RIBOSOME-INACTIVATING PROTEINS: A Plant Perspective

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Abstract Ribosome-inactivating proteins (RIPs) are toxic N-glycosidases that depurate the universally conserved α-sarcin loop of large rRNAs. This depurination inactivates the ribosome, thereby blocking its further participation in protein synthesis. RIPs are widely distributed among different plant genera and within a variety of different tissues. Recent work has shown that enzymatic activity of at least some RIPs is not limited to site-specific action on the large rRNAs of ribosomes but extends to depurination and even nucleic acid scission of other targets. Characterization of the physiological effects of RIPs on mammalian cells has implicated apoptotic pathways. For plants, RIPs have been linked to defense by antiviral, antifungal, and insecticidal properties demonstrated in vitro and in transgenic plants. How these effects are brought about, however, remains unresolved. At the least, these results, together with others summarized here, point to a complex biological role. With genetic, genomic, molecular, and structural tools now available for integrating different experimental approaches, we should further our understanding of these multifunctional proteins and their physiological functions in plants.

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INTRODUCTION

Proteins with selective toxicity have been investigated for use in ways as varied as murder weapons by mystery writers (38) and espionage agents (39, 92), to transgenic plant protection by biologists (110, 112, 117), “silver bullet” therapies by cancer researchers (53, 96, 139, 145, 171, 183), and biological weaponry by military groups (39, 200). One class of such proteins, ribosome-inactivating proteins (RIPs), are found in genera throughout the plant kingdom as well as in certain fungi and bacteria. These proteins act as N-glycosidases to modify large rRNAs and render them incapable of sustaining further translation. The $K_{cat}$ for nonplant ribosomes is greater than $10^3 \text{ min}^{-1}$ for the RIPs abrin (from Abrus precatorius) and ricin (from Ricinus communis; 139). Thus, a single molecule has the potential to kill a cell.

Because of their selective toxicity, RIPs have been primary candidates for the toxic moiety of immunotherapeutics (139). As a result, much of the RIP literature reflects attempts to isolate and characterize RIPs from new plant sources and to exploit these RIPs as anticancer agents (8, 56, 173). Numerous other studies have focused on enzymology, uptake of lectin-associated RIPs into target cells, and subsequent transport to ribosomal targets in the cytosol (116, 140, 162). These investigations have provided a broad knowledge base for understanding biochemical and medicinal properties of RIPs. Less prevalent, however, are investigations into the biological function of RIPs in plants. In recent years, such investigation of RIP activities has increased, especially as tools for gene isolation and transgenic expression became available. These studies have led to an improved understanding of RIP gene expression and activity against pathogens but have also uncovered new enzymatic activities that are suggestive of RIP biology being quite complex. This review summarizes work related to RIP activities and considers unresolved issues in how these activities may affect plant metabolism and protection.

BACKGROUND

Historically, RIPs have been linked to plant defense, with reports appearing as early as 1925 describing inhibition of viral infection by extracts of pokeweed [Phytolacca americana; summarized in (83)]. Pioneering work was performed by L. Barbieri, F Stirpe, and coworkers who assayed extracts from over 50 plants and found that most had translational inhibitory activity in vitro (8, 56, 173). Subsequent purification of the inhibitory proteins led to their identification as RIPs. As searches for additional RIPs were carried out, they were found not only in a few exotic plants but also in crop plants such as wheat, maize, and barley (40).
see comprehensive reviews by Barbieri (8) and Stirpe et al (173) for detailed lists of RIPs, their source plants, and abundance in various organs]. For many years after the first RIPs were characterized, the mechanism by which they inactivated translation was not known. In a major breakthrough, Endo and coworkers showed that the enzymatic activity of RIPs was an N-glycosidation to remove a specific adenine corresponding to residue A4324 in rat 28S rRNA (49, 50). This adenine lies within a 14-nucleotide region that is known as the α-sarcin loop and is conserved in large rRNAs from bacteria to humans (122). A GAGA sequence in which the first A is the RIP substrate forms the core of a putative tetraloop surrounded by a short base-paired stem (41, 65, 143). Irreversible modification of the target A residue blocks elongation factor (EF)-1- and EF-2-dependent GTPase activities and renders the ribosome unable to bind EF-2, thereby blocking translation (133).

Because this translational inhibitory activity is toxic, RIPs were seen as having great potential for use as selective cell-killing agents and interest in them shifted toward medical exploitation [for review see (139, 145, 171, 173)]. With the development of monoclonal antibodies as tools for identifying and targeting cell surface markers, researchers gained the ability to couple antibodies to RIPs and thus deliver the toxic protein directly to specific cells. The potential for using RIPs as cell destructive agents in the immunotoxins stimulated intense efforts to isolate and characterize such proteins from many different plant sources [reviewed in (53, 145)]. Unfortunately, RIP-derived immunotoxins are not perfect clinical tools. For example, they are generally highly antigenic and promote immune responses in animals receiving prolonged treatment with the immunotoxins [reviewed in (8)]. A second problem is with vascular leak syndrome, a deleterious side effect that limits clinical efficacy as a cancer therapy (96). Nevertheless, refined approaches to inhibit toxicity are showing promise (7, 76) and a number of clinical trials are ongoing (53, 96).

**CLASSIFICATION OF RIPs**

RIPs are classified into three groups based on their physical properties (Figure 1) (127). Type 1 RIPs, such as pokeweed antiviral protein (PAP), saporin (from soapwort, *Saponaria officinalis* L.), and barley (*Hordeum vulgare*) translation inhibitor, are monomeric enzymes, each with an approximate Mr of 30,000 (3, 8, 82). They are basic proteins that share a number of highly conserved active cleft residues and secondary structure within the active site region (8, 81, 124, 126) but are distinctly different in overall sequence homology and posttranslational modifications (71). To date, most RIPs that have been characterized fall into the type 1 class (8).

Type 2 RIPs, like ricin and abrin, are highly toxic heterodimeric proteins with enzymatic and lectin properties in separate polypeptide subunits, each of approximate *M*<sub>r</sub> of 30,000 (138, 139, 175). One polypeptide with RIP activity (A-chain) is linked to a galactose binding lectin (B-chain) through a disulfide bond (138, 139, 175). The lectin chain can bind to galactosyl moieties of glycoproteins and/or glycolipids...
Figure 1  Alignment of RIPs showing a comparison of primary structures. Filled, stippled, and hatched boxes denote regions absent in the active enzymes (shown as blank boxes). The N-terminal signal peptides on type 1 and type 2 RIPs target the proteins to the endomembrane system. Once there, they move to various subcellular compartments such as vacuoles, protein bodies or the periplasmic space. The N-terminal extensions of type 3 RIPs do not appear to have targeting functions because the proteins remain in the cytosol.

found on the surface of eukaryotic cells (105, 140, 158, 172, 180) and mediate retrograde transport of the A-chain to the cytosol (16, 139, 160, 191). Once it reaches the cytosol, the RIP has access to the translational machinery and readily disrupts protein synthesis. The type 2 RIPs have been very useful for studies of endocytosis and intracellular transport in mammalian cells (reviewed in 74, 114, 116, 161, 162).

Type 3 RIPs are synthesized as inactive precursors (proRIPs) that require proteolytic processing events to occur between amino acids involved in formation of the active site (127). These RIPs are much less prevalent than type 1 or type 2 RIPs. To date, type 3 RIPs have been characterized only from maize and barley (14, 34, 151, 196), although several close relatives of maize, including sorghum, appear to accumulate type 3 RIPs in seeds as judged by size and immunological cross-reactivity with antibodies to maize proRIP (75, 165). In maize, proRIPs are acidic proteins that are cleaved in vivo to release amino acids from an internal
region as well as short segments at both NH$_2$- and COOH-termini. These processing events result in proteins with tightly associated polypeptide subunits of $M_r$ 16,500 and 8,500 (196). The two RIPs that have been characterized in maize are both type 3 proteins (13, 14, 196). Barley, in contrast, has one type 1 RIP and one type 3 RIP. The latter, called JIP60, is induced by jasmonic acid and activated after proteolytic cleavage to remove internal and COOH-terminal domains (34). The internal insertions of JIP60 occur at approximately the same position within the primary amino acid sequence as in the maize RIPs. However, the JIP60 has a much larger C-terminal region (>20 kDa) and shares homology with eukaryotic initiation factor 4\gamma subunits (34).

The function of the extra domains in the type 3 RIPs is not known. Once they are removed, the processed active protein is similar in charge and enzymatic activity to type 1 RIPs (75, 95, 196). For maize, the extra domains are unlikely to be 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 (14, 75, 95). For barley, however, induction of JIP60 was reported to coincide with a decrease in protein synthesis followed by a decrease in polysome size (151). Thus, the possibility of JIP60 accumulating as a proenzyme to protect barley ribosomes cannot be ruled out.

RIBOSOME SUSCEPTIBILITY TO RIP INACTIVATION

The enzymology of RIPs has been well characterized, more so than their biological function. As a result, the role of RIPs in planta remains open to speculation. One factor contributing to the difficulty in ascertaining biological function occurs because many RIPs seem to share few properties aside from the capacity to depurinate ribosomes at the $\alpha$-sarcin loop. In fact, even this activity varies among different substrates. RIPs such as PAP are very active against both animal and plant ribosomes, but RIPs from cereals generally have low activity against plant ribosomes \([117a; reviewed in (70, 71)]\). An exception to this rule is the RIP of wheat leaves, which can modify plant ribosomes at concentrations where the seed RIP does not (119).

Large differences in efficiency are observed between depurination of RNA and ribosomes. For example, $10^4$-fold more ricin is needed to depurinate oligonucleotides and $10^3$-fold more to depurinate wheat ribosomes than to kill mammalian cells (30, 48, 114). A few RIPs have activity against prokaryotic ribosomes, but usually very large amounts of RIP are necessary for these targets (31).

One difference among RIPs contributing to their translational inhibitory activity is the requirement for cofactors. Gelonin from *Gelonium multiflorum*, barley RIP, PAP, and tritin-S from wheat require ATP for maximal activity (27, 156, 170), but other RIPs such as bryodin-R from *Bryonia dioica*, mormordin from *Momordica choratea*, and saporin do not (27). Brigotti and coworkers have demonstrated that tRNAs affect translational inhibitory activity, with the specificity of the stimulation
Tritin-S was fairly nonspecific in being stimulated equally by different tRNAs, whereas gelonin was stimulated only by tRNA\textsuperscript{Trp} from mammalian and avian cells and not by other tRNAs or tRNA\textsuperscript{Trp} from yeast (20–23). Given the variety of assays and assay conditions used for RIP analysis, some differences observed in previous studies may be explained by cofactor requirements. It is not known, however, whether the need for cofactors simply represents a mechanism for increasing enzyme efficiency or if it is linked to additional biological activities.

An important long-standing question is: “What factors contribute to the resistance or susceptibility of a ribosome to RIP inactivation?” To date, the accumulated results are at least as confounding as clarifying. Endo’s discovery that RIPs depurinate the A\textsuperscript{4324} residue from the strictly conserved \(\alpha\)-sarcin loop eliminated the possibility of target site differences between susceptible and resistant ribosomes and left little doubt that factors other than nucleic acid sequences must be the key determinants (49). Chaddock et al (32) approached the question by swapping domains of ricin (which has little effect on plant or bacterial ribosomes) and PAP (which is highly effective at modifying plant and bacterial ribosomes). Surprisingly, rather than gaining the capacity to attack prokaryotic ribosomes, the chimeric molecules lost the promiscuous RIP activity associated with PAP. Vater et al (192) used chemical cross-linking to identify interactions between ricin A-chain and rat liver ribosomal proteins L9 and P0 (previously known as L10e). This finding was intriguing because L9 and P0 are located in the region of the ribosome called the acidic stalk, which is involved in interactions with elongation factors (153, 188). Furthermore, Vater et al suggest that sequence divergence among these proteins is sufficient to account for a lack of prokaryotic recognition by ricin (192). An additional component of the acidic stalk, P3, appears to be unique to higher plants and may contribute to structural differences that affect RIP interactions with plant ribosomes (4, 5).

Hudak et al (79) recently discovered a different interaction, this one occurring between the yeast ribosomal protein L3 and PAP. A mutant with two amino acid substitutions in yeast L3 is resistant to PAP and the mutant L3 protein loses the capacity to be cross-linked to PAP. Because the altered amino acid residues are highly conserved among fungal, mammalian, and plant cells, the RIP-L3 interaction is not likely to be a determinant of species-specific sensitivity or resistance to RIPs. Nevertheless, the RIP-L3 interaction appears to be necessary for the enzymatic modification of ribosomes by RIPs.

An intriguing concept that ribosome susceptibility to RIPs may be a dynamic process rather than an innate property arose from studies with the type 3 barley RIP, JIP60. 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. In contrast, water-treated controls had no change in polysome size (150, 151). These data should be interpreted with caution, however, as ribosomes were not assayed for depurination at the \(\alpha\)-sarcin loop. Further questions about whether the \(N\)-glycosidase activity
is responsible for polysome cleavage arise because of a report that JIP60 is not competent for translational inhibition unless proteolytic processing with at least two cleavage events has occurred (34).

WHAT PURPOSE(S) DO RIPs SERVE IN PLANTS?

The diversity among RIPs and their activities toward different targets calls into question how far one can extrapolate results in attempting to characterize RIP activity in planta. A presumed role in plant defense could be argued from circumstantial evidence gained both in vitro and in vivo. Data with fungal and insect systems are covered here and antiviral activities are discussed later in the review.

In bioassays, two type 1 RIPs from *Mirabilis expansa* roots were active at microgram levels against several soilborne bacterial species, the first such demonstration of antibacterial activity from a plant RIP in bioassays. In addition, these RIPs were active against a wide variety of both pathogenic and nonpathogenic fungi including *Fusarium* and *Trichoderma* species (195). Surprisingly, in some cases fungal species from the same genus showed differential sensitivity. For example, *Pythium irregulare* was sensitive, whereas *P. ultimum* was resistant.

Treatment of fungal spores with proteolytically activated maize RIP, but not its proRIP form, produced morphological alterations in hyphae of *Aspergillus flavus* but induced autolysis of *A. nidulans* (132). Addition of the type 1 barley RIP was inhibitory to fungal growth on solid media when tested against *Trichoderma reesei* (155). Inhibition of growth in liquid media, however, was minimal with barley RIP alone but increased dramatically when chitinase was also included (100).

This apparent defense role against pathogens also extends to certain insect pests as assayed in feeding studies. Insect bioassays with ricin and saporin showed extreme toxicity to larvae of two *Coleoptera* species but had no detrimental effect on *Lepidoptera* (57). Cinnamomin, a type 2 RIP from seeds of the camphor tree *Cinnamomum camphora*, was toxic to larvae of mosquito (*Culex pipines pallens*) and cotton bollworm (*Helicoverpa armigra* (207)). In both studies, the capacity of insect gut homogenates to hydrolyze the RIP was associated with a decrease in effectiveness of the RIP against insects. Thus, the apparent resistance seen in these studies may simply indicate that the RIP was degraded before it could be internalized into target cells. Another demonstration of insect susceptibility came from two type 2 RIPs of *Eranthis hyemalis* that were highly toxic (90%–100% mortality at 1.0 mg/ml) to the southern corn rootworm *Diabrotica undecimpunctata howardii* in feeding trials (97). Toxicity to insects has also been shown for maize proRIP and active RIP, which deterred feeding of adult beetles of *Carpophilus freemani*, *C. lugubris*, *Stelidota germinata*, and *Sitophilus zeamais* in assays in which the insects were given a choice of diets. In contrast, only active RIP was toxic to caterpillars in no-choice assays (46). Cabbage loopers (*Trichoplusia ni*), which do not feed on maize, were most severely affected, whereas Indian meal moths
which commonly feed on stored maize grain, showed no significant difference from controls. Fall armyworms (*Spodoptera frugiperda*, preferential leaf feeders), corn earworms (*Helicoverpa zea*), and corn borers (*Ostrinia nubilalis*; preferential immature seed feeders) had intermediate levels of susceptibility. Thus, differences in susceptibility of caterpillar species to active RIP varied in apparent accordance to host adaptation (or lack thereof), but factors contributing to the differential susceptibility or resistance of these insects have not been identified.

The fungal and insect bioassays offer the advantage of testing a single protein for its effect. Furthermore, tests can be done with concentrations of the protein equivalent to those found in particular plant organs (46). However, the use of a purified protein creates an artificial situation because the amount of RIP that would actually be released from cells during an endogenous insect-plant or fungal-plant interaction can only be approximated. Ectopic expression of RIPs in transgenic plants can solve this problem by allowing exposure of the pest or pathogen to the RIP only during interactions with the plant. Several such studies with different RIP genes confirm that these proteins can provide protection against plant pathogens [reviewed in (70)]. The type 1 barley RIP expressed by a 35S-CaMV promoter or a wound-inducible promoter in tobacco conferred some reduction to disease symptoms caused by the fungus *Rhizoctonia solani* (112). Addition of a signal sequence to target the RIP to the endomembrane system improved resistance in transgenic plants producing detectable levels of the RIP (84). Transgenic wheat plants, however, exhibited variable (0–40%) resistance against infection by the fungal pathogen *Erysiphe graminis* (19b). The significance of this resistance is questionable because the highest resistance did not correlate with the highest expression of the transgene. Expression of PAP, as well as an enzymatically inactive mutant, conferred fungal resistance and also led to overproduction of pathogenesis-related (PR) proteins in transgenic tobacco (209). This coordinate expression of RIP and PR proteins appears to be specific for the PAP-producing transgenic tobacco, as PR protein induction has not been seen in organs of nontransformed plant species even when RIPs accumulate to 10–20% of the soluble protein. Maize proRIP, when expressed in tobacco, was implicated as a defense protein against the fungus *R. solani* (117). Whether this resistance required proteolytic processing of the maize proRIP to an active N-glycosidase was not determined. However, tobacco containing a deletion construct of the kernel proRIP equivalent to the processed, active form of the enzyme was more resistant than control plants to insect feeding (P Dowd & R Boston, unpublished observations). Knowledge gained from ectopic expression studies with transgenic plants is valuable from the standpoint of agricultural biotechnology applications. But finding that RIPs can decrease susceptibility to damage by pathogens does not preclude additional and even more important roles for those proteins. Discovery of such roles would likely involve comparative analysis of plants in which RIP function has been removed (such as by mutation, insertional inactivation, or cosuppression). To date, gene knockout studies have not been reported for RIPs, but data are available from maize in which
expression of the abundant seed proRIP is regulated by the transcriptional activator Opaque-2 (44, 69, 88, 123, 169). Levels of proRIP and storage proteins are both decreased (more than 100-fold for proRIP) in o2 mutants (12, 169; AD Mehta & RS Boston, unpublished results) and kernels from mutant plants have increased susceptibility to fungal attack (111) and insect feeding (64). The increased susceptibility in the absence of the proRIP is consistent with its having a defense function, but the experimental results cannot be attributed to the maize proRIP a priori because Opaque-2 regulates transcription of many genes that may contribute to a complex mutant phenotype. Thus, definitive loss-of-function analyses are needed.

Taken together, data from mutants, transgenic plants, bioassays, and feeding studies suggest that defense is at least one component of RIP biology. Given that amino acids important for active site cleft formation, but few others, are conserved in all RIPs for which sequence information is available, it could be argued that retention of enzymatic activity is the most important selective pressure for RIP genes and that accumulation of RIPs in storage organs offers an advantage in plant defense. Developing seeds accumulate large quantities of endosperm- or cotyledon-specific proteins for use as nutrient sources during germination. These resources must be protected during grain fill and early stages of seedling growth when the seed is especially vulnerable to both mechanical damage and competition for storage reserves between the embryo and pests and pathogens. Thus, an abundant RIP could provide seeds with both nutritional benefits and protection against pathogen invasion. As discussed above, the maize proRIP accumulates under control of the same transcriptional activator, Opaque-2, that is responsible for directing gene expression for the major storage proteins in the seed. Saporin activity (8) is also highest in mature seeds, as is ricin and the type 1 barley RIP (100, 114). Accumulation of RIPs is not limited to seeds but also occurs in other storage organs. Vivanco et al have characterized RIPs from Andean root crops and reported levels up to 20% of the soluble root proteins of *Mirabilis expansa* (195). Likewise, trichosanthin accumulates to levels estimated at more than 20% of the soluble protein in storage roots of *Trichosanthes kirilowii* (163) and a RIP is the most abundant protein in Iris bulbs (190a). Thus, coordinate accumulation of RIPs and storage proteins would appear to put in place a preformed deterrent to damage by invading pests and pathogens.

But RIPs also accumulate in other organs, and recent reports link expression of some RIP genes to various stresses and even circadian control. A clone for JIP60, the type 3 RIP from barley, was originally identified from a cDNA library of jasmonic acid–induced genes (17). Expression of this gene is not linked strictly to jasmonic acid but is also induced by osmotic stress and desiccation (151). Similarly, RNA encoding the *Phytolacca insularis* antiviral protein, PIP2, accumulated in leaves exposed to jasmonic acid. RNA for this RIP also increased after treatment of plants with abscisic acid or mechanical wounding (169a). RIP activity from leaves of pokeweed and *Hura crepitans* increased during osmotic stress and also as a function of leaf age when green leaves were compared to senescent leaves (174).
Interestingly, in pokeweed, RIP activity increased in response to cold treatment and heat shock, whereas in *H. crepitans*, RIP activity increased in response to heat shock but not cold shock (174).

A RIP from the ice plant *Mesembryanthemum crystallinum* is expressed at low levels in unstressed plants but is induced by salt stress (154). RIP RNA levels from both stressed and unstressed ice plants fluctuated diurnally, with the highest levels appearing during the middle of the light cycle at midday (154). A diurnal effect was also observed with the kernel-specific maize proRIP gene. In this experiment, however, the proRIP RNA accumulated to highest levels during the dark phase of a 24-h clock with 14-h light periods (A Viotti, P Ciceri & R Schmidt, personal communication). Interestingly, a second maize proRIP gene that had been previously found to be expressed at low levels throughout the plant (13) also appears to be regulated by stress. In this case, the proRIP gene was induced 30–40-fold from nearly undetectable levels by both shading and drought stress in postpollinated ear and kernel samples (T Helentjaris, personal communication).

Stress induction of RIP genes is not limited to abiotic stresses but also occurs in some plants in response to biotic signals. For example, trichosanthin, a type 1 RIP from root tubers of *Trichosanthes kirilowii*, accumulated up to 19-fold more in roots of plants cocultivated with microorganisms than in sterile soil or culture medium (202). Beetin 27 and 29, type 1 RIPS from sugar beet leaves, were induced by several different viruses (58). The beetin 27 and 29 RIPS were also induced by H2O2 and salicylic acid, two signaling components of systemic acquired resistance in plants. Taken together, these observations suggest that RIPS may play a more dynamic role in the plant than was previously thought. However, insufficient information is currently available to determine whether or not stress-induction is a widespread phenomenon among RIPS. As more data become available from genomic investigations of gene expression, we may well uncover additional information about RIP gene expression and identify pathways common to its regulation in different plants.

**OTHER RIP ENZYMATIC ACTIVITIES**

The site-specific *N*-glycosidase activity has long been the defining characteristic of RIPS. However, additional activities have also been reported. The most widespread of these is a polynucleotide:adenosine glycosidase that depurinates DNA as well as RNA from sites other than the A4324 of rRNA (10, 11, 11a, 102, 130). This activity was found for all of 52 RIPS surveyed by Barbieri and coworkers (10). Nuclease activity of several RIPS against supercoiled DNA and both single- and double-stranded DNAs was detectable but relatively inefficient compared to the reaction on ribosomes (45, 106, 107, 129, 130, 157, 197). However, Nicolas et al suggest that the *K*_cat against oligoribonucleotides is comparable to that of multisubunit DNA glycosylases that normally act on damaged DNA bases (130a). Prior to having access to RIP DNA and amino acid sequences, researchers were hampered in
investigating RIP activities by the possibility that they were working with impure protein preparations. As a result, there was confusion and concern about the reliability of claims of alternative activities and controversy as to whether they existed at all (42). Part of the difficulty in analyzing other activities comes from some but not all apparently sharing active site residues with the \( N \)-glycosidase such that mutations affect not just one but multiple activities. Additionally, assay conditions can vary and the alternative functions have yet to be tied to functions that occur in planta.

Some discrepancies in activities reported by different research groups may even be due to variations in the actual proteins assayed. For example, PAP cleaved double-stranded supercoiled DNA, but a point mutant of PAP (PAPx) that cannot depurinate ribosomes failed to nick the same supercoiled plasmid templates (197). In contrast, recombinant PAP produced in \textit{Escherichia coli} retained \( N \)-glycosidase activity but showed no capacity to nick supercoiled DNA (42). These studies, while apparently in conflict, may also reflect differences of the proteins synthesized in the three systems. For example, Tumer’s group assayed PAP produced in pokeweed and the mutant PAPx produced in transgenic tobacco, whereas Day and coworkers assayed PAP produced in \textit{E. coli} from a clone in which sequences corresponding to the carboxy terminal 29 amino acids of the natural protein had been deleted.

Most likely, some additional activities observed experimentally do reflect the presence of proteins other than RIPs. Valbonesi et al (190) and Fong et al (50a) separated a previously reported RNase activity from momordin II (125), but residual RNase activity was still detected (50a). Likewise, Day et al (42) used gel filtration chromatography to separate DNase activity from \( N \)-glycosidase activity of ricin. In addition, these authors found differences in DNase and \( N \)-glycosidase activities of PAP with heat and DEPC treatments that they attributed to the presence of at least two different proteins.

These examples point out the need for stringent control of RIP purity, but they do not rule out at least some RIPs having enzymatic activities in addition to depurination at the \( \alpha \)-sarcin loop. Recently, Wang et al (198) used NMR to obtain a high-resolution solution structure of MAP30, a RIP from bitter melon. MAP30 preparations cleave supercoiled DNA and also exhibit DNA glycosylase/apurinic/apyrimidinic (AP) lyase activity (103, 198). These authors assigned DNA and RNA \( N \)-glycosidase activities to one site and AP lyase activity to a different but contiguous subsite of the protein. In this model, DNA would be depurinated by MAP30 after which it would be cleaved by the AP lyase activity. Wang et al also showed that divalent cations interact with negatively charged regions of MAP30 and are likely to facilitate interactions with DNA. Thus, the cations would have a role in enhancing MAP30 activity instead of being associated with contaminating nucleases (42, 198). A sequential lyase activity was also suggested by Ogasawara et al (137). These authors, however, showed cleavage of the \( \alpha \)-sarcin loop by a separate RNA apurinic site-specific lyase following depurination of the RNA by gypsophilin, a RIP from \textit{Gypsophila elegans}. 
Antiviral Activity

Antiviral activities of RIPs have been known for ~75 years [reviewed in (8, 70, 185)]. They were presumed to provide a plant defense function by acting on ribosomes of infected plant cells [reviewed in (8)]. This conclusion was supported by links between susceptibility of host ribosomes to N-glycosidase activity and antiviral activity (182) as well as the absence in viral RNAs of any sequence similarity to the N-glycosidase target site of the α-sarcin loop. In addition, viruses that were incubated with PAP lost infectivity but recovered it once the PAP was removed (184). Antiviral activity is not confined to plant cells but is also seen for both RNA and DNA viruses of animal cells and even retroviruses of yeast (2, 102, 104, 148, 187, 189, 205).

The antiviral effect has been noted for a number of type 1 and type 2 RIPs, but the mechanism of antiviral activity has not been resolved [reviewed in (8, 197a)]. Several lines of evidence, however, suggest that antiviral activity can be separated from depurination at the α-sarcin loop (2, 78, 104, 121, 205). The most definitive demonstration was made by Tumer et al who assayed a deletion mutant of a PAP gene in transgenic tobacco. Even though the mutant protein was unable to depurinate the α-sarcin loop, it still conferred viral resistance against Potato virus X (186).

Hudak et al (80) recently extended this work with the report of a novel activity of PAP to depurinate selectively RNAs that had a 5′ terminal m7GpppG cap. In this study, capped and uncapped mRNAs were transiently exposed to PAP or PAP mutants and assayed for translational competence in vitro. Significant reductions were seen in translation of capped mRNAs that had been exposed to PAP. In contrast, PAP had no significant effect on uncapped mRNAs. Analysis of the capped mRNAs after incubation showed that they had been degraded at high concentrations of PAP and depurinated presumably at multiple sites at low PAP concentrations. From these observations, the authors concluded that the capped mRNAs could serve as targets for depurination. When the assays were carried out with PAP mutants lacking the capacity to depurinate ribosomes at the α-sarcin loop, selective translational inhibition of capped RNAs still occurred for mutants that had been altered in N- or C-terminal regions. However, the capped RNAs remained translationally competent after treatment with protein resulting from a point mutation in the putative active site of the enzyme. Thus, although the N-glycosidase activity was important for inactivation of capped RNAs, ribosome depurination was not.

Direct depurination of capped viral RNA could explain the antiviral activity of PAP against the Sindbis-like viruses, Tobacco mosaic virus (TMV), Brome mosaic virus (BMV), and Cucumber mosaic virus (CMV), all of which have 5′ terminal caps (60, 178). It falls short of providing a comprehensive explanation of the PAP antiviral activity, however, because many viruses inhibited equally well lack 5′ cap structures. For example, Chen et al assayed Potato virus Y (PVY), which has a 5′ terminal protein in place of a m7GpppG cap, on Chenopodium amaranticolor
(35). Application of PAP to C. amaranticolor leaves inhibited formation of local lesions by PVY and the extent of inhibition was even greater than for the capped viruses TMV and CMV (35). PAP also inhibited infection by the uncapped animal viruses influenza virus, poliovirus, and herpes simplex virus [a DNA virus (2, 189)].

Molecules other than capped viral RNAs have also been shown to be targets for antiviral activity. Lee-Huang and colleagues demonstrated activity of MAP30 and GAP31 (from Gelonium multiflorum) against HIV integrase at doses tenfold lower than those needed for translational inhibition (78, 103). Furthermore, they showed that limited proteolysis of the RIPs removed translational inhibitory activity but did not affect the capacity of the RIPs to inhibit HIV integration, proliferation of tumor cells, or HIV protein production in infected lymphocyte cells (78, 103). Strong inhibition of HIV-1 integrase in vitro was also seen for gelonin, luffin, α-momorcharin, β-momorcharin, saporin, and trichosanthin but not agrostin (3a).

In addition to the data from enzymatic analyses and bioassays, structural studies support a conclusion of RIPs interfering with viral integration as well. Wang et al used NMR to examine interactions between MAP30 and the HIV long-terminal repeat (LTR) DNA and concluded that the MAP30 binds to the LTR perhaps in competition with the viral integrase in vivo (198).

Taken together, these studies are suggestive that the antiviral properties of RIPs are quite complex. Furthermore, the mechanism of viral inhibition may vary among different viruses and different RIPs. It is clear, however, that development of sensitive assays for detecting depurination and other modifications of nucleic acids has expanded the enzymatic activities that can be assigned to RIPs. Structural studies as well as studies of mutants have demonstrated the usefulness of these approaches in defining the various protein domains and determining their importance for the different activities. With the availability of cloned DNAs for many RIPs, questions of endogenous or contaminating activities can be answered. Finally, as enzymatic activities are identified in vitro, analysis can move to plants for investigation of whether these activities are relevant under physiological conditions.

ENTRY OF RIPs INTO CELLS

Assuming that ribosome depurination is at least one biologically important activity of RIPs, then the RIPs must gain access to the ribosome-containing cytosol of target cells. Many RIPs likely evolved to have individualized methods for protecting their host cell ribosomes from inactivation while at the same time providing a means by which the protein could come into contact with target cells. This requirement would have to be met regardless of whether RIPs are acting as defense proteins against heterologous ribosomes and/or viral RNAs or regulating cell metabolism by modifying orthologous ribosomes. Here, we first address ways by which synthesis and sequestration of RIPs may protect the host cell and then consider the situation where RIPs must breach at least two barriers—the host cell membrane and the cell membrane of the heterologous target cell.
Secreted and Compartmentalized RIPs

RIPs are localized extracellularly as well as in compartments that vary among different host cells. Most RIPs appear to have N-terminal signal sequences that target them for cotranslational entry into the endomembrane system (Figure 1) (71). The endomembranes then provide a barrier between the RIP and the orthologous ribosomes. RIPs, however, can be transported bidirectionally—both forward and in reverse through the endomembrane system. As a result, additional protective steps are necessary to prevent subsequent retrograde transport of a newly synthesized RIP back to the ribosome-containing cytosol (retrograde transport of RIPs is discussed later). One strategy is synthesis as inactive pro-proteins. PIP2, saporin, sechiumin (from Sechium edule), trichoanguin (from Trichosanthes anguina), and trichosanthin are all type 1 RIPs with short carboxy terminal extensions that are encoded by the corresponding genes but not found on proteins isolated from plants (18, 28, 36, 37, 169a, 204) (Figure 1). The C-terminal extensions are similar in sequence to those found on vacuolar proteins and may act as vacuolar targeting signals (128, 194). Recombinant trichosanthin and sechiumin that contain the C-terminal amino acid peptides are ∼fivefold less active in translational inhibition assays than the corresponding native proteins (204, 208). Thus, in addition to targeting, the extensions may prevent these RIPs from becoming active prior to reaching the storage organelles.

The importance of sequential processing and localization of RIPs away from ribosomes was recently demonstrated by studies to express various ricin constructs in transgenic tobacco protoplasts and determine their cellular fates (55). Ricin, a type 2 RIP, undergoes a complex maturation process. It is produced in castor bean as a precursor polypeptide (preproricin) that contains a signal peptide as well as the A (enzymatic) and B (lectin) chains joined by a short linker peptide (26, 98) (Figure 1). Proricin is generated by cleavage of the N-terminal signal peptide from preproricin and transported from its site of synthesis at the ER to vacuoles via the Golgi complex (113). Only after proricin has reached the vacuole is the linker peptide cleaved to produce the mature active protein. When the ricin A-chain was expressed alone in transgenic tobacco protoplasts, it could be detected in ER, but its continued expression was toxic (55). This toxicity indicates that the active A-chain can most likely be transported from ER into the cytosol where it would interact with ribosomes. When the native preproricin construct was assayed, processing, glycosylation, and targeting to the vacuole occurred normally and no toxicity was seen (166). Thus, it appears that to prevent toxicity to the host cell, ricin, and most likely a number of other type 2 RIPs, must be produced as proproteins and targeted to the vacuole prior to activation by cleavage of the peptide linker between the A- and B-chains.

Besides those RIPs localized in vacuoles, there are others such as PAP and some forms of saporin that are secreted to a final destination in the cell wall matrix (28, 149; also see 71). Like RIPs localized in vacuoles, these proteins would be physically separated from orthologous ribosomes. Furthermore, the host cell would not be a barrier for activity against target cells.
Cytosolic RIPs

Exceptions to the RIPs that are sequestered from host ribosomes are those from cereals. These RIPs lack obvious signal sequences and appear to reside in the cytosol where they would constantly come in contact with host ribosomes (66, 100, 196). In general, cereal RIPs have little if any activity against plant ribosomes at concentrations found in vivo. The apparent lack of ribosome sensitivity to RIPs from the same plant extends to type 3 RIPs even after they have been proteolytically activated (14, 34, 95, 182). The cytosolic RIPs are also distinct in that they apparently lack antiviral activity (182), but they retain antifungal activity (100, 117, 132, 155). At present, the significance of the specialized localizations and ribosome specificity is unclear.

For vacuolar or cytosolically localized RIPs the question arises as to how they exit the host cell. One plausible explanation is that lysis of the plasma or vacuolar membranes upon cell attack by a pest or pathogen would release the RIP. For those type 3 RIPs requiring proteolytic activation, lysis of cellular membranes would likely release both the inactive proRIPs and the proteases required to activate them. Alternatively, activation could occur as a result of proteolysis by enzymes produced by an invading pest or pathogen.

RIP Interactions with Membranes and Receptors

Once a RIP had been released from the host cell, the next barrier would be encountered at the target cell where uptake would require binding of the RIP to the cell surface, transport into the cell, and finally entry into the cytosol. The presence of the lectin B-chain of type 2 RIPs allows binding to galactosyl moieties found on the surface of most eukaryotic cells. Studies of this binding and subsequent uptake of the disulfide bonded A-chain have been reviewed recently and thus are not covered in detail here (74, 114, 161, 162). Instead, we focus on uptake of type 1 and type 3 RIPs for which the uptake process remains mostly unknown.

Type 1 and 3 RIPs lack the lectin chain that facilitates entry and accounts for the extreme toxicity of type 2 RIPs to intact cells (176). Some type 1 RIPs such as gelonin (176), dianthin (177), and Momordica charantia inhibitor (9) are glycosylated and presumably bind to carbohydrate receptors on the cell membrane (reviewed in 8, 87). Similar binding has been shown for the ricin A-chain that carries mannose oligosaccharides capable of binding to mannose receptors even in the absence of the B-chain (51, 54, 91, 152, 167).

Carbohydrate binding is not the only means for recognition and entry, however, nor perhaps the most efficient. Comparison of toxicity of unglycosylated saporin and the glycosylated RIPs, dianthin and gelonin, to a variety of animal cells failed to show a correlation between toxicity and the presence of carbohydrates (8, 15). A better correlation was seen between toxicity and binding to members of the low-density lipoprotein (LDL) receptor family, which interact with a wide spectrum of ligands that are internalized via clathrin-coated pits (59). Saporin and
trichosanthin (also unglycosylated) bind specifically to α-macroglobulin and megalin receptors (29, 33). Saporin was more toxic to macrophages and liver cells that had LDL receptors than to T lymphocytes that did not. Additional cell surface receptor–RIP interactions will likely be discovered as better tools become available for tracking these proteins. In fact, trichosanthin was recently shown to bind to different chemokine receptors and stimulate chemotaxis and G-protein activation of cultured animal cells (206). Equivalent activation, however, was also seen when a mutant trichosanthin with 4000-fold lower ribosome-inactivating activity was used (131, 206). Whether these data reflect a very low threshold for RIP action or an activity unrelated to N-glycosidation is unresolved.

Although interactions between RIPs and receptors at the cell surface would seem to indicate a protein-mediated uptake, receptor-independent mechanisms of membrane binding are also supported by experimental evidence. Studies with reconstituted liposomes have implicated negatively charged phospholipids in conferring selective binding and uptake of the fungal RIP α-sarcin (141, 142). Alpha-sarcin (89, 203) is similar to many type 1 RIPs in that it is a single-chain nonglycosylated protein with no strikingly hydrophobic zones to allow for crossing of the membrane bilayer. Furthermore, binding to protein receptors has not been documented, yet α-sarcin was found to associate with phospholipid vesicles (141). This association has been attributed to aggregation of α-sarcin into oligomeric forms in the presence of negatively charged phospholipids (142). The type 1 RIP, saporin, was shown to promote fusion of membranes containing negatively charged phospholipids, but uptake into liposomes was not assayed (68, 108).

The diversity of proteins and phospholipids able to interact with type 1 RIPs makes it hard to offer a single model for introduction into cells. For type 3 RIPs, even less is known, including whether these proteins interact with cells as the proRIP zymogens, the activated (processed) RIPs, or in both forms. Based on studies with type 2 RIPs and with the ricin A-chain, uptake might be expected to be quite complex. For the ricin holoenzyme, the primary means of uptake is endocytosis via clathrin-coated pits with transport through the Golgi apparatus to the ER (161, 162, 168). When clathrin-mediated uptake was inhibited, an alternative method of uptake was revealed (61, 161, 162). This alternative internalization is clathrin independent and highly regulated through complex signaling pathways involving protein kinases, G-proteins, cAMP, and cell polarity (47, 77, 85, 109).

**Retrograde Transport of RIPs**

For RIPs that enter the cell by way of vesicles, further transport is necessary to gain access to cytosolic ribosomes. Type 2 RIPs follow a retrograde transport pathway through endosomes or Golgi to the ER and then the cytosol (115, 116, 162, 168, 199). This retrograde movement has been studied extensively for type 2 RIPs as well as for the ricin A-chain alone, which has often been taken to be a model for the single-chain type 1 RIPs (116, 161, 162, 179). Recent advances in elucidating the terminal steps of retrograde transport are described briefly here even
though their importance for type 1 and 3 RIPs can only be hypothesized at present.

The last step in retrograde transport, exit from the ER, is mediated by a protein conducting translocon in the ER. This translocon serves a dual function in export of unfolded or misfolded proteins from the ER to the cytosol for degradation and in import of nascent polypeptides from the RER into the secretory pathway (24, 120). Polypeptides that are to be exported from the ER are identified as being modified or partially unfolded by quality control proteins such as the ER molecular chaperones that bind to nonnative domains and target the proteins for degradation by the proteasome (24). RIPs have been proposed to subvert this pathway such that as they exit the ER through the translocon, they escape to the cytosol rather than interact with the proteasome (74, 114). Work by Simpson et al (168) provides experimental support for translocon-mediated entry. These authors found that a large proportion (80%) of recombinant ricin A-chain polypeptides produced in wild-type yeast were rapidly degraded, whereas almost no degradation was seen in yeast strains defective in the ER-associated degradation (ERAD) pathway. The ricin A-chain molecules found in the cytosol of wild-type yeast were presumed to have entered by passing through the translocon and escaping degradation by the proteasome.

Use of the translocon by ricin A-chain suggests that it was recognized in the ER as having unfolded or misfolded domains. Consistent with this notion is recent evidence that the conformational stability of ricin A-chain favors an unfolded state in the ER and that refolding in the cytosol could be mediated by ribosomes (1). Because of structural similarities between the ricin A-chain and the type 1 and 3 RIPs, a hypothesis of retrograde transport based on the ricin A-chain seems to be a good basis for investigating entry of type 1 and type 3 RIPs into the cytosol from the ER.

**PHYSIOLOGICAL MANIFESTATIONS OF RIP ACTIVITY**

Although the enzymatic mechanism of RIP activity is well defined, the physiological steps by which ribosome inactivation leads to cell death are not well understood. In cultured mammalian cells, both type 1 and type 2 RIPs have been linked to a programmed cell death, or apoptosis (19a, 63, 99, 146, 147, 201). RIP-treated cells exhibited the morphological features characteristic of apoptosis including condensation and fragmentation of cell nuclei, cytoplasmic densification, breakdown of nuclear DNA into discrete fragments, and mitochondrial membrane alterations (19a, 25, 63, 72, 90, 99, 118, 135, 159, 193, 201). In addition, use of inhibitors and pharmacological agents that perturb signal transduction pathways leading to programmed cell death add further support to the idea that apoptosis is initiated by RIPs. For example, production of reactive oxygen species (ROS) was detected in single cells treated with trichosanthin (205a). Cell viability increased, however, upon preincubation with α-tocopherol, a protective agent against ROS-induced cell
death. Similarly, when the apoptotic protease activities of caspases and serine proteases were inhibited, treatment of cells with ricin failed to bring about programmed cell death (93, 136). Treatment with the thiol antioxidant N-acetylcysteine (NAC) blocked cytotoxicity of ricin and led to the conclusion that redox regulation of proteins via their thiol groups also plays a role in ricin-induced programmed cell death (134). The redox regulation, however, appears to act through a signaling pathway distinct from the protease-mediated pathway because NAC treatment did not affect the generation of caspase activity. Signaling through a third molecule, tumor-necrosis factor-α (TNF-α), has also been implicated in ricin-induced apoptosis as judged by the lack of a ricin effect in cells in which TNF receptors had been blocked (73).

Promotion of RIP-induced cell death through multiple pathways may have important therapeutic implications for RIP-based immunotoxins. For example, many cancer cells are resistant to apoptosis and thus would not likely be killed by action through caspases and serine proteases but might well be susceptible through the thiol or TNF pathway. For plant, fungal, and insect cells, however, the presence of a dual response pathway has not been explored.

Because apoptosis is an indirect effect that occurs downstream of the known RIP enzymatic activities, several investigations were focused on the importance of the ribosome-inactivating activity of RIPs in the apoptotic response. Initial reports had suggested that the B-chain lectin of type 2 RIPs can induce programmed cell death in mammalian cells (67). More recent reports, however, have shown that highly purified B-chain subunits alone cannot induce programmed cell death (193). The role of the B-chain in this process is most likely only as a carrier to allow the A-chain to have access to the cell (135). Site-directed mutagenesis of the type 2 RIP mistletoe lectin 1 showed a strict positive correlation between the enzymatic ribosome-inactivating activity of the A-chain and programmed cell death (99).

Not all type 1 RIPs induce apoptosis, however. Presumably, these differences in response are due to differential uptake of the RIP into cells because conjugation of a nontoxic type 1 RIP, bryodin-1 from Bryonia dioica roots, to an antibody that recognized the cell surface protein CD40, resulted in prompt cytotoxicity in TS1 cells (52).

The necessity of having ribosome-inactivating activity for subsequent apoptosis is consistent with the hypothesis that reduction in protein synthesis is a trigger for programmed cell death in mammalian cells. Indirect evidence for such a hypothesis comes from work with an interferon-induced, dsRNA-dependent protein kinase [PKR; reviewed in (86)]. PKR inactivates the translational initiation factor, eIF2α, by phosphorylating it such that initiation of translation and subsequent protein synthesis are severely inhibited (101, 144). Overexpression of PKR in cultured cells increases their sensitivity to inducers of apoptosis, whereas overexpression of a dominant negative PKR confers resistance to inducers of programmed cell death (6, 43).
In a separate study, Sandvig and coworkers found that either ricin or cyclohexamide blocked protein synthesis in mammalian cell cultures, but only ricin treatment led to programmed cell death. However, if cyclohexamide was added to the ricin-treated cells at the cessation of protein synthesis, programmed cell death did not occur (159). These observations at first seem to run counter to the idea that a reduction in protein synthesis leads to programmed cell death. They can be explained, however, by the finding that cyclohexamide inhibits autophagy, a key degradative step in apoptosis (94, 159). By inhibiting autophagy, cyclohexamide likely blocked the ricin induction of apoptosis following translational inhibition. Thus, the idea that RIPs induce apoptosis by blocking protein synthesis remains viable.

The finding that RIPs can induce apoptosis in mammalian cell cultures is important for defining mechanisms of activity and designing targeted applications for these proteins. Extrapolating these results to effects on whole organisms, however, may be more complex. For example, a side effect of the antibody-targeted use of RIPs as anticancer agents is vascular leak syndrome (VLS) characterized by an increase in vascular permeability that leads to organ failure. Recent results by Baluna et al (6a, 7) suggest that VLS may not be induced by the ribosome-inactivating activity of RIPs but instead by a three–amino acid structural motif that interacts with and damages vascular endothelial cells. Whether RIPs with this motif would also have a detrimental effect on insect, fungal, or plant cells is an interesting but unanswered question. Although fungal bioassays and insect feeding studies point to RIPs from all three structural classes as being toxic, how those RIPs actually affect cells from organisms other than mammals is less clearly defined.

Ectopic expression of PAP and the type 3 barley RIP, JIP60, produced abnormal phenotypes in transgenic tobacco (62, 110). For PAP, only those plants making large amounts of enzymatically active proteins produced morphologically aberrant plants (110, 186). Bioassays to determine the effect of RIPs on insects have primarily depended on gross changes in the growth or survival of the insect and not effects on individual cells (46, 57, 97).

The most common bioassays to determine RIP antifungal activity are based on inhibition of fungal colony growth (100, 155, 164, 195). Recently, however, microscopic examination of fungi has offered some insight into what effect RIP treatment might be having on individual fungal cells. Hyphae of *Trichoderma reesei* treated with two type 1 RIPs from *Mirabilis expansa* were found to be narrower than control hyphae, and to have extensive septum formation and enlarged tips (195). Treatment of *Aspergillus nidulans* spores with an active form of maize RIP led to apparently normal germination but autolysis of hyphae prior to septum formation. The autolysis depended on both ribosome-inactivating activity of the protein and G-protein signaling in the cells (132; K Nielsen, GA Payne & RS Boston, unpublished results). Confocal imaging of maize RIP labeled with the fluorophore Texas Red and nuclei stained with the DNA binding dye DAPI showed localization of RIP within the cell and a decrease in nuclear staining prior
to the autolytic event (K Nielsen, GA Payne & RS Boston, unpublished results). These observations suggest that fungi, like mammalian cells, have a programmed cell death pathway whereby the nuclear degradation observed prior to autolysis in A. nidulans cells is analogous to the programmed cell death observed with RIP treatment of mammalian cells. Taken together, the above observations suggest that the effect of RIPs on individual cells from various organisms may be manifested by similar pathways.

FUTURE PERSPECTIVES

After years of being investigated as tools to be exploited for their toxicity, RIPs are stimulating interest for their biological functions in plants. Transgenic plant technologies have introduced new avenues for targeting RIPs to additional sites within plants, expressing RIPs in plants that lack endogenous RIP activity, and inactivating endogenous RIP genes to determine the breadth of their influence on plant phenotype. Understanding the process of RIP uptake and transport in cells is benefiting from new concepts in retrotranslocation and development of tools for nondestructive imaging (181). These approaches should also be valuable for comparative analysis. In particular, investigation of how type 1 and type 3 RIPs enter cells of potential pests and pathogens lags far behind studies of type 2 RIPs and their effects on animal cells. Recent invention of a photochemical internalization method for introducing membrane-impermeable molecules into cells has removed a major obstacle, namely, the lack of an efficient internalization of the proteins (19, 166a). Much is left to be learned from comparing RIPs from the different structural classes and with different substrate affinities. New insights into antiviral mechanisms have begun to increase our understanding of properties important for interfering with viral infection. Moreover, they have opened investigation into the likelihood that RIP activity may not be limited to ribosomal substrates but may also damage DNA or RNA of pathogen and/or host cells (147a). Discovery of multiple enzymatic activities and dynamic fluctuation of some RIPs in response to biotic and abiotic signals has prompted exploration into the possibility of RIPs participating in maintaining organismal homeostasis, perhaps by facilitating macromolecular turnover (34, 154, 157, 174). The next few years are likely to be an exciting phase for fundamental RIP research as we obtain insights into the functional significance for medicinal and agronomic applications of emerging discoveries.

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