Description, Distribution, Activity and Phylogenetic Relationship of Ribosome-Inactivating Proteins in Plants, Fungi and Bacteria

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Abstract: Ribosome-Inactivating Proteins (RIPs) are enzymes that trigger the catalytic inactivation of ribosomes and other substrates. They are present in a large number of plants and have been found also in fungi, algae and bacteria. RIPs are currently classified as type 1, those formed by a single polypeptide chain with the enzymatic activity, and type 2, those formed by 2 types of chains, i.e. A chains equivalent to a type 1 RIPs and B chains with lectin activity. Type 2 RIPs usually contain the formulae A-B, $(A\text{-}B)_2$ and less frequent $(A\text{-}B)_4$ and polymeric forms of type 2 RIPs lectins. RIPs are broadly distributed in plants, and are present also in fungi, bacteria, at least in one alga; recently RIP-type activity has been described in mammalian tissues. The highest number of RIPs has been found in *Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, Phytolaccaceae* and *Poaceae.* However there are no systematic screening studies to allow generalisations about occurrence. The most known activity of RIPs is the translational inhibitory activity, which seems a consequence of a *N*-glycosidase on the 28 S rRNA of the eukaryotic ribosome that triggers the split of the A_{4324} (or an equivalent base in other ribosomes), which is key for translation. This activity seems to be part of a general adenine polynucleotide glycosylase able to act on several substrates other than ribosomes, such as tRNA, mRNA, viral RNA and DNA. Other enzymatic activities found in RIPs are lipase, chitinase and superoxide dismutase. RIPs are phylogenetically related. In general RIPs from close families share good amino acid homologies. Type 1 RIPs and the A chains of type 2 RIPs from Magnoliopsida (dicotyledons) are closely related. RIPs from Liliopsida (monocotyledons) are at the same time closely related and distant from Magnoliopsida. Concerning the biological roles played by RIPs there are several hypotheses, but the current belief is that they could play significant roles in the antipathogenic (viruses and fungi), stress and senescence responses. In addition, roles as antifeedant and storage proteins have been also proposed. Future research will approach the potential biological roles played by RIPs and their use as toxic effectors in the construction of immunotoxins and conjugates for target therapy.

INTRODUCTION

In the last twenty years, a number of comprehensive reviews appeared [1-11] covering the core research on ribosome-inactivating proteins (RIPs). However, the large number of RIPs disclosed in the last few years together with the different enzymatic activities attributed to these proteins makes it necessary an integrative review on their distribution, activities and phylogenetic relationships. Despite their apparently broad distribution especially in the plant kingdom, there are no systematic screening data supporting their ubiquity.

Ribosome-inactivating proteins are proteins isolated initially from various plant materials and so named because they irreversibly inactivate ribosomes. The first identified RIPs were ricin and abrin, two potent toxins both studied by Paul Ehrlich, but only in 1971 it was found that ricin inhibits eukaryotic protein synthesis [12] and then that it acts by damaging ribosomes [13]. At the same time it was reported that a Pokeweed Antiviral Protein (PAP) from *Phytolacca americana* leaves inhibited protein synthesis through a similar mechanism [14]. Subsequently many other proteins with apparently identical properties were identified, which were divided into two groups: type 1 and type 2 RIPs. Type 1, more numerous, are single-chain, strongly basic proteins of 30 kDa, approx., having enzymatic activity. They inhibit cell-free protein synthesis but are relatively non-toxic to cells and animals. Type 2 RIPs are proteins of 60-65 kDa, in which an enzymatic A chain similar to type 1 RIPs is linked to a slightly larger B chain, a lectin specific for sugars generally with the terminal free D-galactose structure [4]; SNA I has been reported to bind terminal sialic acid [15]. The B chain binds to cell membranes, thus facilitating entry of the RIPs into cells. Thus, this group includes ricin, abrin and other potent toxins found subsequently, but also nigrin b from *Sambucus nigra* [16], ebulin l from *Sambucus ebulus* [17] and other proteins identified more recently, which have similar structure and enzymatic properties, and still are non-toxic as compared with the highly toxic ricin.

The denomination of type 3 RIPs has been proposed for a maize b-32 RIP, which is synthesised as a proenzyme, which is activated after the removal of a short internal peptide segment and leaves two segments of 16.5 and 8.5 kDa [18], and for JIP60, a RIP from barley in which a segment similar to type 1 RIP continues with another segment of similar size without a known function [19]. It seems unjustified to define a new type of proteins on the

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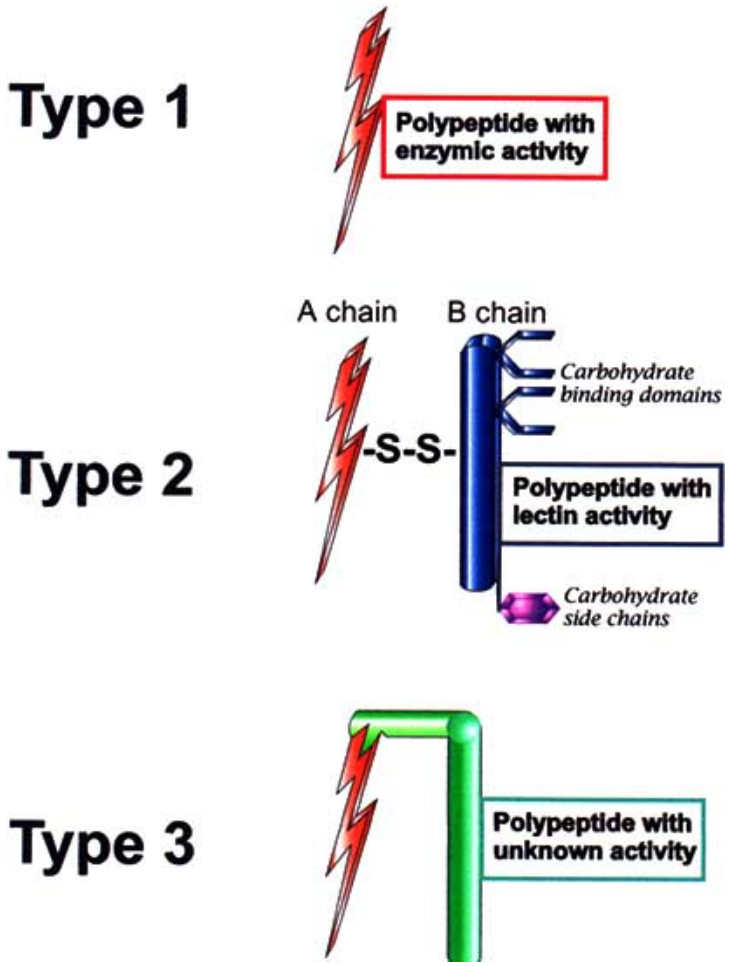

Fig. (1). Schematic representation of the structure of ribosome-inactivating proteins.

basis of single and different cases, and by the time being it seems preferable to consider these two proteins as peculiar type 1 RIPs. The two types of RIPs could be more appropriately differentiated on the basis of the absence (type 1) or presence (type 2) of a lectin chain, which makes a significant difference. A schematic representation of the structure of RIPs of the various types is in (Fig. **1**). This classification allows to fit easily other examples of RIPs. For instance, under this point of view, the four-chain RIPs like SNA I [15] and SNA I´ [20] must be classified as type 2 RIPs. In addition, some proteins may also be included in the type 2 RIP category, like SNRLP 1 and 2 [21] and the basic nigrin b [22], which exhibit the classical two-chain structure but are unable to agglutinate red blood cells, due most probably to some kind of defect in the B chain.

DISTRIBUTION AMONG PLANTS, FUNGI, ALGAE AND BACTERIA

Work done in the last years revealed that RIPs are widely distributed among plants, fungi, algae and bacteria [1-4, 6], and recently a RIP-type activity has been described in mammalian tissues as well [23]. As (Table **I**) shows, a large number of type 1 and type 2 RIPs have been found and studied. They belong to a rather broad range of families apparently unrelated from a phylogenetic point of view. The highest number of RIPs has been found in a small number of families, namely *Caryophyllaceae, Sambucaceae, Cucurbitaceae, Euphorbiaceae, Phytolaccaceae* and *Poaceae*. Nonetheless, this could be merely a consequence of the screening procedure in which it was pursued to find plants with high concentrations of RIP precisely in those families previously shown to contain RIPs rather than to analyse their distribution in the plant kingdom. In this line, screening studies conducted in parallel to the RIP research revealed that approximately one third of the plants examined contain RIPs or RIP-related activities [1-4, 6; unpublished studies]. The low activity, if any, seen in many plant extracts may be due either to low concentration of RIP in a particular extract or simply to the low activity of the RIP present. This could have restricted the focus of the study to a reduced number of plant families known to include plants containing RIPs in several parts and in some cases in large amounts, for instance, *Saponaria officinalis* [24] and *Sambucus nigra* [15, 16, 20-22]. In addition, a large number of RIPs have been isolated from only one tissue of the plant. This could have also reduced the view of RIPs as potential components of yet unknown or not well clarified mechanisms broadly represented in plants and perhaps in other organisms like, for instance, the alleged antipathogenic role played by RIPs. The fact that some RIPs could be induced by different factors like senescence [25], virus infection [26], development [19] and stress [27] supports this belief.

Table I. RIPs from Plants, Algae, Fungi and Bacteria

Family	Species	Source	RIP Name	Mw (kDa)	Yield (mg/100g)	Ref.
Type II						
Magnoliidae						
Lauraceae	*Cinnamomum camphora*	seeds	cinnamomin	61	2.1	[110]
	Cinnamomum porrectum	seeds	porrectin	64.5	-	[111]
Ranunculaceae	*Eranthis hyemalis*	bulbs	EHL *Eranthis hyemalis* lectin	62	-	[112]
Rosidae						
Cucurbitaceae	*Cucurbita foetidissima*	roots	foetidissimin	63	3.5	[113]
Euphorbiaceae	*Ricinus communis*	seeds	ricins (D, E)	63-67	120	[114]
	Ricinus communis	seeds	RCA	130		[4]
Fabaceae	*Abrus precatorius*	seeds	abrins	66-68	75	[115]
	Abrus precatorius	seeds	APA	138		[4]
	Abrus pulchellus	seeds	pulchellin			[203]
Viscaceae	*Phoradendron californicum*	leaves	PCL *Phoradendron californicum* lectin	69	-	[116]
	Viscum album	leaves	MLI(viscumin), II, III	61	7	[117]
	Viscum album	leaves	VAA	120		[4]
	Viscum album coloratum	plant	VCA	62.7		[118]
Dileniidae						
Passifloraceae	*Adenia volkensii*	roots	volkensin	62	0.2	[119]
	Adenia digitata	roots	modeccin 4B and 6B	57-58	20(180)-7	[120]
Asteridae						
Sambucaceae	*Sambucus ebulus*	leaves	ebulin l	56	3.2	[17]
	Sambucus ebulus	rhizome	ebulins r1 and r2	56	1.35-0.56	[121]
	Sambucus ebulus	fruits	ebulin f	56	1.6	[122]
	Sambucus nigra	fruits	nigrin f	58	1.3-0.12	[43]
	Sambucus nigra	fruits	SNA-If	140	-	[123]
	Sambucus nigra	bark	nigrin b	58	254	[16]
	Sambucus nigra	bark	SNAI	136		[15]
	Sambucus nigra	bark	SNAI'	67	-	[20]
	Sambucus nigra	bark	basic nigrin b	64	-	[22]
	Sambucus nigra	bark	SNLRP1, 2	68-62		[21]
	Sambucus nigra	leaves	nigrins l1 and 2	63	-	[7]
	Sambucus nigra	seeds	nigrin s	57.3		[124]
	Sambucus racemosa	bark	basic racemosin b	58		[7]
	Sambucus sieboldiana	bark	sieboldin b	60	2.6	[125]
	Sambucus sieboldiana	bark	SSA	132		[126]
Liliidae						
Liliaceae	*Poligonatum multiflorum*	leaves	PMRIPm and PMRIPt	60-240	0.25	[127]

(Table I). contd.....

Family	Species	Source	RIP Name	Mw (kDa)	Yield (mg/100g)	Ref.
Iridaceae	*Iris hollandica* var. Prof. Blaauw	bulbs	IRAb and IRAr	65-68	-	[46]
Type I						
Magnoliidae						
Lauraceae	*Cinnamomum camphora*	seeds	camphorin	23	0.94	[128]
Caryophyllidae						
Aizoaceae	*Mesembryanthemum crystallinum*	leaves	*Mesembryanthemum crystallinum* RIP1	32.6	-	[27]
Amaranthaceae	*Amaranthus viridis*	leaves	amaranthin	30	-	[129]
	Celosia cristata	leaves	CCP 25	25	-	[130]
Basellaceae	*Basella rubra*	seeds	*Basella rubra* RIP 2 *Basella rubra* RIP 3	30.6-31.2 31.2	13.0 10.4	[131]
Caryophyllaceae	*Agrostemma githago*	seeds	agrostins 2,5 and 6	29.5-30.6	8.4-34.2-18.4	[132]
	Dianthus barbathus	leaves	dianthin 29	29	1.5	[133]
	Dianthus caryophyllus	leaves	dianthin 30 and 32	29.5-31.7	2.0-2.0	[134]
	Dianthus sinensis	leaves	RIP 1,2,3	30.7	-	[135]
	Gypsophila elegans	plant	gypsophilin	28	0.9	[136]
	Lychnis chalcedonica	seeds	lychnin	26.6	160	[137]
	Petrocoptis grandiflora	plant	petrograndin	28.6	-	[138]
	Petrocoptis glaucifolia	plant	petroglaucins 1 and 2	26.7-27.5	1.3-0.3	[139]
	Saponaria ocymoides	seeds	ocymoidin	30.2	124	[140]
	Saponaria officinalis	seeds	saporin S5,S6,S8,S9	29.5	63-268-15-24	[24]
	Saponaria officinalis	leaves	saporins L1, L2	31.6	35-13	[24]
	Saponaria officinalis	roots	saporin R1, R2, R3	30.2-30.9	20-5-9	[24]
	Stellaria aquatica	leaves	stellarin	27.5	0.15	[141]
	Vaccaria pyramidata	seeds	pyramidatin	28	37	[142]
Chenopodiaceae	*Beta vulgaris*	leaves	beetin 27	27		[26]
	Chenopodium album	plant	CAP30B	29		AF230812 [204]
	Spinacia oleracea	root	SOP	31	-	[143]
Nyctaginaceae	*Bougainvillea spectabilis* Willd	leaves	bouganin		3.5	[144]
	Mirabilis expansa R&P	roots	ME1 and 2	27-27.5	-	[145]
	Mirabilis jalapa	roots,t.c.	MAP	27.8	200-60	[74]
	Mirabilis jalapa	seeds	MAP S		22.6	[146]
Phytolaccaceae	*Phytolacca americana*	leaves	PAP I, PAP II	29-30	9.2-3.6	[147]
	Phytolacca americana	seeds	PAP-S1 and S2	29	100-180	[148]
	Phytolacca americana	t.c.	PAP-C	29	17.5	[149]
	Phytolacca americana	roots	PAP-R	29.8	7.0	[137]
	Phytolacca americana	hairy roots	PAP-H	29.5	-	[150]
	Phytolacca dioica	seeds	PD-S1, S2, S3	30.9-32	3.5, 43, 1.7	[151]

(Table I). contd.....

Family	Species	Source	RIP Name	Mw (kDa)	Yield (mg/100g)	Ref.
	Phytolacca dioica	leaves	PD-L1,L2,L3,L4		1.54-0.72-2.48-4.0	[152]
	Phytolacca dodecandra	leaves	dodecandrin	29	3.4	[153]
	Phytolacca dodecandra	t.c.	dodecandrin C	31-32	1.0	[154]
	Phytolacca acinosa		PAP	32.6	-	AY049785
	Phytolacca insularis	leaves	Insularin (PIP)	33	2.4	[155]
	Phytolacca insularis	cDNA	PIP 2	33	-	[156]
Asteridae						
Lamiaceae	*Clerodendrum aculeatum*	leaves	CA-SRI Protein	34	6.4	[157]
	Clerodendrum inerme Gaertn	leaves	CIP-29 and CIP-34	29-34	-	[158]
Rosidae						
Cucurbitaceae	*Benincasa hispida* var. *chieh-qua*	seeds	Hispin	21	0.3	[159]
	Bryonia dioica	leaves	bryodin-L	28.8	0.17	[137]
	Bryonia dioica	roots	bryodins 1 and 2	27-29	0.5	[160]
	Citrullus colocynthis	seeds	colocins 1 and 2	26.3	10-16	[137]
	Cucurbita moschata	sarcocarp	cucurmosin		-	[161]
	Cucurbita pepo	sarcocarp	pepocin	26	2.7	[162]
	Luffa acutangula	seeds	luffaculin	28	10.1	[163]
	Luffa aegyptiaca	seeds	LRIP (Luffin c)	30		[164]
	Luffa cylindrica	seeds	Luffins a and b	28.2-27.2	20-12	[165]
	Luffa cylindrica	seeds	Luffin S	10		[166]
	Luffa cylindrica	seeds	Luffins S1,S2,S3	8		[167]
	Luffa cylindrica	seeds	Luffacylin	8	0.2	[168]
	Marah oreganus	seeds	MOR I and II	28	46.7-8.7	[169]
	Momordica balsamina		momordin II	30		[170]
	Momordica charantia	seeds	momordin I	29.1	150-180	[171]
	Momordica charantia	seeds	α,β momorcharin	29	-	[172]
	Momordica charantia	seeds	charantin	9.7	-	[173]
	Momordica cochinchinensis	seeds	momorcochin S	30.7	61.7	[174]
	Momordica cochinchinensis	roots	momorcochin R	32		[175]
	Momordica grosvenorii	seeds	momorgrosvin	27.7	0.24	[176]
	Sechium edule	seeds	sechiumin	27	0.77	[177]
	Trichosanthes cucumerina var. anguina	seeds	trichoanguin	35	12.4	[178]
	Trichosanthes sp. Bac Kan 8-98	genomic	trichobakin (TBK)	27		[179]
	Trichosanthes cucumeroides	roots	β trichosanthin	28		[180]
	Trichosanthes kirilowii Maximowicz	seeds	Trichokirin	27	5.7	[181]
	Trichosanthes kirilowii		α, β kirilowin	27.5-28.8		[182] [183]
	Trichosanthes kirilowii	seeds	S-Trichokirin	8		[184]

(Table I). contd.....

Family	Species	Source	RIP Name	Mw (kDa)	Yield (mg/100g)	Ref.
	Trichosanthes kirilowii	roots	trichosanthin TAP29 TCS α,β,γ, karasurin trichobitacin	29	14	[185]
	Trichosanthes lepiniate	roots	trichomaglin	24.6		[186]
Euphorbiaceae	*Croton tiglium*	seeds	crotins I and II (2,3)	30.2		[187]
	Euphorbia serrata	leaves	euserratins 1 and 2	26.5-30	0.4-2.3	AF457874 AF457875
	Gelonium multiflorum	seeds	gelonin (GAP)	30	250-300	[188]
	Hura crepitans	latex	*Hura crepitans* RIP	27.5	146	[189]
	Jatropha curcas	seeds	curcin 2	28.1	-	[187]
	Manihot palmata	seeds	mapalmin	32.3	16	[137]
	Manihot utulissima	seeds	manutins 1 and 2	30.7	20-23	[4]
Fabaceae	*Pisum sativum* var. arvense Poir	seeds	Pisavins α,β	20.5-18.7	0.02	[190]
	Pisum sativum var. macrocarpon	legumes	sativin	38	1.4	[191]
Asteridae						
Sambucaceae	*Sambucus ebulus*	leaves	ebulitins α,β,γ	29-32	0.13-0.35-0.57	[44]
	Sambucus nigra	fruits	nigritins f1 and f2	24-23.6	0.4-0.3	[42]
Liliidae						
Agavaceae	*Yucca recurvifolia*	leaves	YLP			[192]
Asparagaceae	*Asparagus officinalis*	seeds	asparin 1, 2	30.5-29.8	5.4-4.9	[193]
Liliaceae	*Muscari armeniacum* L. and Miller	bulbs	musarmins 1,2,3	27-30	1.3-2.0-1.7	[194]
Iridaceae	*Iris hollandica* var. Prof. Blaauw	bulbs	IRIP 1,2,3	29	250 (total)	[195]
Poaceae	*Hordeum vulgare*	seeds	Barley RIP, JIP60	30	139	[196]
	Secale cereale	seeds	RPSI	30	200	[197]
	Triticum aestivum	germ	tritin	30	0.03	[198]
	Triticum aestivum	leaves	tritin L	37		[199]
	Triticum aestivum	seeds	tritin S	32		[199]
	Zea mays	seeds	Maize RIP1, b-32	25	12-20	[18]
Class *Phaeophyceae*						
Laminariaceae	*Laminaria japonica A*	alga	lamjapin	36	0.1	[37]
Class *Basidiomycetes*						
Pleurotaceae	*Pleurotus tuberregium*	mushroom	pleuturegin	38	-	[31]
Tricholomataceae	*Lyophyllum shimeji*	mushroom fruiting bodies	lyophyllin	20		[32]
	Flammulina velutipes	mushroom fruiting bodies	velutin	13.8		[33]
	Flammulina velutipes	mushroom fruiting bodies	flammulin	40		[34]

(Table I). contd.....

Family	Species	Source	RIP Name	Mw (kDa)	Yield (mg/100g)	Ref.
	Hypsizygus marmoreus	mushroom fruiting bodies	hypsin	20		[35]
Pluteaceae	*Volvariella volvacea*	mushroom fruiting bodies	volvarin	29		[36]
Gamma-proteobacteria						
Enterobacteriaceae	*Escherichia coli*	bacteria	Verotoxin 1 (Shiga-like toxin)	70.5		[28]
	Shigella dysenteriae	bacteria	Shiga toxin	70.7		[29]

Classification of Plant Species According to Cronquist [200]; t.c. = tissue culture

As shown also in (Table. **I**), there are a few genuses on which RIPs research has been focused, namely *Luffa, Momordica, Phytolacca*, *Sambucus, Saponaria,* and *Trichosanthes.* In these genuses a number of RIPs, mostly isoforms, have been identified. However, this seems neither indicative of a limited occurrence of RIPs nor of a relatively little important role in plants. Clearly more research on RIPs is needed to ascertain a more appropriate picture on the occurrence and significance of RIPs.

The molecular weights of type 1 RIPs fall in the range of 21-38,000 except for a number of low molecular weight protein synthesis inhibitory proteins also alleged to be RIPs, which fall in the range of 8-13,500. As type 2 RIPs are concerned, the molecular weights of the two-chain proteins fall in the range of apparent Mr 56-69,000 and of the four-chain proteins in the range of apparent Mr 120-140,000.

It was known for many years that RIPs are also present in bacteria. So, the type 1 RIPs, Stx1 and Stx2, have been isolated from *Escherichia coli* [28, 29]. These RIPs act exactly as the plant counterpart [28-30]. Notably, type 1 RIPs have been found in the last few years in fungi [31-36] and at least one in *Laminaria japonica* A [37]. All these findings favour the generally accepted hypothesis that RIPs are enzymes broadly represented and therefore they must play important and as yet undefined biological roles.

From the studies carried until now it seems that type 1 RIPs could be more abundant than the type 2 RIPs. In fact until the discovery of the type 2 RIPs in *Sambucus* [6, 7, 16, 17], only the highly toxic ricin, abrin, viscumin, modeccin and volkensin had been described [1-4]. The indication to conduct the screening for the identification of new type 2 RIPs was the high toxicity of the plant specimens like *Ricinus communis*, *Abrus precatorius*, etc. This may lead to the notion that most type 2 RIPs have passed unperceived most probably due to the intrinsic characteristics of some of the type 2 RIPs now isolated. So, the type 2 RIPs isolated from *Sambucus*, namely nigrins and ebulins [16, 17], have the extraordinary feature that, although being even more active than ricin at the molecular level, they lack the high toxicity of ricin towards cultured animal cells and *in vivo* in rodents. Therefore, they have been named non-toxic type 2 RIPs [7, 16, 17]. For example, the IC_{50} of nigrin b for protein synthesis is 0.03 nM in a cell-free system (rabbit reticulocyte lysate) and 535 nM in Hela cells and the LD_{50} in mice is 12 mg per kg body weight [38, 39]. These values mean that nigrin b is three-fold more active at the ribosomal level than ricin but is 18,000 times less toxic in cultured animal cells and 1,500 times less toxic to mice than ricin. After the acknowledgement that plants can have non-toxic type 2 RIPs, a number of them have been identified and studied (Table **I**).

The low toxicity of *Sambucus* type 2 RIPs has been investigated recently and exemplified with ebulin l. Ebulin l A-chain has the same enzymatic activity as ricin in promoting the depurination of the A_{4324} of the 28S rRNA of the rat liver ribosome thus leading to the inhibition of protein synthesis [17]. Experiments of uptake and internalisation of ricin A-chain and ebulin l suggest that the A chains in general have no special translocation signals to complement the target cell binding promoted by the B chains of type 2 RIPs [40]. Therefore, the reduced cytotoxicity of ebulin l as compared with ricin could be due to deficiencies of the B chain. The molecular cloning, amino acid sequence and the 2.8-A crystal structure of ebulin l revealed changes in key amino acids of the sugar-binding subdomains of the B chain, especially in the high affinity sugar-binding 2γ subdomain in which the Tyr249 of ricin is changed to Phe in ebulin l [41]. This reduces the affinity of ebulin 1 for galactosides and therefore for galactose-containing glycoproteins located at the surface of the plasma membrane.

At least in two *Sambucus* spp., *Cinnamomum camphora* and *Iris hollandica,* type 1 RIPs coexist with type 2 RIPs, and this could be a more general phenomenon. *S. nigra* fruits contain the type 1 nigritins f1 and f2 constitutive and inducible by ripening, respectively [42]. Preliminary studies indicated that both nigritins are accumulated in the skin of the fruits while the type 2 RIP nigrin f [43] is in the flesh. In addition, *S. ebulus* leaves contain the type 1 ebulitins α, β, and γ [44]. Such coexistence of type 1 and type 2 RIPs has been described also in *Cinnamomum camphora* in which cinnamomin (type 2) and camphorin (type 1) have been described; nonetheless, a recent report indicates that the type 1 RIP camphorin is in fact the A chain of the type 2 cinnamomum [45]. *Iris hollandica* also contains type 1 (IRIP 1,2,3) and type 2 (IRAb and IRAr) RIPs [46]. At present we have not yet any idea on the meaning of the simultaneous occurrence of type 1 and type 2 RIPs in the same tissue probably because, despite the hypotheses raised to date, we do not know the biological role of RIPs.

Table II. RIPs Used to Construct the Phylogenetic Tree of Figure 3

Bacteria							
Class	**Subclass**	**Order**	**Family**	**Species**	**RIP**	**Type RIP**	**Swiss-Prot/TrEMBL accession number**
Proteobacteria	Gammaproteo-bacteria	Enteroba-cteriales	Enterobacte-riaceae	*Escherichia coli*	Stx 1 A subunit	bacterial	Q8VV64
Proteobacteria	Gammaproteo-bacteria	Enteroba-cteriales	Enterobacte-riaceae	*Shigella dysenteriae*	Stx A subunit	bacterial	P10149
Magnoliophyta							
Class	**Subclass**	**Order**	**Family**	**Species**	**RIPs**	**Type RIP**	**Swiss-Prot/TrEMBL accession number**
Magnoliopsida	Magnoliidae	Laurales	Lauraceae	*Cinnamomum camphora*	Cinnamomin I	2	Q94BW5
	Rosidae	Cucurbitales	Cucurbitaceae	*Bryonia dioica*	Bryodin I	1	P33185
				Trichosanthes kirilowii	alpha-Trichosanthin	1	P09989
				Trichosanthes cucumerina	Trichoanguin	1	P56626
				Cucurbita moschata	Moschatin II	1	Q8S2R5
				Cucumis figarei	RIP 1	1	Q9FRX4
				Luffa cylindrica	Luffin-alpha	1	Q00465
				Momordica charantia	Momordin I	1	P16094
				Momordica balsamina	Momordin II	1	P29339
		Euphorbiales	Euphorbiaceae	*Ricinus communis*	Ricin	2	P02879
				Gelonium multiflorum	Gelonin	1	P33186
				Jatropha curcas	Curcin	1	Q8S452
		Santalales	Viscaceae	*Viscum album*	Mistletoe lectin	2	Q8RXH6
		Fabales	Fabaceae	*Abrus precatorius*	Abrin c*, APA**	2	P28590, Q9M6E9
	Caryophyllidae	Caryophyllales	Amaranthaceae	*Amaranthus viridis*	Amarandin-S	1	Q96322
			Chenopodiaceae	*Spinacia oleracea*	RIP	1	Q8VWY2
				Beta vulgaris	Betavulgin	1	Q39418
				Chenopodium album	RIP CAP30B	1	Q9AUE3
			Nyctaginaceae	*Mirabilis jalapa*	MAP	1	P21326
				Bougainvillea spectabilis	Bouganin	1	Q8W4U4
			Phytolaccaceae	*Phytolacca americana*	PAP-S	1	P93444
				Phytolacca acinosa	PAP	1	Q941G8
				Phytolacca insularis	RIP 2	1	Q9XFF8

(Table II). contd.....

Class	Subclass	Order	Family	Species	RIPs	Type RIP	Swiss-Prot/TrEMBL accession number
			Caryophyllaceae	*Dianthus caryophyllus*	Dianthin 30	1	P24476
				Dianthus chinensis	RIP 1	1	Q93Y66
				Saponaria officinalis	Saporin 6	1	P20656
			Aizoaceae	*Mesembryanthemum crystallinum*	RIP1	1	P93261
	Asteridae	Dipsacales	Sambucaceae	*Sambucus nigra*	Nigrin b*, SNAI**	2	P33183, Q41358
				Sambucus sieboldiana	Sieboldin b*, SSA**	2	Ref. , D25317(GenBank)
				Sambucus ebulus	Ebulin l	2	Q9AVR2
		Lamiales	Lamiaceae	*Clerodendrum aculeatum*	CA-SRI protein	1	P93077
Liliopsida	Commelinidae	Poales	Poaceae	*Zea mays*	proRIP-alfa chain	1	P28522
				Oryza sativa	RIP 1	1	Q9AS85
				Triticum aestivum	Tritin	1	Q07810
				Secale cereale	Protein synthes.inhibit.	1	JC5848 (GenBank)
				Hordeum vulgare	RIP 1	1	P22244
	Liliidae	Liliales	Liliaceae	*Muscari armeniacum*	Musarmin 3	1	Q8L5M4
			Liliaceae	*Polygonatum multiflorum*	PMRIPm	2	Q9M654
			Iridaceae	*Iris hollandica*	IrisRIPA3*,IRAb**,IRAr***	1(*) y 2	O04358, Q8W2E7, Q8W2E8

ENZYMATIC ACTIVITIES OF RIPS

RIPs display a number of enzymatic activities, most of them may be merely a reflection of their activity on the RNA and other are completely new. These activities have been reviewed recently [10, 47]. Below we described the activities that seem to be more relevant according to the substrate implicated.

Anti-Ribosomal Activity

Despite the bulk of research done on RIPs since the discovery of ricin [11] it was in 1987 that Endo's group reported the molecular mechanism of action of ricin involved in the inhibition of protein synthesis. Ricin and other RIPs are N-glycosidases that break the *N*-glycosidic bond that links the A_{4324} to the polyphosphate backbone of the 28S rRNA of the rat liver ribosome (Fig. **2**) [48-54]. This adenine is located in a loop which is highly conserved in the different species, named the ricin/sarcin loop, and reported to be essential for the interaction of the elongation factors G and 2 with prokaryotic and eukaryotic ribosomes, respectively [1-4]. In this line, it has been shown that the stabilisation of EF-G on *E. coli* ribosomes with the antibiotic fusidic acid protects it readily from the depurination by inhibitory RIPs [55].

RIPs inactivate also the ribosomes from insects [56], fungi [57] and in some cases the ribosomes from plants, bacteria and archaebacteria through a similar mechanism of action acting in mammalians [55, 58-72]. Even though there are no published systematic studies on the sensitivity of ribosomes to RIPs, the available data indicate that mammalian ribosomes are much more sensitive than the plant and bacterial ribosomes [1-4]. A screening on the relative sensitivity of ribosomes from *Escherichia coli, Agrobacterium tumefaciens, Brevibacterium lactofermentum* and *Streptomyces lividans* revealed that they are sensitive to a few type 1 RIPs but not to others or to the type 2 RIPs [60, 61]. This has hindered the molecular cloning and expression of such inhibitory RIPs in the common bacterial expression systems [73-74].

The target loop for RIPs is also the target for other protein synthesis inhibitors like the RNases from the α-sarcin family, namely α-sarcin, restrictocin and tricholin [75-77], which hydrolyse the phosphodiester bond at the 3' end of the G_{4325}. Some RIPs trigger the hydrolysis of one adenine per ribosome whereas others can hydrolyse also other adenines from the ribosome; a property that does not seem to be correlated with the inhibitory capacity of the RIP [78].

A new type of enzyme has been found in some plant species that produce RIPs, i.e. the so-called "ribosomal RNA apurinic site specific lyase (RALyase)". This enzyme promotes in a specific way the hydrolysis of the

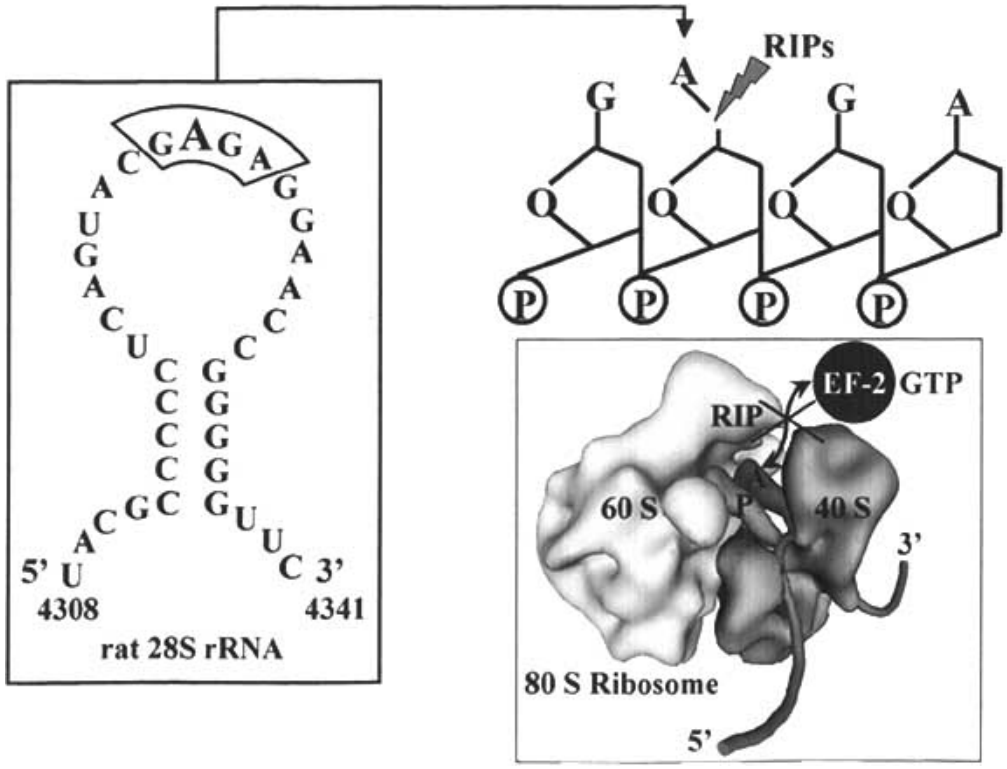

Fig. (2). The mechanism of action of ribosome-inactivating proteins. All RIPs cleave an adenine base (A4324 in rat) from rRNA of the large ribosomal subunit, rendering ribosomes unable to bind the elongation factor 2. Some RIPs remove more that one adenine residue.

phosphodiester bond at the 3' end of the apurinic site generated by the action of the autologous RIP. It has been suggested that this enzyme might form part of a RNA repair system similar to that found for DNA [79-81], which would be acting in plants producing RIPs and whose ribosomes would be accidentally inactivated by the action of RIPs [80].

Adenine Polynucleotide Glycosylase

Research in the last years revealed that RIPs can eliminate adenines not only from ribosomes but also from any kind of nucleic acid namely rRNA, tRNA, mRNA, viral RNA and even DNA [82-88]. Such activity lead to rename RIPs with the more significant and systematic denomination of adenine polynucleotide glycosylases [82-84, 89]. This broad-substrate enzymatic activity might be responsible for some properties that have been attributed to RIPs during the last years like antiviral activity [3-8], senescence promotion [25] and apoptosis by DNA modification [90].

Specific Action on mRNA

Specific effects of RIPs on mRNA different from the adenine polynucleotide glycosylase activity have described recently for PAP. PAP is able to bind to "cap" structures of messenger and then to promote mRNA depurination in a 5'-3' sense, thus inactivating mRNA [91]. This allows PAP also to regulate the level of expression of its own mRNA [92]. In addition, it has been reported that PAP specifically inhibits Ty1-directed +1 ribosomal frameshifting and retrotransposition in *Saccharomyces cerevisiae* [93].

Other Actions on DNA

RIPs through the adenine polynucleotide glycosylase activity are able to release adenines from, and to promote the inactivation of, any kind of DNA. Recent research revealed some other activities on DNA like nuclease [94], glycolyase [95] and topoisomerase [96] activities. However, these studies opened an interesting controversy since it has been also described that these activities disappeared upon careful and extreme purification of RIPs. Therefore, such activities seem to be due essentially to contaminants present in the RIP preparations [97].

Lipase and Membrane Disordering Activities

Interactions between RIPs and biological membranes has been described in the last years. The most intense research has been done with ricin and related proteins. It has been described that ricin A-chain binds to negatively charged phospholipid vesicles and destabilises lipid bilayers [98]. Work carried out with the type 2 RIP cinnamomin indicates that the protein forms cation channels in the lipid bilayers [99]. More recent studies indicated that ricin displays lipase activity, which could be related with the translocation through the intracellular membranes [100, 101]. Such lipase activity seems to be related to specific amino acid residues belonging to both the A and the B chains, and it has been argued that merely the mutation to alanine of catalytic serine 221 on the A-chain strongly reduced ricin lipase activity [102]. The absence of equivalent amino acids in some type 2 RIPs like ebulin l and VVA could be the molecular basis of the lack or the lower toxicity of such proteins as compared with ricin [102].

Bifunctional Chitinase-RIP Protein

It has been reported that *Trichosanthes kirilowii* cell cultures produces bifunctional plant defence enzymes with chitinase and RIP activities [103]. To date there are no studies concerning to the occurrence of the bifunctional proteins despite the potential relevance of this new type of protein that could play a role in the resistance to pathogens.

Other Enzymatic Activities

RIPs have been cited also as inhibitors of other enzymes. Among them the inhibition of poly(ADP-ribose) polymerase involved in the DNA repair has been reported [104]. Such inhibition is exerted on the poly(ADP-ribose) moiety bound to the enzyme, and it has been suggested that such effect could be the basis of the transforming, apoptotic and perhaps the antiviral activities attributed to some RIPs.

Another enzyme whose activity is inhibited by RIPs is the HIV-1 integrase. Lee-Huang and co-workers found that some RIPs are able to inhibit the three partial reactions involved in the HIV-1 activity, namely: i) 3'-terminal processing; ii) HIV-1 cDNA-integration in the acceptor DNA; iii) des-integration of the pro-viral DNA. According to Lee-Huang, these proteins are the first known inhibitors

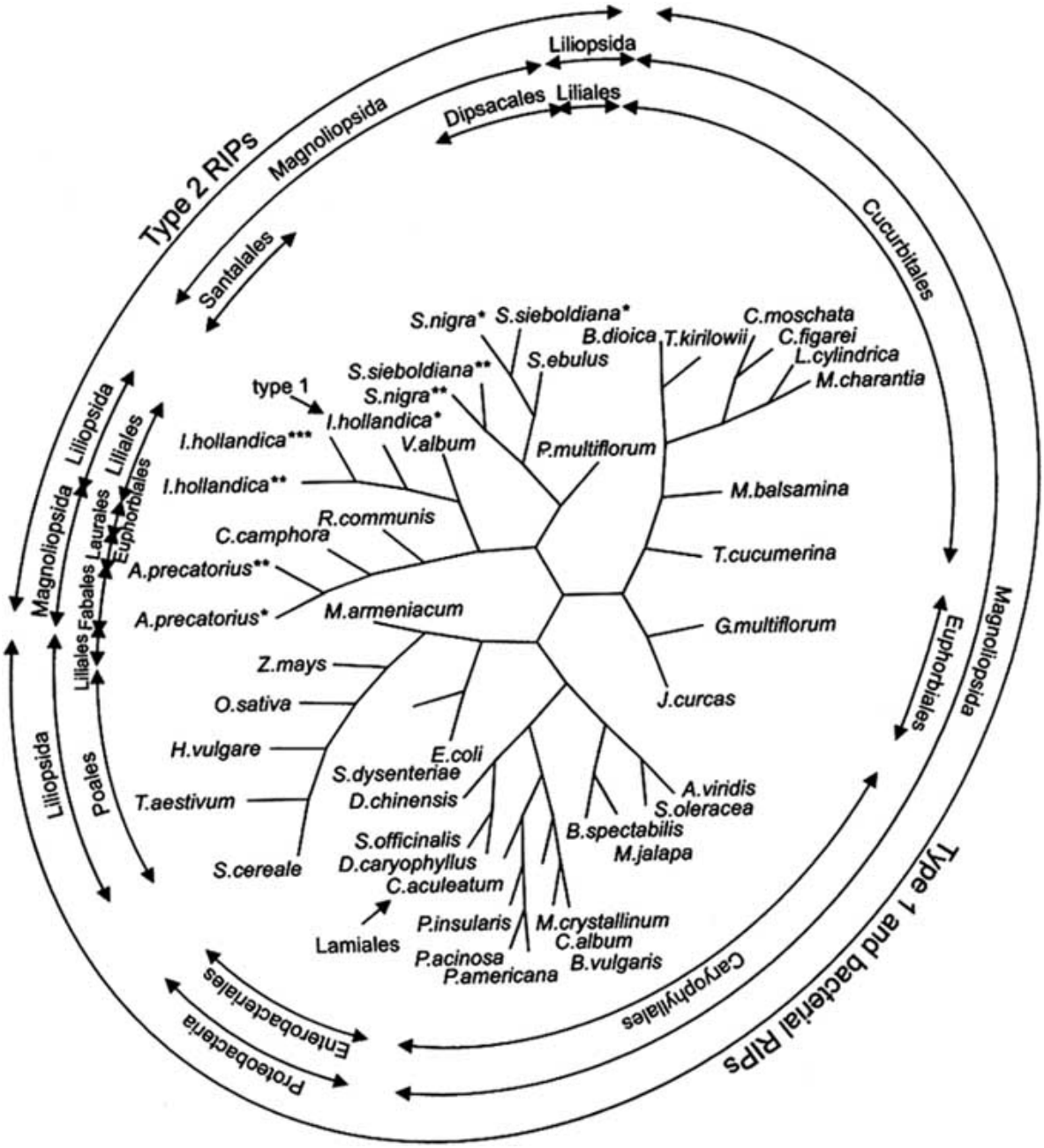

Fig. (3). Dendrogram of the phylogenetic relationship of type 1 RIPs and the A chain of type 2 RIPs. Amino acid sequences of type 1 RIPs, the A chain of type 2 RIPs and the A chains of bacterial RIPs were aligned using CLUSTAL W [201] and the phylogenetic tree was generated using the neighbor-joining method [202]. Asterisks refer to the proteins listed in table II.

of the HIV-1 integrase and could be tools to inhibit the viral replication [105-107].

Other enzymatic activities, which have been reported to be sensitive to RIP are telomerase [108] and superoxide dismutase [109]. Further studies must be undertaken to ascertain whether such effects are a general attribute of RIPs or instead they are merely peculiarities.

PHYLOGENETIC RELATIONSHIP

To date the cDNAs coding for a large number of type 1 and type 2 RIPs have been completely sequenced. (Fig. **3**) shows a phylogenetic tree that relates the type 1 RIPs and the type 2 RIPs A-chains obtained by comparison of the sequences of some representative RIPs. In general, we selected a significant RIP of each one of the known RIP-containing species to simplify the figure and also because the RIPs obtained from the same species, in general, show a higher homology among them than with RIPs of other species. For instance, the RIPs of *Saponaria officinalis* show amino acid homologies comprised between 83 and 98%. Usually, RIPs of the same taxon have related amino acid sequences, thus indicating their presence in parent species and that they evolve in parallel to the differentiation of the corresponding plants. Thus, all RIPs from class Magnoliopsida (dicotyledons) are closely related. RIPs of the family Cucurbitaceae appear as a very homogeneous group, with amino acid homologies in the range 44 - 86%, which are clearly different from the RIPs of other families. Some of these RIPs have been described as powerful abortifacient agents. Euphorbiaceae, Amaranthaceae and Nyctaginaceae show amino acid sequence homologies in the range of 31 - 49%. In this group we included also the RIP of a Chenopodiacea (*Spinacia oleracea*). On the other hand, the RIPs of the families Aizoaceae, Chenopodiaceae and Phytolaccaceae appear also related with amino acid homologies in the range of 32 - 76%. In this group are included the protein CA-SRI from *Clerodendrum aculeatum* (family Lamiaceae) and betavulgin (beetin; family of Chenopodiaceae).

All type 1 RIPs from the class Liliopsida (monocotyledons) appear related. In this group, the RIPs of the family Poaceae appear as a homogeneous group with amino acid homologies in the range of 20 - 92%. On the other hand, the A chain of all type 2 RIPs are also related, which would indicate that they have a common origin. The exception is the type 1 RIP from *Iris hollandica,* which is related with the type 2 RIPs of the same species, perhaps because it could have been formed by a deletion of the B chain from a pre-existing type 2 RIP. (Fig. **4**) shows a phylogenetic tree of type 2 RIPs. The RIPs of the same species are also related. Exceptions to this are the type 2, four-chain RIPs from *Sambucus* (SNA I, SNA If and SSA) and the type 2, two-chain RIPs related to them (SNAI', SNLRPs 1 and 2). These are less related to the rest of the other known RIPs from *Sambucus* than to RIPs from *Polygonatum*, *Viscum*, *Cinnamomum*, *Abrus*, *Ricinus* and *Iris.* This would indicate that among *Sambucus* RIPs there are proteins that have a different origin. Work carried in parallel to research on RIPs revealed that some lectins present in *Sambucus* seem to be formed by deletion of the A-chain of the type 2 RIPs (ie. SNA-IV and SNA-IVl) [7].

ROLE PLAYED BY RIPS

Despite the intense research on RIPs carried out in the last 25 years, no sound picture may be presented on the role played by RIPs in the RIP-producers, plants, fungi, algae or

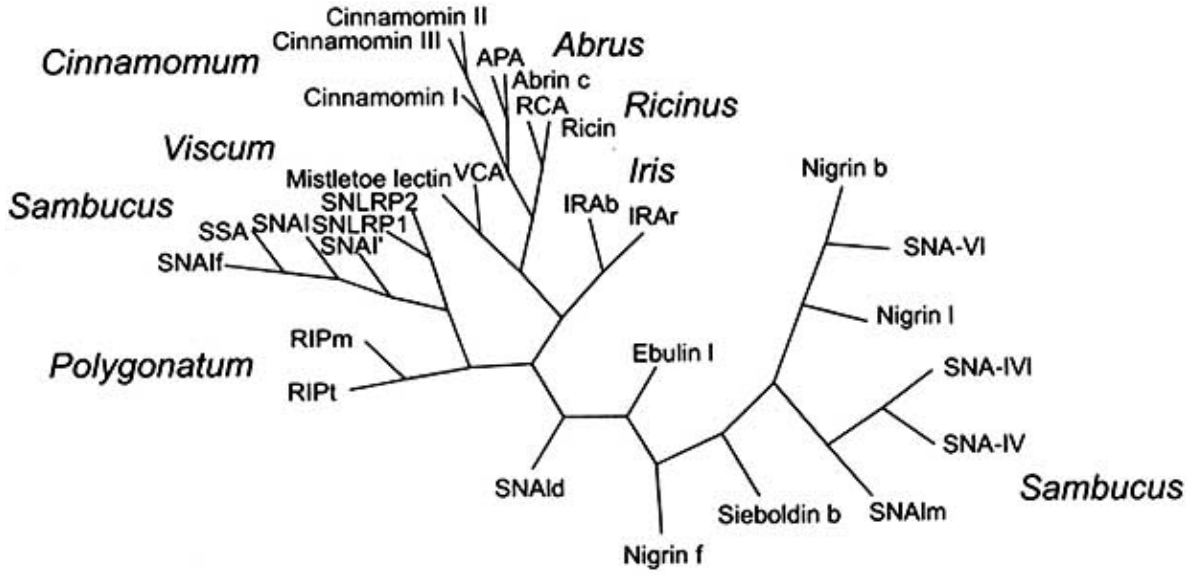

Fig. (4). Dendrogram of the phylogenetic relationship of type 2 RIPs. The alignement of amino acid sequences and the construction of the phylogenetic tree were as in Figure **3**. Swiss-Prot/TrEMBL accession numbers: Cinnamomin I Q94BW5, Cinnamomin II Q94BW4, Cinnamomin III Q94BW3, APA Q9M6E9, Abrin c P28590, RCA P06750, Ricin P02879, IRAb Q8W2E7, IRAr Q8W2E8, Ebulin l Q9AVR2, Nigrin b P33183, SNA-Vl Q945S2, Nigrin l Q8GT32, SNA-IVl Q945S4, SNA-IV O04366, SNAlm Q8GTA5, Sieboldin b [126], Nigrin f O04367, SNAld Q8GTA6, RIPt Q9M653, RIPm Q9M654, SNAIf O22415, SSA D25317 (GenBank), SNAI Q41358, SNAI' P93543, SNLRP1 O04072, SNLRP2 O04071, Mistletoe lectin Q8RXH6, VCA Q8W243.

bacteria. There are nonetheless several hypotheses reviewed recently that to explain potential roles played by RIPs [10]. RIPs have been proposed as antiviral agents, as antifungal agents, as storage proteins, as antifeedant agents, as elements of the plant senescence programme, in development, in saline stress, etc. However, none of these hypotheses has been fully supported and the potential biological role remains elusive. We believe that probably RIPs play pleiotropic effects that could be orchestrated by yet unknown mechanisms.

FUTURE RESEARCH

Research on RIPs has received special attention in recent years due to their intrinsic interest as components of unknown biological mechanisms and also to their use as toxic moieties of conjugates and immunotoxins for the target therapy of important diseases. There are a number of open questions concerning the role played by RIPs in the RIP-producers and on their use for medical application. The recently described role for PAP as a component of the mRNA complex reading system in plants together with the antipathogenic intrinsic role attributed to RIPs in the last times, but never demonstrated clearly, add further interest to these enzymes. The potential role of RIPs and structurally related lectins as nitrogen storage system has not yet been properly assessed, especially in those cases in which RIPs undergo seasonal fluctuations. Moreover, the variation of RIP concentrations in different tissues and the presence of RIP isoforms remain to be explained in mechanistic terms. A potential role in plant development cannot be excluded. Concerning the RIPs molecular mechanism of action, the broad specificity of many RIPs on mammalian, plant and bacterial ribosomes raises the question of whether RIPs contain specific binding and catalytic domains for the different ribosomes, mRNAs and even DNAs. Since many RIPs do not inhibit protein synthesis either in bacteria or in plants, their cloning and expression and the construction of transgenic plants resistant to fungi and viruses will facilitate the elucidation of the RIP structure-function relationships. Future research will attempt to address all these (and other) biological questions concerning the function(s) of RIPs in the plant, fungi and bacteria and, perhaps, in animal biology.

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