as ricin can take advantage of this to move from the plasma membrane, via the Golgi and the ER to the cytosol. There are multiple retrograde routing steps which are utilised by different toxins. “Fast” routes through the Golgi can be utilised by the dedicated bacterial ER-trafficking toxins such as STx and SubAB. However, to a first approximation, ricin appears to be an endosomal trafficker and only a small proportion of cell-bound toxin can access retrograde routes to the ER, trafficking slowly and inefficiently through the Golgi stack. In the ER, reductive separation releases the active A chain from the inactive holotoxin. The ER-cytosol dislocation step is currently cryptic for these toxins, but since this is preceded by an unfolding step, there is a clear requirement for re-folding in the cytosol to gain a catalytic conformation. This final step for ricin is dependent upon cytosolic chaperones, underlining the central role of Hsc70 in cellular protein folding.

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Ribosome-Inactivating Protein-Containing Conjugates for Therapeutic Use

Giulio Fracasso, Fiorenzo Stirpe, and Marco Colombatti

Abstract A number of plant proteins inhibit protein synthesis by irreversibly inactivating the 60S ribosomal subunit in a catalytical, that is, enzymatic, manner. For this property, they are called ribosome-inactivating proteins (RIPs). Several RIPs are utilized in the preparation of therapeutic heteroconjugates (immunotoxins), obtained either by chemical conjugation of a vehicle molecule to an RIP or by genetic fusion of a targeting molecule and an RIP. In the present review, we will focus on the properties of RIPs and of their immunotoxins. The most recent advancements in this domain will be reported in the following paragraphs.

1 Introduction

There are a number of recent reviews on ribosome-inactivating proteins (RIPs) (Van Damme et al. 2001; Girbés et al. 2004; Hartley and Lord 2004; Stirpe 2004; Stirpe and Battelli 2006), and therefore only the main features will be dealt with here.

RIPs have been divided into two main groups: type 1, single chain proteins with enzymatic activity, and type 2, consisting of two unequal chains cross-linked by disulphide bond(s) – an A chain with enzymatic activity and a B chain with the properties of a lectin with specificity for sugars. A barley protein (JIP60) consisting of an A-type chain linked to a peptide with an unknown function has been identified and designated as a type-3 RIP (Peumans et al. 2001) (Fig. 1). The B chains of type 2 RIPs bind to carbohydrates (in general galactosyl residues) on the surface of

G. Fracasso and M. Colombatti
Department of Pathology, University of Verona, Policlinico "G.B. Rossi", P.le L. Scuro 10, 37134 Verona, Italy
e-mail: marco.colombatti@univr.it
F. Stirpe
Via S. Petronio Vecchio 19, 40125 Bologna, Italy

J.M. Lord and M.R. Hartley (eds.), *Toxic Plant Proteins*, Plant Cell Monographs 18,
DOI 10.1007/978-3-642-12176-0_12,

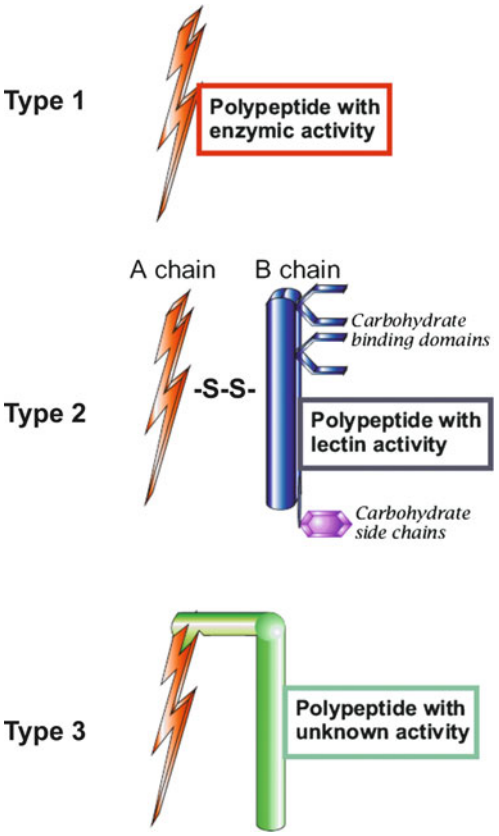

Fig. 1 Schematic representation of the structure of ribosome-inactivating proteins (from Stirpe 2004)

virtually all cells, allowing and facilitating the entry of the whole molecule into the cells. Hence, some type 2 RIPs are potent toxins, ricin being the best known while others are not so toxic (see below). Type 1 proteins, lacking a B chain, enter into cells with difficulty and, consequently, have a relatively low toxicity. Type 1 RIPs, however, are very toxic if linked to molecules capable of delivering them inside the cell.

Ricin has been known for more than a century, and pokeweed antiviral protein (PAP) was the first type 1 RIP identified by its inhibitory effect on protein synthesis (Irvin 1975). Subsequently, a number of RIPs of either type were identified, and many were purified and characterized (for a recent review see Girbés et al. 2004). More recently, some two-chain lectins were identified, which consist of A and B chains with lectin and enzymatic activity typical of the corresponding chains of type 2 RIPs; they are, however, endowed with low cytotoxicity, comparable to that of type 1 RIPs (see below) and are commonly referred to as nontoxic type 2 RIPs.

A list of known type 2 RIPs is shown in Table 1. The more numerous type 1 RIPs are listed in Girbés et al. (2004), and a list of those used to prepare immunotoxins will be provided below.

Table 1 Toxic and nontoxic type 2 ribosome-inactivating proteins

Toxic		Nontoxic	
Name	Source	Name	Source
Abrin	*Abrus precatorius* seeds	Ebulin 1	*Sambucus ebulus* leaves
Ricin	*Ricinus communis* seeds	Nigrin b	*Sambucus nigra* bark
Viscumin	*Viscum album* leaves	Sieboldin-b	*Sambucus sieboldiana* bark
Modeccin	*Adenia digitata* root	*Momordica charantia* lectin	*Momordica charantia* seeds
Volkensin	*Adenia volkensii* root	Ricinus agglutinin	*Ricinus communis* seeds
Type 2 lectin	*Adenia goetzei* caudex	Iris agglutinin (IRA)	*Iris hollandica* bulbs
Lanceolin	*Adenia lanceolata* caudex	Cinnamomum	*Cinnamomum camphora* seeds
Stenodactylin	*Adenia stenodactyla* caudex		
Aralin	*Aralia elata* shoots		
Riproximin	*Ximenia americana* powder		

RIPs are officially denominated "rRNA *N*-glycosidases" (EC 3.2.2.22, Endo 1988), as it was found that they remove a specific adenine residue (A_{4324} in rat liver rRNA) from eukaryotic rRNAs. They also depurinate DNA and other polynucleotides (Barbieri et al. 1997), and the denomination "adenine polynucleotide glycosylases" was also proposed (Barbieri et al. 2001).

2 Distribution

RIPs are present in many plants, including some edible ones (Barbieri et al. 2006). More type 1 RIPs are known than type 2, and the notion was put forward that they could be ubiquitous. In some plants, they are present in many or even in all tissues examined (e.g., saporins in *Saponaria officinalis*, Ferreras et al. 1993) and in cultured cells, whereas in other plants they are confined to one or few tissues (e.g., ricin in the seeds of *Ricinus communis*). In many plants, the expression of RIPs was found to be enhanced in senescent tissues, or in conditions of stress or infection (reviewed in Stirpe and Battelli 2006).

RIPs were also found in mushrooms and algae, and are produced by some bacteria (Shiga and Shiga-like toxins (Reisbig et al. 1981; Obrig 1997)). In animal cells and tissues, an enzymatic activity, which removes adenine from DNA as RIPs do (see below), was found, and like RIPs in plants (Girbés et al. 1996), it is higher in virally infected and stressed cells (Barbieri et al. 2001).

2.1 Enzymatic Activity

Ricin was found to inhibit protein synthesis using whole cells (Lin et al. 1971) and cell-free systems (Olsnes and Pihl 1972), and so do the toxic type 2 RIPs, some ten of which are known (Stirpe and Battelli 2006). Type 1 RIPs and the isolated A chains of type 2 RIPs have lower effects on the protein synthesis of whole cells, in which they enter with difficulty, but are very potent in cell-free systems, where they cause an irreversible damage of ribosomes by removing a single adenine residue (A_{4324} in rat liver rRNA) from a GAGA sequence in a highly conserved loop at the top of a stem in 28S rRNA (Fig. 2) (reviewed by Endo 1988). This activity is common to all RIPs examined (Stirpe et al. 1988). It is noteworthy that some, but not all, RIPs require various cofactors for maximal inhibitory activity on translation (Carnicelli et al., 1992), e.g. gelonin requires a specific tRNA (Brigotti et al. 2002).

Some RIPs, however, remove more than one adenine residue per ribosome (Barbieri et al. 1994) and all RIPs examined remove adenine residues from DNA and other polynucleotides (Barbieri et al. 1997; Nicolas et al. 1998). The latter property seems to be another characteristic specific to RIPs, to the point that it was used to detect new RIPs (Pelosi et al. 2005). Also, ricin and other RIPs remove adenine from the poly(A) tail of poly(ADP-ribosylated poly(ADP-ribose) polymerase (activated PARP, Barbieri et al. 2003). PAP depurinates capped mRNA (Hudak et al. 2002). A lyase activity of RIPs was also reported (reviewed in Van Damme et al. 2001; Aceto et al. 2005), which in some cases was due to contamination by nucleases (reviewed in Stirpe 2004).

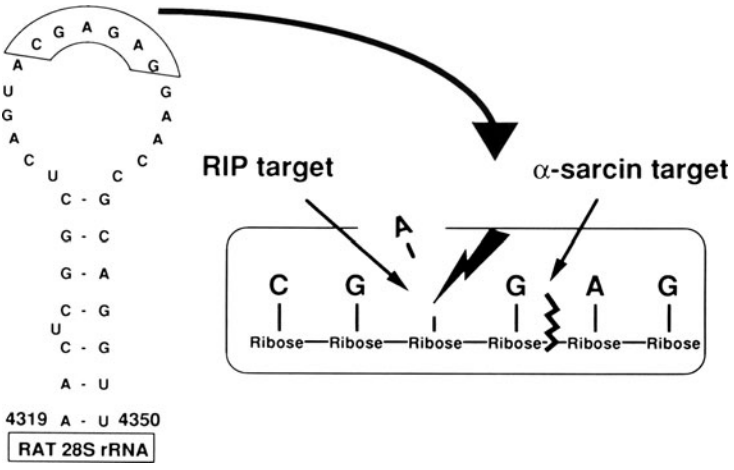

Fig. 2 Schematic representation of the enzymatic action of ribosome-inactivating proteins on rRNA. RIPs cleave a single adenine base (A_{4324} in 28S rat rRNA) at a site adjacent to the site of attack by α-sarcin, which cleaves the phosphodiester bond between G_{4325} and A_{4326} in rat 28S rRNA (from Stirpe 2004)

2.2 Toxicity

It was mentioned above that some type 2 RIPs are potent toxins, and indeed for some years it was assumed that all type 2 RIPs were highly toxic. The high toxicity was explained by the combined action of the two chains: the B chain binds to galactosyl-terminated residues on the surface of most cells, allowing and facilitating the entry of the toxins into the cells, where the A chain is separated and can exert its enzymatic activity, damaging ribosomes and possibly other structures, with consequent cell damage and death.

A number of lectins (*Ricinus* agglutinin, lectins from *Sambucus*, camphor tree and iris) are type 2 RIPs, with the same enzymatic and lectin properties as those of ricin and related toxins, and still have very low cytotoxicity (reviewed in Van Damme et al. 2001; Girbés et al. 2003; He and Liu 2003). The reasons for the difference are largely unknown, and could be in the binding to, and entry into, cells, the intracellular pathway, as well as the excretion from, and resistance to destruction by cells. Inside cells, some RIPs traffic to the Golgi apparatus and from there to the cytoplasm, some others are degraded by lysosomes, and some are expelled from the cells (reviewed by Sandvig and van Deurs 2002). Differences in each of these destinations and processes could result in different cytotoxicity. Indeed, nigrin b, a nontoxic type 2 RIP from *Sambucus nigra* bark (Girbés et al. 1993), enters cells equally as well as ricin, but is more rapidly and extensively degraded in, and excreted from, cells (Battelli et al. 1997), whereas volkensin, which has a higher toxicity than ricin, is excreted by cells mostly nondegraded, thus being still active and capable of entering other cells (Battelli et al. 2004). Studies with ricin have clarified that in order to reach their cytosolic substrates, ricin as well as several other toxins (Lord et al. 2003) undergoes retrograde transport to the endoplasmic reticulum (ER) before translocating across the ER membrane. To achieve this export, these toxins exploit the ERAD (ER-associated protein degradation) pathway but must escape, at least in part, the normal degradative fate of ERAD substrates in order to intoxicate the cell. Toxins that translocate from the ER have an unusually low lysine content that reduces the likelihood of ubiquitination and ubiquitin-mediated proteasomal degradation. Regarding intracellular trafficking, it was also observed that despite the fact that the 3D-fold and residues responsible for the *N*-glycosidase activity are well conserved between saporin and ricin A-chain (RTA), it appears that these two toxins follow different intracellular routes in mammalian intoxicated cells: RTA cytotoxicity is blocked by brefedin A (BFA), a fungal agent disrupting the Golgi complex, and can be enhanced by the addition of a C-terminal KDEL ER retrieval motif. In contrast, neither treatment with BFA nor addition of a KDEL motif has any significant effect on saporin-mediated cytotoxicity (Vago et al. 2005). Although several lines of evidence suggest that saporin (and other type I RIPs) can cross cellular membranes, the site and mechanism(s) of their translocation may differ from the one used by the catalytic subunits of type II RIPs, with ERAD pathways playing little or no role in their productive intoxication paths (Vago et al. 2005; Geden et al. 2007).

3 Properties of RIPs

The properties of type 1 and type 2 RIPs are summarized in Table 2.

Once inside cells, RIPs depurinate rRNA, and this causes a "ribotoxic stress response" which is characterized by activation of several protein kinases (Iordanov et al. 1997). This in turn causes the release of TNF and other proinflammatory cytokines observed in cells and animals poisoned with ricin and other toxic type 2 RIPs. (reviewed in Stirpe and Battelli 2006).

Both type 2 (e.g., ricin (Griffiths et al. 1987), viscumin (Büssing 1996), abrin (Hughes et al. 1996; Narayanan et al. 2004) and high doses of type 1 RIPs (e.g., saporin, Bergamaschi et al. 1996) cause apoptosis and subsequently, or at higher doses, severe necrosis both in cultured cells and in the organs of poisoned animals. In animals, the liver is most often affected, with differences among both RIPs and animals, and inflammation may be present, severe in the case of ricin poisoning (review in Battelli 2004). Inflammation seems to have an important role in the pathogenesis of ricin toxicity, since its inhibition attenuates the lesions and reduces mortality of ricin-poisoned mice (Mabley et al. 2009). It was reported that apoptosis does not directly correlate with the protein synthesis inhibition (Hu et al. 2001; Suzuki et al. 2000; Brigotti et al. 2002) and saporin-6 and its mutants induce caspase-dependent apoptosis in U937 cells via the mitochondrial or intrinsic pathway, its *N*-glycosidase activity and protein synthesis inhibition being not required for apoptosis induction (Sikriwal et al. 2008).

Interestingly, all toxic type 2 RIPs are transported retrogradely along peripheral nerves (reviewed in Wiley and Lappi 1995), but only modeccin and volkensin (Wiley and Stirpe 1988) and stenodactylin (Monti et al. 2007) are transported retrogradely when injected in the central nervous system (CNS). The reasons for these differences are not known, and may reside in the characteristics of the B chains.

RIPs are also allergenic. The allergenicity of ricin is well known from observations in factories producing castor oil (Thorpe SC et al. 1988). Formation of IgE has been observed in mice after administration of ricin (Thorpe et al. 1989) and of several type 1 RIPs (Zheng et al. 1991), and in laboratory personnel working with RIPs (Szalai et al. 2005).

Table 2 Properties of ribosome-inactivating proteins

RIPs	Structure	Molecular mass (kDa)	Inhibition of protein synthesis (IC_{50}[a]) (nM)		Toxicity to mice (LD_{50}) (mg/kg)
			Cell-free[b]	Hela cells	
TYPE 1	One chain	26–32	<0.01–4.0	170–3,300	0.95–44
TYPE 2					
Toxic	Two chains	60–65	43–88	0.0003–1.7	0.001–0.008
Nontoxic	Two chains	56–63	0.6–>100	0.54–15,000	1.4–>40

[a]The IC_{50} is the concentration inhibiting 50% protein synthesis in target cells.
[b]Cell-free system (rabbit reticulocyte lysate).

3.1 Other Biological Properties

PAP, the first known type 1 RIP, was identified as an antiviral protein (reviewed by Irvin 1975). Subsequently, PAP and other RIPs were found to have antiviral activity against both plant and animal viruses (reviewed by Battelli and Stirpe 1995). The antiviral activity was thought to be due to an easier entry of RIPs into infected cells which were killed, with consequent arrest of viral replication. More recently, however, it was suggested that RIPs could act by damaging directly viral RNA (review in He et al. 2008).

Also, RIPs have some fungicidal (Vivanco et al. 1999; Ng 2004) and insecticidal activity (reviewed by Bertholdo-Vargas et al. 2009).

3.2 Possible Uses

Some practical applications of RIPs of both types, either as such or modified, have been envisaged in medicine and agriculture.

For their activity against viruses, type 1 RIPs have been tested as antiviral agents in plants, animals, and humans. In agriculture, plants transfected with RIP genes showed increased resistance to viral and fungal infection, although they were damaged if the RIP was expressed above a certain level (reviewed in Stirpe and Battelli 2006).

Trichosanthin (McGrath et al. 1989) and PAP (Zarling et al. 1990) inhibit HIV replication in vitro, but the attempts to treat HIV-infected patients were unsuccessful (Byers et al. 1994).

In old traditional Chinese medicine, extracts of tubers of *Trichosanthes kirilowii* were used to induce abortion (Anonymous 1976). This is due to an RIP, trichosanthin, (Yeung et al. 1988), which is currently used in China to induce early and midterm abortion with over 95% success rate and minimal side effects. It was found that momordin and other RIPs have abortifacient activity (Ng et al. 1992). Trichosanthin is highly toxic to trophoblasts and choriocarcinoma-derived cells (Battelli et al. 1992) and causes necrosis of syncytiotrophoblastic cells and fragmentation of placental villi, with consequent large areas of necrosis in the placenta and death of the fetus (Anonymous 1976). The protein was also proposed for the therapy of hydatidiform moles and choriocarcinoma (reviewed in Ng et al. 1992; Shaw et al. 1994). Recently, a unique new intracellular delivery route has been demonstrated for trichosanthin, its well documented invasive properties being able to hijack exosome-mediated intercellular trafficking (Zhang et al. 2009).

In experimental medicine, RIPs have been conjugated to several molecules (lectins, hormones, growth factors, and especially antibodies) capable of delivering them to cells to be selectively eliminated. This matter will be dealt with extensively below.

Ricin, being a potent and easy to obtain poison, has been used for suicidal and homicidal purposes, and there are fears that it could be used as a weapon for warfare or terrorism in the form of powder or aerosol to be inhaled (Waterer and Robertson 2009). The toxin is included among the potential biological weapons by the United States Center of Disease Control and Prevention.

3.3 Role in Nature

The persistence throughout the evolution of these proteins suggests that they may have an important function, useful to the organisms producing them. Several hypotheses have been considered, such as that they could be storage proteins in seeds, or that they may have a defensive role against predators or parasites and fungal, bacterial, and/or viral infections. While these functions cannot be excluded, their higher expression in senescent, wounded, or stressed tissues suggested that they could play a more physiological role and they may also be involved in the mechanism of programmed cell death.

4 RIP-Based Immunotoxins

Application of native RIPs has been explored for the treatment of several diseases (see also Sect. 3.2); it soon became clear however that the potent cytotoxic effect mediated by RIPs could be made more specific and powerful if they were linked (either chemically or via gene fusion) to a targeting molecule.

The general term immunotoxins, first introduced to describe chemical conjugates of RTA and antibodies (Jansen et al. 1982), was subsequently extended to include, however inaccurately, other types of targeted RIPs making use of nonantibody vehicle molecules (e.g., cytokines, peptides, aptamers). For the sake of simplicity, we will also adopt henceforth the term immunotoxins to describe all types of vehicle–toxin combinations, irrespective of the targeting molecule.

4.1 Chemical Immunotoxins

Chemical immunotoxins are obtained by linking RIPs to a vehicle molecule by means of a cross-linking agent. Such immunotoxins are often as cytotoxic as type 2 RIPs. The synthesis is generally accomplished by modifying the carrier molecule and the RIP portion of the conjugate lacking available –SH groups with a heterobifunctional reagent that introduces and activates disulfide groups. When hemitoxins are used (e.g., RTA or abrin A-chain), a free SH group becomes available because of the reduction of the disulfide bond holding the A and the B subunits

together. The linkage used to join the targeting molecule and the toxin must meet the following criteria: (1) it should not impair the binding capacity of the targeting molecule (i.e., antibody, growth factor, cytokine, or hormone); (2) it must allow the active toxin component to enter the cytosol and kill the cell; for this reason cross-linkers introducing nonreducible thioether bonds are not preferred; and (3) for in vivo use, the link must be stable enough to remain intact while the immunotoxin is transported through the tissues to its intended site of action. Among the most frequently used heterobifunctional reagents are *N*-succinimidyl-3-(2-pyridylthio) propionate (SPDP) and 2-iminothiolane (2-IT) (Carlsson et al. 1978; Lambert et al. 1985). Second generation cross-linkers introducing hindered disulfide linkages were later generated to improve stability of the conjugates in vivo. These include sodium *S*-4-succinimidyloxycarbonyl-α-methyl benzyl sulphonate (SMBT) and 4-succinimidyloxycarbonyl-α-methyl-α(2 pyridyldithio) toluene (SMPT) (Thorpe et al. 1987).

The most used conjugates were initially made by conjugating through a disulfide bond the catalytic A subunits of type 2 RIPs such as ricin or abrin, each of which had been separated from its binding B domain by reduction (Krolick et al. 1980; Blythman et al. 1981). Even without its binding domain, RTA was taken up nonspecifically by macrophages and hepatic nonparenchimal Kupffer cells (Fulton et al. 1988). This uptake was due to glycosylated side residues of RTA binding to mannose receptors on the liver cells (Bourrie et al. 1986). The most appropriate technique for reducing nonspecific uptake of RTA was through chemical deglycosylation. Deglycosylated RTA (dgA) immunotoxins had a significantly prolonged lifetime in mice, leading to an improved therapeutic index (Blakey et al. 1987; Fulton et al. 1988). Because the ricin B-chain facilitates the cytotoxicity of RTA-containing immunotoxins (Ramakrishnan et al. 1989), whole ricin has been targeted after chemically blocking its oligosaccharide binding sites to prevent nontarget cell binding. Carbohydrate binding sites of ricin were blocked with ligands prepared by chemical modification of glycopeptides containing triantennary *N*-linked oligosaccharides (Lambert et al. 1991). The resulting "blocked ricin" (bR) was then chemically conjugated to antibodies to make immunotoxins (Table 3).

A further strategy of cross-linking often used with type 1 RIPs utilizes the bifunctional cross-linker 2-iminothiolane; the RIP is first reacted with 2-iminothiolane and the free sulfydryl group protected with Ellman's reagents. The derivatized RIP is then reduced with mercaptoethanol and added to an unreduced derivatized antibody.

4.2 Recombinant Immunotoxins

Recombinant DNA technology has led to the cloning of several RIPs and to the development of RIP-containing constructs of immunotoxins. Most of the RIPs used to obtain recombinant immunotoxins have been cloned following the initial cloning of the RTA gene in *Escherichia coli* by O'Hare et al. (1987). Indeed, in the

Table 3 Clinical applications of RIPs-based immunotoxins

Agent	Antigen	Toxin	Disease	Reference
Ber-H2-SO6	CD30	Sap6	HD	Falini et al. 1992
Ki-4-dgA	CD30	dgA	HD	Schnell et al. 2002
RFT5-dgA	CD25	dgA	HD	Engert et al. 1995; Schnell et al. 2000
RFB4-dgA	CD22	dgA	B-NHL, CLL	Amlot et al. 1993; Sausville et al. 1995
RFB4-Fab'-dgA	CD22	dgA	B-NHL	Vitetta et al. 1991
HD37-dgA	CD19	dgA	B-NHL	Stone et al. 1996
Anti-B4-bRicin	CD19	bR	B-NHL	Grossbard et al. 1999
DA7	CD7	dgA	T-NHL	Frankel et al. 1997a, b
T101-RTA	CD5	RTA	B-CLL	Hertler et al. 1988, 1989
B43-PAP	CD19	PAP	ALL	Uckun 1993
H65-RTA	CD5	RTA	Cutaneous T-cell lymphoma	LeMaistre et al. 1991
Anti-B4-bR	CD19	bR	Myeloma	Grossbard et al. 1998
454A12-rRA	TFR	rRTA	CSF cancer	Laske et al. 1997
260F9-RTA	Tumor antigen	rRTA	Breast cancer	Weiner et al. 1989 Gould et al. 1989
79IT/36-RTA	Tumor antigen	RTA	Colon carcinoma	Byers et al. 1989; LoRusso et al. 1995
XOMAZYME-MEL	Tumor antigen	RTA	Melanoma	Spitler et al. 1987; Gonzalez et al. 1991; Selvaggi et al. 1993
N901-bR	CD56	bR	SCLC	Epstein et al. 1994; Fidias et al. 2002
BDI-1-MD	Tumor antigen	Momordin	Bladder cancer	Yu et al. 1998
CD5-IC	CD5	RTA	Rheumatoid arthritis	Strand et al. 1993; Olsen et al. 1996
CD5 Plus	CD5	RTA	SLE	Stafford et al. 1994
MDX-RA	Antigen of the human lens epithelial cells	RTA	Posterior capsule opacification	Clark et al. 1998

Sap6, saporin, isoform 6; dgA, deglycosylated Ricin A-chain; bR, "blocked" ricin; RTA, ricin A-chain; rRTA, recombinant ricin A-chain; HD, Hodgkin's disease; B-NHL, B-cell non-Hodgkin's lymphoma; CLL, chronic lymphocytic lukemia; ALL, acute lymphoblastic leukemia; CSF, cerebrospinal fluid; SCLC, small cell lung carcinoma; SLE, systemic lupus erythematosus

following years, saporin (Benatti et al. 1989), abrin (Wood et al. 1991), PAP (Kataoka et al. 1993), dianthin (Legname et al. 1993), gelonin (Nolan et al. 1993), and bryodin (Gawlak et al. 1997) were expressed in heterologous systems and obtained in recombinant form.

The availability of recombinant RIPs in turn paved the way to the subsequent development of numerous recombinant immunotoxins. Many examples of such immunotoxins can be found in other sections of this review in relation to other aspects of the work conducted with both chemically synthesized and recombinant

RIPs and RIP-immunotoxins function and applications. In the present section, only some representative recombinant RIP-immunotoxins will be mentioned.

One of the aspects that had to be considered when designing fusion toxins with type 1 RIPs or type 2 hemitoxins is the requirement for intracellular release of the catalytic domain to the cytosol. In an attempt to construct a fusion toxin containing RTA from which free A-chain could be generated, interleukin-2 (IL-2) was fused to recombinant RTA through a linker that contained a proteolytic cleavage site for diphtheria toxin or for clotting factor Xa (Cook et al. 1993). Although the recombinant toxin could be cleaved extracellularly, it could not selectively target cells because the ligand and the toxin were no longer connected. Later, IL-2 was fused to a mutant form of PAP, but the fusion toxin was not purified and was not highly cytotoxic (Dore et al. 1997). Ligands fused to RIPs have produced recombinant immunotoxins with significant cytotoxic activity, including one containing an antiCD40 antibody and bryodin (Francisco et al. 1997), one containing urokinase binding domain and saporin (Fabbrini et al. 1997), and one containing human fibroblast growth factor and saporin (Tetzke et al. 1997). For these molecules, however, it is not known whether the recombinant molecule entered the target cells intact, or the ligand was unstable after internalization, allowing the catalytic domain alone to translocate to the cytosol. The ability of stable ligands to predictably separate from the catalytic domain is a crucial feature of recombinant immunotoxins (Kreitman 1997) and a unique feature found only in native bacterial toxins.

4.3 In Vitro Cytotoxicity

The cytotoxicity of type 1 RIP-based immunotoxins appears to be generally more variable than that of corresponding immunotoxins made with type 2 RIPs or with bacterial toxins (Kreitman 1997). This has been attributed to the lack of a B chain which appears to facilitate the translocation of the enzymatic subunit across cell membranes in addition to its function in cell surface binding. This has prompted a number of investigations aimed at exploring potentiating substances that could be coadministered with the RIP-based immunotoxins to enhance their cytotoxic potential and strategies designed to augment their cytotoxic effect against target cells.

4.4 Enhancement of Cytotoxicity

4.4.1 Lysosomotropic Amines and Carboxylic Ionophores

Lysosomotropic amines and carboxylic ionophores are able to increase dramatically the cytotoxic potency of weakly cytotoxic immunotoxins. In some instances, even noncytotoxic immunotoxins may acquire considerable cytotoxic potency.

These compounds accelerate the cell intoxication process and greatly reduce the number of immunotoxin molecules required for cytotoxicity. They may act by several mechanisms, including inhibition of lysosomal hydrolases, traffic alteration along the endosome-Golgi route, and inhibition of the extracellular recycling of internalized material.

4.4.2 Ammonium Chloride (NH_4Cl)

NH_4Cl is one of the most extensively studied reagents used for enhancing RIP-immunotoxin activity. Raising the pH within acidic organelles (e.g., lysosomes and endosomes) to which the immunotoxins are routed is considered to be one of the mechanisms involved in increasing immunotoxin cytotoxicity (Poole and Ohkuma 1981). It is likely that the lipophylic NH_3 can diffuse across the plasma and lysosomal membranes and become protonated to NH_4^+ within the intracellular organelles, where its entrapment causes a pH increase, thus inhibiting the function of acidic proteolytic enzymes. Using RTA-based immunotoxins, it was found that NH_4Cl could only increase the cytotoxicity of the immunotoxin when the pH was raised to above 7 and that NH_4Cl acts on internalized molecules for a very short time, suggesting that this enhancer affects an early intracellular step (Casellas et al. 1984; Ravel and Casellas 1990). It is however intriguing that the cytotoxic effect of immunotoxins made with other RIPs (i.e., saporin, gelonin) could not be potentiated upon treatment with NH_4Cl (Siena et al. 1988; Goldmacher et al. 1989; Battelli et al. 1998). Differences in trafficking may partly explain these contradictory observations.

4.4.3 Chloroquine

Chloroquine is a well known drug used for the therapy of malaria and, being a clinical drug, might be more suitable for use in combination with immunotoxins in patients. Chloroquine can enhance the cytotoxicity of RTA-based immunotoxins up to 2,500-fold (Casellas et al. 1984). However, as observed for NH_4Cl, immunotoxins made with type 1 RIPs are in general much less sensitive to the potentiating effect of chloroquine (Ramakrishnan and Houston 1984; Goldmacher et al. 1989; Lizzi et al. 2005).

4.4.4 Other Lysosomotropic Amines (Methylamine, Amantadine)

Poole and Ohkuma (1981) have shown that weakly basic substances also can increase the intralysosomal pH in a concentration-dependent manner. Methylamine is a weak base which affects the intralysosomal pH. A concentration of 10 mM enhanced the activity of an anti-CD5 immunotoxin on CEM cells by over 13,000-fold (Casellas et al. 1984). The drug 1-adamantanamine hydrochloride

(amantadine) also is a potent enhancer of the cytotoxic activity of anti-CD5 RTA-based immunotoxins against peripheral blood T cells (Siena et al. 1987) and can restore the activity of an anti-IgM–saporin conjugate whose efficacy is impaired by the presence of human bone marrow (Bregni et al. 1988). Amantadine may be more advantageous than NH_4Cl because it is a licensed drug used for the prophylaxis of the influenza; although the in vitro concentration used in the study cited above (1 mmol/L) may be difficult to achieve in the blood of patients, it may nevertheless be used to purge the bone marrow of patients from malignant cells or from allogeneic mature T cells which are often responsible for graft-versus-host-disease (GVHD) reactions.

4.4.5 Carboxylic Ionophores

Carboxylic ionophores such as monensin are well studied reagents able to enhance immunotoxin efficacy. Monensin, grisorixin, lasalocid, and nigericin are all able to enhance the effect of RIPs-based immunotoxins (especially those made with RTA); however, other ionophores such as nonactin, valinomycin, and calicimycin have no effect on immunotoxin toxicity. The present section will focus essentially on the effects brought about both in vitro and in vivo by monensin, which is the most widely used and described carboxylic ionophore for immunotoxin potentiation.

Monensin is a molecule capable of ion complexation through a cyclic form stabilized by hydrogen bonding between the carboxyl and hydroxyl groups (Mollenhauer et al. 1990). Monensin is able to collapse Na^+ and H^+ gradients across cell membranes and may increase the pH of acidic vesicles like lysosomes through the exchange of Na^+ for H^+. As an immunotoxin potentiator, monensin was first described in 1984 (Raso and Lawrence 1984; Casellas et al. 1984) and was found to function at very low concentrations (nanomolar range) in vitro yielding significant increase in the cytotoxicity of RTA-based immunotoxins, with IC_{50s} in the range 10^{-12}–10^{-14} M. When assayed to evaluate the potentiation of antibody–gelonin conjugates, however, its effects were close to nil (Goldmacher et al. 1989). As shown by Raso and Lawrence (1984), monensin accelerates the kinetics of target cell intoxication. Lysosomotropic amines and carboxylic ionophores raise the pH and so it has been suggested that they may act by reducing the rate of degradation of the immunotoxin (Casellas et al. 1982, 1984). However, Raso and Lawrence (1984) and Jansen et al. (1992) have shown that monensin potentiates RTA immunotoxins at concentrations that do not affect lysosomal pH, suggesting that an alternative mechanism may be operating. In fact, at micromolar concentrations monensin increases the pH in the lysosomes; however, vacuolization of the Golgi and enhancement of immunotoxins can be obtained at 100-fold lower concentrations of 50 nM (Jansen et al. 1992). Indeed, studies have shown that lysosomotropic amines can delay the delivery of immunotoxins to lysosomes Carrière et al. 1985), keeping them longer inside peripheral endosomes and possibly diverting them to other subcellular compartments which facilitate their escape to the cytosol. Along these lines, Ravel et al. (1992) demonstrated that the presence of

NH_4Cl or monensin, both dramatically enhancing the effects of RTA immunotoxins, did not affect the rate of internalization or the intracellular localization of the immunotoxin, suggesting that these activators could act at a postendocytotic level on a limited number of immunotoxin molecules.

To evaluate the possible in vivo potentiation of RTA immunotoxins by monensin, and our group (Colombatti et al. 1990) have used monensin chemically cross-linked to the carrier protein human serum albumin (HSA). This compound can enhance the immunotoxin effect to the same extent as it does in in vitro experiments. However, since disulfide cross-linked HSA–monensin is rapidly inactivated by human serum, we (Candiani et al. 1992) synthesized thioether cross-linked HSA–monensin conjugates, which are resistant to treatments with reducing agents (e.g., glutathione, dithiothreitol) and show potentiating activity identical to that of free monensin and disulfide HSA-linked monensin achieving a potentiation of 45–35,000-fold. Immunotoxin potentiation by both disulfide- and thioether-linked types of HSA–monensin conjugates is inhibited by whole human serum, the serum blocking factor(s) residing mostly in a Mr 40,000–90,000 protein fraction (Candiani et al. 1992). In support of this finding, Jansen et al. (1992) found that a serum glycoprotein of an approximate molecular mass of 45 kDa (sGP3.5) was responsible for plasma inhibition of monensin potentiating effect on immunotoxins. The protein sGP3.5 could be involved in the physiological regulation of intracellular trafficking.

Other formulations of monensin were subsequently explored to allow easier application in vivo. Incorporation of monensin in unilamellar vesicles (liposomes) (Griffin et al. 1993), and monoclonal antibody-targeted liposomes (Singh et al. 1994) were 100-fold more efficacious in immunotoxin potentiation as compared to nontargeted monensin–liposomes.

4.4.6 Antagonists of Ca^{++} Channels and Other Compounds

Ca^{++} channel blockers and their derivatives have been studied to evaluate their ability as immunotoxin enhancers. They can often provide several logs increase of immunotoxin efficacy. Their mechanism of action does not appear to be associated with the Ca^{++} channel function but might be related to the prevention of lysosomal degradation of the immunotoxin.

4.4.7 Verapamil and Its Derivatives

Verapamil was shown to enhance the cytotoxicity of anti-EGFR RTA-immunotoxins up to 40-fold (Akiyama et al. 1984). On the other hand, no influence was observed on an anti-CD22–RTA conjugate (van Horssen et al. 1999). It is likely that the potentiation afforded is in relation with the target antigen or with the histotype of the target cell.

4.4.8 Perhexiline and Indolizines

Perhexiline maleate is another Ca^{++} channel antagonist and is able to enhance immunotoxin cytotoxicity. An anti-CD5 RTA-immunotoxin could be enhanced 30–20,000-fold on human T leukemia cells (Jaffrezou et al. 1990). It is thought that perhexiline may act by altering the membrane lipid composition through its inhibitory action on acid sphingomyelinases leading to modifications in the intracellular trafficking of the immunotoxins.

A novel class of Ca^{++} blockers (indolizines SR33557 and SR33287) demonstrated potentiating effects greater than those observed with verapamil using an anti-CD5 RTA-immunotoxin (Jaffrezou et al. 1992).

All-*trans* retinoic acid was also found to specifically increase receptor-mediated intoxication of RTA-immunotoxins by more than 10,000-fold. Direct microscopical examination demonstrates that the Golgi apparatus undergoes morphological changes upon treatment with retinoic acid, suggesting that it may alter the intracellular trafficking of internalized immunotoxins.

4.4.9 Ricin B-Chain

Many immunotoxins obtained by linking the enzymatic effector subunit of the type 2 RIP ricin (i.e., RTA) to targeting molecules have been described (Colombatti 2002). However, the cytotoxicity of RTA-based immunotoxins is generally more variable than that of the corresponding immunotoxins made with the whole toxin (Colombatti 2002). This has been attributed to the lack of the B-chain which may facilitate RTA translocation across the cell membrane in addition to its cell surface binding.

Specific cytotoxic agents were therefore prepared by linking intact ricin to antibodies in a manner that produces obstruction of the galactose-binding sites on the B chain of the toxin and thereby diminishes the capacity of the conjugate to bind nonspecifically to cells (Thorpe et al. 1984; Cattel et al. 1988). The "blocked" ricin conjugates combine the advantages of high potency, which is often lacking in antibody–RTA conjugates, with high specificity, which previously was lacking in intact ricin conjugates. Moreover, several strategies were also explored to supply isolated B-chain to RTA-containing preparations. Addition of free B-chain in vitro (Youle and Neville 1982; McIntosh et al. 1983), or ricin-B-chain coupled to antitarget antigen antibody or to antimouse antibodies recognizing the cell surface (Vitetta et al. 1983, 1984) enhanced activity. To overcome the possible limitations inherent in using ricin B-chain-containing samples in vivo, ricin B-chain was chemically modified to reduce its ability to bind terminal galactose residues on the cell surface (Vitetta 1986). More recently Frankel et al. (1997) applied molecular biology techniques to fuse IL-2 and a ricin B-chain variant, expressed in insect cells, with modifications of aminoacid residues in each of the three galactose-binding subdomains.

4.4.10 Viruses

Viruses utilize specialized envelope structures to enter the cytosol of the infected cells. Following binding to the cell surface receptors, viruses traffic to acidic intracellular compartments (e.g., endosomes) where domains of the viral coat are activated, thereby triggering the interaction of viral proteins with the organelles' membranes and the disruption of the endosomal membrane.

In a simplistic approach, adenoviruses were first used to enhance the cytotoxic effects of RTA coupled to antitransferrin receptor antibodies (FitzGerald et al. 1983) or to anticolon carcinoma cell antibodies (Griffin et al. 1987). Two fusogenic peptides of the influenza virus (HA23 and HA24) were employed to enhance the efficacy of anti-HIV immunotoxin by mixing the immunotoxins with peptide preparations (Tolstikov et al. 1997). Peptide HA23 enhanced the activity of the immunotoxin by four- to fivefold. Greater potentiation was achieved by fusing a vesicular stomatitis virus (VSV)-derived peptide to the cDNA coding for RTA (Chignola et al. 1995). Three chimeric proteins were successively obtained by fusing together the dianthin gene and DNA fragments encoding for the following membrane-acting peptides: the N-terminus of protein G of the vesicular stomatitis virus (KFT25), the N terminus of the HA2 hemagglutinin of influenza virus (pHA2), and a membrane-acting peptide (pJVE) (Lorenzetti et al. 2000). Genetic fusion of these membrane-acting peptides to enzymatic cytotoxins resulted in the acquisition of new physico-chemical properties and in the enhancement of the cytotoxic potential of immunotoxins constructed with the modified dianthin.

4.4.11 Saponins

Saponins are plant glycosides that consist of a steroid, steroid alkaloid, or triterpenoid aglycone and one or more sugar chains that are covalently linked by glycosidic binding to the aglycone. Heisler et al. (2005) and Fuchs et al. (2009) investigated whether saponins are able to enhance the efficacy of an immunotoxin consisting of epidermal growth factor (EGF) linked to saporin. Preapplied saponin enhances the target cell-specific cytotoxic effect, dependent on the cell line, between 3,560- and 385,000-fold with a maximum IC_{50} of 0.67 pM. Nontarget cells are not affected at the same concentration. Thus saponins not only preserve the target specificity of the chimeric toxin but also broaden the therapeutic window with simultaneous dose lowering. A drawback of saponins in tumor therapy is their nontargeted spreading throughout the whole body and their innate toxicity.

5 Animal Studies

Before evaluating the clinical safety and efficacy of an immunotoxin, preclinical investigations are carried out in animal models. An enormous literature has been produced in the past on these matters. This subject has been dealt with in a number

of excellent reviews (Kreitman 1997; Wong et al. 2005) and only general aspects will be treated here.

To determine whether an immunotoxin might be effective in vivo, murine models are most commonly produced in which mice are grafted with human xenografts of tumor cell lines. In this regard, solid tumors have been found in general more difficult to treat than disseminated leukemia of the same cell line (van Horssen et al. 1996) and this is reproduced in the greater clinical response of hematological tumors as compared to that of solid tumors (see also below). Animal models are hampered however by great limitations that may lead to overestimation of the potential beneficial effects of immunotoxin treatment in humans. In fact, once antitumor activity is found to be appreciable in vitro, it is still not clear that the agent would result in responses in patients. One reason for this is that cell lines may grossly overestimate the number of antigen binding sites/cells in patients. Thus primary tumor cells freshly isolated from patients often display a much lower number of target antigens/cell. It must also be considered that regulation of antigen expression is sometimes crucially dependent upon conditions and microenvironments which are found exclusively in vivo. Therefore, primary tumor cells are often tested ex vivo to determine sensitivity to the immunotoxin and to predict possible outcomes in vivo. Another problem with murine models is that much greater unwanted toxicity may occur in patients than in mice because unwanted cross-reactions appearing in humans may not be detected in mice and the murine target antigen may not even bind the immunotoxin in the same manner as the human antigen, resulting in different pharmacokinetics and pharmacodynamics of the agent. Indeed, early clinical trials with immunotoxins were sometimes discontinued because of significant toxicity stemming from unpredicted cross-reactions with essential organs (Weiner et al. 1989, Gould et al. 1989). For this reason, nonhuman primates that display the antigen on their normal cells are used for toxicity experiments. Even so, expensive experiments of this type are often not predictive of human toxicity.

One additional problem with immunotoxin treatment that emerged clearly in animal models is the development of resistant mutants after treatment of animals with only one immunotoxin, as demonstrated in an AKR mouse model, where Thy1.1-negative mutants caused fatal relapses in 20% of the animals treated with an immunotoxin to the Thy1.1 antigen of mouse lymphoma cells (Thorpe PE et al. 1988). Use of immunotoxin cocktails, that is, a combination of two or more immunotoxins against different antigens on the same target cell, reduces the likelihood of mutant cell escape. Analysis of malignant cells re-established ex vivo from tumors that had relapsed after therapy with one immunotoxin showed high sensitivity toward immunotoxins directed against a different target antigen. In addition, 90% of mice treated with a cocktail of two or three immunotoxins after tumor challenge had continuous complete remissions, as compared with only 40% of animals treated with the same dose of a single immunotoxin (Engert et al. 1995). This concept has also been confirmed recently in a severe combined immunodeficiency (SCID) mouse model (Flavell et al. 2001) where it was demonstrated that treatment with a cocktail of anti-CD7 and anti-CD38 saporin-based immunotoxins

was clearly superior to the treatment with one single immunotoxin in eliminating grafted human T-ALL cells. New developments achievable through the use of recombinant DNA techniques will allow the engineering of bispecific or multi-specific immunotoxins for a more thorough eradication of target tumor cells.

Many valuable results were obtained in the recent past using saporin-containing immunotoxins and other conjugates directed against cells of the nervous system. This concept of "molecular" surgery has been extensively reported in various excellent reviews (Wiley and Lappi 2003; Lappi and Wiley 2004). Among the main results reported, Alzheimer's disease was reproduced by selective destruction of the basal forebrain structures (Wiley et al. 1995). Moreover, conjugates of saporin and substance P have been used to selectively eliminate sensory neurons (reviewed in Wiley and Lappi 2003), and their potential use for treating patients with chronic debilitating pain has been proposed (Ralston 2005; Wiley and Lappi 2005).

The remainder of this review will focus on RIP-based immunotoxins tested in patients to date.

6 Ex Vivo Bone Marrow Purging with Immunotoxins

Bone marrow transplantation (BMT) is used in the treatment of leukemias and lymphomas which have failed, or are likely to fail, first-line chemotherapy. An increasing number of patients with metastatic disease of various solid tumors receive autologous BMT as well. While the development of several GVHDs is the major complication in allogeneic BMT, relapses due to infusion of bone marrow or peripheral stem cells contaminated with residual malignant cells are one of the most challenging problems in autologous transplantation. Ex vivo treatment of the patients' stem cells with monoclonals, immunotoxins, magnetic beads, or cytostatic drugs before reinfusion ("purging") is expected to reduce the frequency of relapses after autologous BMT.

A large number of studies were conducted to evaluate the feasibility of purging bone marrow with RIPs-based immunotoxins in the past decades. The reader is referred to an excellent review for a more detailed description of different immunotoxins used and the various settings studied (Frankel et al. 1996). Here we will just mention the first studies made with RIP-containing immunotoxins as examples. Immunotoxins directed against the CD5 and CD7 T-lineage differentiation antigens were used to purge autografts of patients with high-risk T-lineage acute lymphoblastic leukemia (Uckun et al. 1990). In general, the combination of different methods for bone marrow purging (e.g., simultaneous treatment with immunotoxins and chemotherapeutic agents) is more effective in killing residual tumor cells. Relapses which occur after effective bone marrow purging are often caused by insufficient pretransplant conditioning (Uckun et al. 1990).

Before a patient receives an allogeneic BMT, healthy donor bone marrow is often purged of T-lymphocytes in order to prevent GVHD. Immunotoxins have been successfully used for T-cell depletion in allografts, yet extensive T-cell

elimination has a negative impact on engraftment of donor marrow and disrupts the graft-versus-leukemia effect (Blazar et al. 1991).

Benefits of purging tumor cells from the bone marrow, however, have not been reliably confirmed in phase II and III trials in several hematologic diseases (Alvarnas and Forman 2004). Limitations of purging include possible loss of progenitor cells, delayed engraftment, and qualitative immune defects following transplant. Further studies will be needed to clarify these points.

7 Clinical Studies

A number of studies have evaluated the effects and potentials of RIP-based immunotoxin treatment in the clinics. However, in spite of the great number of RIPs discovered in the past, only a handful of them have been studied as a potential macromolecular therapeutic in human diseases (Table 3).

Only investigations that have used RIP-based immunotoxins will be dealt with here. The reader is referred to excellent reviews for descriptions of immunotoxins made with bacterial toxins (e.g., diphtheria toxin, Pseudomonas exotoxin A) and their effects in human diseases (Pastan et al. 2007).

Experimental studies suggest that immunotoxins have optimal efficacy when inoculated as a single short course of treatment in patients with minimal disease (Ghetie et al. 1994). However, phase I clinical trials are designed to test the safety of a drug and can be carried out in patients who generally are affected by diseases not responding to conventional therapies and with bulky tumors. Only when the side effects, immunogenicity, maximum tolerated dose (MTD), and pharmacokinetic parameters have been established, can the immunotoxin proceed to phase II trials where patients with less advanced disease can be selected. In phase II trials, therapeutic efficacy can be tested and if a response rate between 20 and 40% is achieved (partial or complete remission) the drug under study will further proceed into phase III clinical trials. To date several immunotoxins have completed phase II/III trials and their efficacy, although it was found encouraging in many occasions, is still under evaluation. In the following paragraphs we will review the major achievements obtained thus far with RIP-based immunotoxins in a clinical setting. The main results in terms of response to therapy will be reported here. Toxicity phenomena and other drawbacks of immunotoxin treatment will be cumulatively described in a separate section (see below).

7.1 Hematologic Tumors

Hematologic malignancies are most suitable for treating with immunotoxins, because malignant cells are often intravascular and directly accessible to intravenously administered drugs, or concentrated in lymphoid organs where access to

macromolecules is less problematic than in other tissues. Additionally, patients affected by hematologic malignancies often lack sufficient immunity to reject the heterologous macromolecules administered. It is surprising, however, that in spite of this, only a handful of antigens have been targeted with RIP-based immunotoxins in hematologic patients. These are summarized in Table 3.

7.1.1 Hodgkin's Lymphoma

In Hodgkin's disease, Hodgkin and Reed–Sternberg cells consistently express the antigen CD30. An anti-CD30 monoclonal antibody (Ber-H2) was chemically cross-linked to saporin (S06) and the immunotoxin was given to four patients with advanced refractory Hodgkin's disease (Falini et al. 1992). In three, there was rapid and substantial reduction in tumor mass (50–75%). Clinical responses were transient (6–10 weeks).

An anti-CD30 immunotoxin (Ki-4-dgA, constructed by chemical linkage of the antibody Ki-4 to (dgA)) was evaluated by Schnell et al. (2002) in patients with relapsed $CD30^+$ lymphoma. Clinical response in the 15 evaluable patients included one partial remission, one minor response, and two stable diseases. The immunotoxin, however, was less well tolerated than other immunotoxins of this type, possibly because of the low number of $CD30^+$ peripheral blood mononuclear cells, and in part because of binding of the immunotoxin to soluble CD30 antigen and the resulting circulation of immunotoxin/CD30 complexes.

The anti-CD25 immunotoxin RFT5-dgA was administered to patients with relapsed Hodgkin's lymphoma with previous heavy treatment in two clinical trials (Engert et al. 1995; Schnell et al. 2000); in spite of the suboptimal conditions of the patients to ascertain the efficacy of treatment with an immunotoxin (relapse, high tumor cell burden, unsuccessful previous therapies), RFT5-dgA was found to be of moderate clinical efficacy (in a total of 32 patients, four partial responses, two minor response, eight stable disease, and nine progressive disease were observed).

7.1.2 Non-Hodgkin's Lymphoma

Anti-CD22 immunotoxins were evaluated in non Hodgkin lymphomas (NHL). Fab'-RFB4.dgA was the first immunotoxin to be tested in advanced refractory B-cell NHL of low, intermediate, or high grade type (Vitetta et al. 1991). Although patients presented with large tumor masses and were heavily pretreated, 38% achieved a partial remission. The partial remission rate was even higher in patients with >50% CD22-positive tumor cells in lymphnode or bone marrow biopsies. IgG-RFB4.dgA made with whole IgG antibodies was used in similar phase I studies (Amlot et al. 1993; Sausville et al. 1995) in refractory B-NHL patients. The cumulative results of these studies were that in 42 patients in total one complete response, nine partial responses, three minor responses, and six stable diseases were observed.

Because of its smaller size, Fab' immunotoxins were expected to penetrate into solid tumors more easily than immunotoxins made with whole IgG. However, antitumor activity of Fab' immunotoxins was generally inferior because of reduced target cytotoxicity and shorter half-life.

Anti-CD19 immunotoxins were also studied in similar trials; the immunotoxin IgG-HD37.dgA, however, was less efficacious than IgG-RFB4.dgA (Stone et al. 1996), in accordance with a lower IC_{50} and a less impressive performance in mice (Ghetie et al. 1992). Of 23 evaluable patients one complete response and one partial response were observed. In a subsequent study with the same immunotoxin (Messmann et al. 2000), unpredictable clinical courses including deaths were observed. A tendency of the immunotoxin to aggregate and inaccuracies in shipping, storage, and handling may explain such results leading to the conclusion that non-aggregate-forming formulations should be carefully pursued prior to clinical trials.

A phase II trial was undertaken to determine the safety, toxicity, and potential efficacy of the B-cell restricted anti-CD19 immunotoxin anti-B4-bR (Anti-B4-bR) administered as adjuvant therapy to patients in complete remission after autologous BMT for B-cell NHL (Grossbard et al. 1999). The 4-year disease-free survival and overall survival were estimated at 56 and 72%, respectively. This study demonstrated that anti-b4-bR can be administered safely to patients as adjuvant therapy early after autologous BMT for B-cell NHL. The toxicities were tolerable and reversible.

The anti-T-NHL immunotoxin to CD7 (DA7) consisting of deglycosylated ricin A chain coupled to a mouse monoclonal antihuman CD7 antibody (Frankel et al. 1997) was also studied in 11 patients with T-cell lymphoma (>30% CD7+ malignant cells). Two partial responses and one minimal response were seen. Patients with minimal lymphoma burden or T-cell large granular lymphocyte (LGL) leukemia showed the best responses.

7.1.3 Leukemia

An immunotoxin b43(anti-CD19)PAP made by linking the PAP toxin to an anti-CD19 monoclonal (Uckun 1993) was evaluated in patients affected by therapy refractory B-lineage ALL. Four complete remissions and one partial response were observed, as well as a rapid reduction in the numbers of leukemic cells in circulation in five additional patients. Interestingly, no significant organ toxicity was noticed and the patients did not develop an immune response to either the PAP toxin or the murine monoclonal antibody.

In two studies where the CD5 antigen was taken as the target, patients affected by B-CLL refractory to alkylating agents were treated with the immunotoxin T101-RTA (Hertler et al. 1988, 1989). All patients had a rapid fall in the white blood cell count of less than 2–4 h duration after each immunotoxin infusion, most likely secondary to the antibody portion of the immunotoxin. No sustained benefit could be demonstrated in any patient, possibly because in the absence of enhancing agents, the leukemic cells of all four treated patients were resistant to T101-RTA at concentrations up to 2 μg/ml in vitro.

7.1.4 Multiple Myeloma

B-cell restricted immunotoxin anti-B4-bR was administered to five patients with previously treated multiple myeloma (Grossbard et al. 1998). No patient demonstrated a significant decline in the disease during therapy.

7.1.5 Cutaneous Lymphoma

The CD5 antigen is heterogeneously expressed on cutaneous T-cell lymphoma tumor cells, but is not expressed on normal cells except lymphocytes. A phase I trial was therefore conducted in which 14 patients with cutaneous T-cell lymphoma progressive on other therapies were treated with H65-RTA (an anti-CD5 monoclonal coupled to RTA) (LeMaistre et al. 1991). Partial responses lasting from 3 to 8 months were documented in four patients. Interestingly, the immunotoxin could be repeatedly administered safely, even in the presence of anti-immunotoxin antibodies, with significant response.

7.2 Cerebrospinal Fluid Spread of Tumors

Leptomeningeal neoplasia occurs in 5–20% of all cancer patients and results in a very poor prognosis, with a median survival of only a few months. Studies indicate that 35–40% of all breast cancer patients experience CNS involvement at some point in their disease and that 5% experience leptomeningeal involvement. In addition, primary CNS tumors and leukemias can spread diffusely to the leptomeninges, thwarting efforts at treatment. Therefore, a pilot study of intraventricular therapy with the immunotoxin 454A12-rRA in eight patients with leptomeningeal spread of systemic neoplasia (six breast carcinomas, one melanoma, and one leukemia) was conducted (Laske et al. 1997). The immunotoxin 454A12-rRA is a conjugate of a monoclonal antibody against the human transferrin receptor and recombinant RTA. In four of the eight patients, a greater than 50% reduction of tumor cell counts in the lumbar cerebrospinal fluid occurred within 5–7 days after the intraventricular dose of 454A12-rRA; however, no patient had the CSF cleared of tumor, and evidence of tumor progression was demonstrated in seven of the eight patients after treatment.

7.3 Solid Tumors

7.3.1 Small-Cell Lung Cancer (SCLC)

In patients affected by SCLC, relapse and resistance to established chemotherapy regimens often cause death. N901-blocked ricin (N901-bR), a murine monoclonal antibody-bR immunotoxin, was studied as a potential therapeutic for SCLC

(Epstein et al. 1994) in 21 patients. N901-bR targets CD56, present on SCLC and cells of neuro-ectodermal origin. In this study, one patient had a documented partial response and six patients demonstrated stable disease. A further phase II trial was designed to evaluate the efficacy and toxicity of the immunotoxin N901-bR in patients with SCLC who achieved a complete or near-complete response following chemotherapy and/or radiation (Fidias et al. 2002). Nine patients enrolled in the study before it closed following a treatment-related death. Seven patients had extensive-stage disease and entered the study with a more than 90% reduction of their original tumor. Two patients with limited-stage SCLC had no evidence of disease at study entry. Toxicity and a massive immune response to the administered immunotoxin reduced the effects of the treatment in one patient with limited stage disease.

7.3.2 Bladder Cancer

Bladder tumors are relatively more accessible to passive administration of therapeutic macromolecules. Indeed, Yu et al. (1998) describe an ingenious application of an antibladder tumor monoclonal (BD1-I) chemically cross-linked to momordin for the therapy of bladder carcinomas. The immunotoxin was introduced via a catheter into the bladder and 18 patients were treated. The authors concluded that intravesical administration is very safe and effective.

7.3.3 Breast Tumors

Four women with metastatic breast cancer were treated by Weiner et al. (1989) with the immunotoxin 269F9-rRTA. The trial however was quickly suspended because patients treated with a continuous infusion schedule developed significant neurological toxicities. Also in a study by Gould et al. (1989) five patients treated with antibreast cancer immunotoxin with continuous infusion experienced severe toxic effects, including marked fluid overload and debilitating sensorimotor neuropathies. In this case, immunohistochemistry suggested that the 260F9 monoclonal targeting of the Schwann cells may have induced demyelination and subsequent neuropathy.

7.3.4 Colon Carcinoma

In other clinical trials, the application of anticolon carcinoma immunotoxins (Byers et al. 1989; LoRusso et al. 1995) was studied. The mAb 791T/36, recognizing a Mr 72,000 antigen on the surface of colon carcinoma cells was conjugated to RTA. Seventeen patients with metastatic colorectal cancer were treated in a phase I trial. Biological activity, manifest as mixed tumor regression, was seen in five patients (Byers et al. 1989). The immunoconjugate XMMCO-791/RTA (RTA linked to a

murine mAb 791T which binds a glycoprotein of 72 kDa, expressed on human colorectal carcinoma, ovarian carcinoma, and osteogenic sarcoma) was used by LoRusso et al. (1995) in a phase I trial. Twelve patients with metastatic colorectal carcinoma were treated. No antitumor activity was seen.

7.3.5 Melanoma

Spitler et al. (1987) conducted a trial of a murine monoclonal antimelanoma antibody–RTA immunotoxin (XOMAZYME-MEL) in 22 patients with metastatic malignant melanoma. Encouraging clinical results were observed (one complete response and nine mixed response/stabilization of the disease), even after a single course of a low dose of immunotoxin.

In a study by Gonzalez et al. (1991), 20 patients with metastatic melanoma were treated with XOMAZYME-MEL. There was one durable complete response of 12+ month duration and one brief mixed response lasting 3 months. In a subsequent study by Selvaggi et al. (1993) of four patients treated with XOMAZYME-MEL, one experienced partial lymphnode remission for 5 months and a second patient had stable mediastinal disease for 20 months.

8 Autoimmune Diseases

The targeting of T cells can be envisaged in attempts to treat autoimmune diseases because autoreactive T cells are thought to be involved in their pathogenesis. In the following paragraphs, results obtained in rheumatoid arthritis (RA) and in systemic lupus erythematosus (SLE) are reported.

8.1 RA

The safety and activity of an immunoconjugate of RTA and anti-CD5 monoclonal antibody (anti-CD5 IC), with and without concomitant methotrexate and/or azathioprine, was evaluated for the treatment of RA in 79 patients with active disease (Strand et al. 1993). Response rates were 50–68% at 1 month and 22–25% at 6 months. Transient depletion of CD3/CD5 T cells was observed on days 2 and 5 of treatment, with reconstitution on day 15 or day 29. This initially suggested activity of anti-CD5 IC in active RA but when the efficacy of the treatment was evaluated in a total of 104 evaluable patients in a multicenter, double-blind, multiple-dose, placebo-controlled study, CD5-IC failed to produce marked or prolonged T cell depletion and was not more effective than placebo in ameliorating disease manifestations (Olsen et al. 1996).

8.2 SLE

CD5 Plus (an anti-CD5 monoclonal linked to RTA) was used in patients with SLE to determine the safety and clinical and biological effects (Stafford et al. 1994). Six patients (four with glomerulonephritis and two with thrombocytopenia) were studied. Improvement was documented in two patients with nephritis; no effect on thrombocytopenia was observed. The conclusion was that anti-CD5 ricin A chain immunoconjugate is well tolerated in patients with SLE, causes modest T cell depletion which may persist for months, and may have some clinical efficacy in lupus nephritis.

9 Other Applications

9.1 Corneal Opacification

The safety and effectiveness of an immunotoxin (MDX-RA) designed to inhibit posterior capsule opacification (PCO) was evaluated in 63 eyes of 63 patients having extracapsular cataract extraction by phacoemulsification (Clark et al. 1998); these patients were enrolled in a Phase I/II clinical investigation. The immunotoxin consists of a murine monoclonal antibody (4197X) that binds specifically to human lens epithelial cells conjugated to RTA. It was found that the immunotoxin was well tolerated and was effective in reducing PCO for up to 24 months after cataract surgery.

10 Problems and Opportunities in the Future Development of Immunotoxins

10.1 Selection of Patients

It is generally believed that immunotoxins could be most useful in the elimination of small aggregates of target cells, as is the case in the "minimal residual disease." So far, however, immunotoxins have been administered only to patients at an advanced stage of disease, that is, with large tumor masses and with limited possibilities of benefiting from a biological therapy. Considering the low penetration of immunotoxins into solid tumors, even minor responses in these patients are impressive. If, however, immunotoxins would be used in an adjuvant setting, after surgical debulking or following established conventional type of regimens (e.g., radiotherapy and chemotherapy) small clumps of cells or even isolated tumor cells remaining might be completely removed by the administration of immunotoxins.

Furthermore, various applications can be envisaged which could be more practicable than the use of systemically administered immunotoxins. Encouraging results have been obtained in the local administration of antibrain tumor immunotoxins (Laske et al. 1997). Further, immunotoxins can be administered intravesically for the therapy of bladder cancer. In this way, immunotoxins may not enter into contact with the systemic circulation (or only negligibly) reducing the risk of an immune response and minimizing the risk of toxicity and side effects, thus allowing the use of higher dosages against cancer cells. RIP-containing immunotoxins (Thiesen et al. 1987; Battelli et al. 1996) and a fibroblast growth factor–saporin conjugate (Tetzke et al. 1997) specific for bladder tumors have been assayed, and clinical trials with immunotoxins have been conducted with promising results (Yu et al. 1998; Zang et al. 2000).

10.2 Immunogenicity

Taking into account the results obtained in a wide range of clinical trials, the incidence of immunogenicity after a single course of immunotoxin treatment ranges from 50 to 100% for solid tumors, and from 0 to 40% for hematologic malignancies. Antibodies that neutralize the cytotoxic effects can be detected by determining whether serum containing them can block the cytotoxicity of the immunotoxin against cultured cells. The presence of neutralizing antibodies in the patients' serum lowers the levels of biologically active immunotoxin and compromises efficacy. The method most useful for other biologic agents, such as interferon (Reddy 2004) and L-asparaginase (Graham 2003), is PEGylation, which not only reduces immunogenicity but also prolongs half-life. This approach has been evaluated also for ricin, demonstrating a lower binding of antiricin antibodies after PEGylation without altering its enzymatic properties (Hu et al. 2002), and in the case of isolated RTA, the method may even increase its therapeutic potential (Youn et al. 2005). PEGylating a toxin, however, appears more challenging than PEGylating simpler molecules, because the disturbance of sites on an enzymatic toxin may reduce activity. Moreover, PEGylation does not completely prevent an immune response.

Immunologic studies have identified a large number of T-cell and B-cell epitopes on RTA, suggesting that "humanization" of the molecule or elimination of immunodominant epitopes would be extremely difficult. Indeed, use of human T-cell lines and T-cell clones allowed the identification of residue I175, which is part of the active site of the molecule, as a crucial residue for the epitope(s) recognized by two HLA-DRB1 alleles. Failure of T-cell clones to recognize RIPs showing sequences similar but not identical to that of RTA further confirmed the role of I175 as a key residue in immune recognition of RTA (Tommasi et al. 2001) but also encourages sequential use of immunotoxins with different RIPs in treatment courses. Use of a peptide scan approach and the sera of patients treated with

antibody–RTA conjugates allowed the identification of a continuous B-cell epitope recognized by all patients studied, located within the stretch L161-I175 of the RTA primary sequence, close to a the T-cell epitope (Castelletti et al. 2004). The ability of anti-L161-I175 antibodies to recognize folded RTA and to affect its biological activity by inhibiting RTA-immunotoxin cytotoxicity in vitro revealed that they may exert an important role in immunotoxin neutralization in vivo. No similar studies were conducted for other RIPs, however.

Recently, bouganin, a type I ribosome inactivating protein isolated from the leaf of *Bougainvillea spectabilis*, was mutated to remove the T-cell epitopes while preserving the biological activity of the wild-type molecule (Cizeau et al. 2009). A genetic fusion with an anti-EpCAM Fab moiety was then produced and in vivo efficacy demonstrated using a human tumor xenograft model in SCID mice. However, effects of T-epitopes removal on immune recognition in vivo was not evaluated.

10.3 Side Effects

A variety of unwanted toxicities have been observed with immunotoxins that have limited the dose and hence the efficacy. The most common toxicity is vascular leak syndrome (VLS), which is characterized by symptoms related to extravasation of fluid into the interstitial space, such as hypoalbuminemia, edema, weight gain, hypotension, tachycardia, and weakness. These side effects are not surprising, given that a cytotoxic protein must interact with endothelial cells to exit the blood vessels. Studies have shown that RTA binds the fibronectin receptor on the endothelial cells, acting as a "disintegrin" and increasing intercellular permeability (Soler-Rodriguez et al. 1993). Other studies have suggested that specific residues on RTA and IL-2 can bind to endothelial cells through a (x)D(y) motif and elicit VLS by a mechanism independent of the normal toxin-induced cell death (Baluna et al. 1999). Such studies led to a mutant form of RTA showing less VLS in animals (Smallshaw et al. 2003).

Hepatotoxicity, a side effect frequently observed also with recombinant immunotoxins, is attributed to the binding of basic residues on the antibody to negatively charged hepatic cells (Schnell et al. 1998). Hepatotoxicity appears to be related to cytokine release, possibly by the Kupffer cells of the liver (Schnell et al. 2003).

Renal toxicity due to immunotoxins is less well defined and could be nonspecific at least in part because the kidneys are among the main routes of excretion of immunotoxins.

Toxicities due to unpredicted cross-reactions of the immunotoxin with crucial tissues were observed in the first trials with immunotoxins and were reported above. More accurate studies of specificity prevented these side effects in subsequent studies.

11 Conclusions

Several years of study in the basic science domain as well as in the clinics have confirmed that RIPs represent an invaluable tool for the improvement of human health and the setting up of new treatment regimens in many diseases.

A few general conclusions can be drawn for RIPs and their applications in vivo at the present time: (1) RIP-based immunotoxins have a wide range of potential applications in many human diseases; (2) clinical trials have established the minimum tolerated dosages and the pharmacology of many types of synthetic and recombinant immunotoxins, suggesting that continuous infusions might be preferable over the bolus administration. This, however, may increase the risk of side effects; (3) for many types of diseases, immunotoxins are not likely to work by themselves but may be a useful, sometime crucial, adjuvant of more established forms of treatment. Selection of patients that may best benefit from a combined treatment with immunotoxins may yield greater efficacy. Particularly in cancer, poorly vascularized, bulky, solid tumors may not be suitable for immunotoxin-based therapies. Circulating cells and well-vascularized lymphomas appear to be better targets; (4) in most of the trials (except those conducted in immunosuppressed lymphoma patients) anti-immunotoxin antibodies were generated. In some cases these antibodies were neutralizing, but in all cases they decreased the $t_{1/2}$ of the immunotoxin in the blood, thus reducing its therapeutic potential. Nevertheless, there were meaningful responses even in the presence of such antibodies. So far, attempts to decrease the antibody responses by the use of immunosuppressive drugs in humans have been unsuccessful, in spite of the results obtained in animal models. The many RIPs that have been described so far, the use of human or humanized targeting molecules, and the development of molecular biology techniques may allow to by-pass the immune response against the toxin moiety by swapping the toxin and selecting the one most appropriate for the applications under investigation; (5) in spite of the limitations described in the present review, the number of patients achieving partial or complete remission from immunotoxin treatment of cancer has reached impressive ratios. This can be considered as a substantial achievement, particularly considering that trials were mostly conducted in terminal patients failing all other established therapies and that many reported trials were Phase I trials, aimed at evaluating essentially safety of in vivo administration. If compared with the performance of more conventional anticancer therapies, it must be noticed that more than 90% of chemotherapeutic drugs used today produced less than 5% partial or complete remissions in Phase I clinical trials. Good progress has been made in the therapeutic use of RIP-based immunotoxins and the advent of recombinant immunotoxins will certainly represent a further advancement.

Acknowledgments The help and advice of Dr. Serena Fabbrini (Istituto di Biologia e Biotecnologia Agraria, CNR, Milano, Italy) are gratefully acknowledged.

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