Enzymatic Activities of Ribosome-Inactivating Proteins

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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

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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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