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Maize Ribosome-Inactivating Protein (b-32)

Homologs in Related Species, Effects on Maize Ribosomes, and Modulation of Activity by Pro-Peptide Deletions

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The ribosome-inactivating protein (RIP) from maize (*Zea mays* L.) is unusual in that it is produced in the endosperm as an inactive pro-form, also known as b-32, which can be converted by limited proteolysis to a two-chain active form, αβ RIP. Immunological analysis of seed extracts from a variety of species related to maize showed that pro/αβ forms of RIP are not unique to maize but are also found in other members of the Panicoideae, including *Tripsacum* and sorghum. Ribosomes isolated from maize were quite resistant to both purified pro- and αβ maize RIPs, whereas they were highly susceptible to the RIP from pokeweed. This suggests that the production of an inactive pro-RIP is not a mechanism to protect the plant's own ribosomes from deleterious action of the αβ RIP. RIP derivatives with various pro-segments removed were expressed at high levels in *Escherichia coli*. Measurement of their activity before and after treatment with subtilisin Carlsberg clearly identified the 25-amino acid intradomain insertion, rather than the N- or C-terminal extensions, as the major element responsible for suppression of enzymatic activity. A RIP with all three processed regions deleted had activity close to that of the native αβ form.

Many plants produce RIPs, a unique class of proteins that are exceptionally potent inhibitors of eukaryotic protein synthesis (Stirpe et al., 1992; Barbieri et al., 1993). RIPs catalytically inactivate eukaryotic, and in some cases prokaryotic, ribosomes by cleaving the N-glycosyl bond of a single specific adenine residue in the ribosomal RNA (A_{4324} in the case of rat liver ribosomes; Endo et al., 1988). Depending on the species of plant, RIPs can be expressed in leaves, roots, sap, or seeds, often at very high levels. The physiological function of RIPs is at present unclear, although evidence is accumulating that they have a role in plant defense. Leah et al. (1991) have shown that a RIP from barley seeds inhibits the growth of fungal pathogens, particularly when combined with seed chitinases and glucanases. A defensive role against the mechanical transmission of plant viruses has also been proposed (Chen et al., 1991; Bonness et al., 1994). These results have been extended by the observation that transgenic tobacco plants expressing a RIP from barley exhibit increased tolerance to fungal infection (Logemann et al., 1992), and plants expressing a RIP from pokeweed have decreased susceptibility to viral infection (Lodge et al., 1993).

RIPs have been classified into two types (Stirpe et al., 1992): type-1 RIPs are the most prevalent; over 40 have been described. They are typically single-chain, basic polypeptides of 25 to 32 kD with relatively low toxicity to intact cells because they do not readily cross cellular membranes. The rarer type-2 RIPs have arisen from a gene fusion between a type-1 RIP domain and a lectin-like domain. The lectin domain (or B chain) can bind to cell surfaces and mediate the delivery of the RIP (or A chain) into the cytosol of the cell. The RIP A chain can then rapidly and irreversibly inactivate ribosomes to ultimately kill the cell. Thus, most type-2 RIPs described to date are potent cytotoxins, the best known example of which is ricin from castor bean seeds. However, two type-2 RIPs have recently been described that are not cytotoxic, although they have a RIP/lectin structure (Girbes et al., 1993b, 1993c).

We have previously described the purification, characterization, and molecular cloning of a unique type-1 RIP from the endosperm of maize (*Zea mays* L.) (Walsh et al., 1991). Unlike other RIPs, maize RIP accumulates in the seed as an inactive 34-kD precursor (pro-RIP), which is converted into an active form by proteolytic processing. This involves the removal of 16 amino acids (1763 D) from the N terminus, several amino acids from the C terminus, and, surprisingly, 25 amino acids (2708 D) from the center of the RIP polypeptide. The result of this unique series of proteolytic processing steps is a two-chain, activated form of the RIP that we have called αβ RIP. The two chains are tightly associated but are not covalently linked. Activation of pro-RIP occurs during germination by the action of endogenous proteinases, but can also be performed in vitro by a variety of nonspecific proteinases such as papain and subtilisin Carlsberg. The pro-RIP that we identified by purifying RIP activity from maize endosperm and characterizing the protein responsible for the activity and then isolating its precursor form proved to be homologous to b-32, the Opaque-2-regulated polypeptide of previously

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Abbreviations: IC_{50}, concentration resulting in 50% inhibition; RIP, ribosome-inactivating protein.

unknown function (DiFonzo et al., 1986, 1988; Lohmer et al., 1991). Bass et al. (1992) subsequently reported low levels of RIP activity associated with preparations of b-32. However, their study did not clearly identify and differentiate between inactive precursor forms (pro-RIP or b-32) and proteolytically activated forms of the RIP ($\alpha\beta$ RIP).

Intrigued by the unique maize pro-form of this widespread class of proteins, we asked the following questions regarding maize RIP biology:

1. Do analogous pro-RIP/$\alpha\beta$ forms of RIP exist in relatives of maize, or is the pro-RIP unique to Z. *mays*?

2. Is the pro-form of maize RIP a mechanism to protect maize ribosomes from the activity of the RIP, i.e. are maize ribosomes susceptible to activated maize RIP?

3. Which specific propeptide segment of the pro-RIP is the molecular "switch" that inactivates this otherwise potent enzyme; the N-terminal segment, as in many other zymogenic forms of enzymes, or the intradomain insertion?

In this work, we have determined that pro- and $\alpha\beta$ forms of RIP are not unique to maize but are found in other members of the Panicoideae. We have assessed the activity of both maize pro-RIP and fully activated $\alpha\beta$ RIP on ribosomes from maize and other species to establish what role the precursor may play in suppressing RIP activity in vivo. In addition, we have shown that maize pro-RIP can be expressed at high levels in a soluble form in *Escherichia coli*, and through a series of genetic deletions we have identified the peptide segments of the pro-RIP that are responsible for suppressing enzymatic activity in the precursor. These observations are discussed in terms of the in vivo function of RIPs.

MATERIALS AND METHODS

Plant Material

Seeds of *Tripsacum dactyloides*, *Zea mays mexicana*, *Zea mays parviglumis*, *Zea luxurians*, *Sorghum bicolor* (KFS-1), *Coix lachryma-jobi*, and *Zea mays* (Pioneer 3110) were a gift from Dr. Neil Cowen (DowElanco, Indianapolis, IN).

Genetic Manipulations

Standard methods of DNA purification, restriction enzyme digestion, agarose gel analysis, DNA fragment isolation, ligation, and transformation were as described by Ausubel et al. (1987) and Sambrook et al. (1989). Enzymes used for genetic manipulations were from Pharmacia LKB Biotechnology (Piscataway, NJ), BRL (Gaithersburg, MD), or New England Biolabs (Beverly, MA). Buffers and protocols used were provided by the manufacturer. All genetic manipulations were done in *Escherichia coli* strain DH5α from BRL.

PCR

A Perkin-Elmer Cetus Thermocycler (Norwalk, CT) was used for PCR amplifications. A typical run consisted of a 1-min denaturation step, a 2-min annealing step, and a 3-min extension step. Temperatures used were 94°, 37° or 50°, and 72°C, respectively. After 25 cycles, the reaction was held at 72°C for 7 min for extension of unfinished products. The reaction conditions for amplification were those recommended by Perkin-Elmer Cetus (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs, and 2.5 units of *Taq* DNA polymerase or AmpliTaq). Reactions were performed in four separate tubes, each containing 100 ng of template DNA in 0.1 mL of buffer. Primers were synthesized on an Applied Biosystems PCR Mate or 380A DNA synthesizer and were purified on acrylamide gels. About 50 pmol of each primer were included in each reaction. The sequences of the primers are shown in Figure 1.

Construction of Maize RIP Derivatives

pDE600 and pDE601/pro-RIP

The cDNA encoding maize pro-RIP in pUC19 (Walsh et al., 1991) was engineered for bacterial expression using PCR. Primer RIP-1 (Fig. 1) contains termination codons in all three reading frames to halt translation of any vector-encoded polypeptides, upstream of a Shine-Dalgarno sequence followed by the maize pro-RIP ATG. Primer PIR-4 spans the cDNA/pUC 19 junction. The engineered, amplified product was purified from an agarose gel and ligated into the filled-in *Hin*dIII site of the expression vector pGEMEX-1 (Promega, Madison, WI) to create pDE600. Initial expression experiments indicated that co-expression of pro-RIP with the vector-encoded gene 10 product resulted in insoluble aggregates. Removal of the gene 10 coding region (excision of the 918-bp *Xba*I fragment between the T7 promoter and RIP cDNA) to create plasmid pDE601 eliminated this difficulty. Plasmid pDE601 served as the backbone for all other constructions.

pDE602/RIP-ΔN

The RIP gene contained on pDE602 contains a deletion of the N-terminal leader sequence (residues 1–16 of pro-RIP) resulting in polypeptide RIP-ΔN. It was made by replacing the *Nco*I-*Eco*RI fragment of pDE604 with that of pDE601.

5' Primers (RIP)

RIP-1 5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGGCCGAGATAACCCTAGAGCCGAG 3'

RIP-2 5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGAAAAGAATAGTGCCAAAGTTCACTG 3

RIP-3 5' ACCGTCACCATGGGCCGCGCCGAAATGACCAGGGCCGTCAACGACCTGGCGAAGAAGAAGAAGG
CGGCTGACCCACAGGCCGACACGAAGAGC 3'

RIP-8 5' AAGGGTCTGGAGACCGTCACCATG 3'

3' Primers (PIR)

PIR-4 5' TATATAGCATGCCGGCCAGTGAATTCGGCACG 3'

PIR-5 5' GCATTGATCAGGCCTCGTCGTCGTCG 3'

PIR-6 5' ATATATATATGAATTCGCCAGGTCGTTGACGGCCCTGG 3'

PIR-7 5' CGGATCCAGCAGTAGCGGCAGCGGCAGTAG 3'

PIR-9 5' TATATAGGATCCGGCAGTAGTTTGATTCTTAACGAG 3'

Figure 1. PCR primers used for engineering maize RIP for bacterial expression.

pDE603/RIP-ΔI

Plasmid pDE603 encodes maize RIP-ΔI with the insertion region deleted (residues 162–186 of pro-RIP). The vector fragment was prepared by cutting pDE601 with *Nco*I (cuts approximately midpoint in the cDNA) and *Stu*I (cuts at the 3′ end of the coding region). The large vector fragment was purified for ligation with the PCR-engineered 3′ insert fragment. The insert was generated by using pDE601 as template in an amplification reaction using primers RIP-3 and PIR-5. RIP-3 directs deletion of the insertion region of RIP and backs up to the unique *Nco*I site. The PCR product was cut with *Nco*I and *Stu*I and gel purified. Ligation with the pDE601 *Nco*I-*Stu*I vector fragment generated pDE603.

pDE604/RIP-ΔNI

The plasmid pDE604 encodes a maize RIP-ΔNI that is deleted for both the N-terminal leader and insertion sequences (residues 1–16 and 162–186, respectively, of pro-RIP). The vector fragment was prepared by cutting pDE603 with *Not*I (cuts between the T7 promoter and cDNA ATG) and treating the ends with T4 DNA polymerase to eliminate single-strand overhangs at the ends. The DNA was then restricted with *Nco*I and gel purified away from the 5′ coding region of the cDNA. The insert was prepared by PCR amplifying a pDE603 template with the primers RIP-2 and PIR-6. The resulting PCR product was cut with only *Nco*I to generate a 3′ sticky end. The 5′ end of the fragment was left blunt for ligation into the filled-in *Not*I site of pDE603. Ligation of the 5′ truncated PCR product and the vector pDE603, cut with *Not*I and *Nco*I, produced plasmid pDE604.

pDE605/RIP-ΔNIC

Plasmid pDE605 encodes a maize RIP-ΔNIC that has deletions of the N-terminal leader and insertion sequences and a segment from the C terminus (residues 1–16, 162–186, and 295–301, respectively, of pro-RIP). The vector was pDE604 cut with *Nco*I and *Stu*I and gel purified from the 3′ coding segment. The insert fragment was a PCR product using pDE603 as template with primers RIP-3 and PIR-7. The amplified fragment was cut with *Nco*I, gel purified, and ligated into pDE603 cut with *Nco*I and *Stu*I. The resulting plasmid is pDE605.

pDE606/RIP-ΔNICSN1

Plasmid pDE606 encodes a maize RIP-ΔNICSN1 that is identical to that of pDE605/RIP-ΔNIC except that five additional amino acids are deleted from the carboxyl terminus (residues 1–16, 162–186, and 290–301, respectively, of pro-RIP). The 3′ half of the RIP gene present on the *Nco*I-*Bam*HI fragment of pDE605 was replaced with a PCR-derived fragment using pDE605 as template and primers RIP-8 and PIR-9. The PCR fragment was cut with *Nco*I and *Bam*HI and ligated directly into the pDE605 vector to create plasmid pDE606.

RIP Expression in *E. coli*

The expression system used was based on the T7 system described by Studier et al. (1990). The expression strain JM109(DE3) is lysogenic for the T7 RNA polymerase gene under *lac* promoter control. Typically, JM109(DE3) (Promega) was transformed with one of the pDE600 plasmids the day before an expression experiment. The freshly transformed cells were harvested from plates and transferred to Luria broth (5×10^7 cells/mL). The cultures were induced immediately with 1 to 10 mM isopropyl-β-thiogalactoside and incubated with vigorous shaking for 2 to 4 h before harvest by centrifugation. Cell pellets were stored at −20°C.

Cells were disrupted by two freeze-thaw cycles and suspension in 2 volumes of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100 [v/v], 1 mg/mL lysozyme, 100 μg/mL DNase, and 100 μg/mL RNase). The cells were allowed to incubate in lysis buffer for 15 min at 37°C. The extract was centrifuged at 4000*g* for 10 min at room temperature. The supernatant was collected and stored at −20°C prior to purification.

RIP Purification and Characterization

The recombinant polypeptides were purified from the bacterial extracts by precipitating protein at 65% ammonium sulfate and dialyzing the resulting pellet into an appropriate buffer for ion-exchange HPLC. Mono Q chromatography with 20 mM Tris-Cl buffer, pH 7.8, was used for recombinant pro-RIP purification and Mono S chromatography with 10 mM sodium phosphate buffer, pH 7, was used for purification of the remainder of the RIP derivatives, except RIP-ΔN, for which 10 mM sodium phosphate, pH 6, was used. Columns were eluted with a NaCl gradient. Typically, a lysate derived from 1×10^{10} cells yielded 5 to 10 mg of purified recombinant protein. Methods for gel electrophoresis, immunoblot analysis, determinations of protein concentrations, and RIP activity have been described previously (Walsh et al., 1991).

Activation by subtilisin Carlsberg was performed in 0.2 M Tris-Cl, pH 7.8, for 1 h using a RIP:subtilisin ratio of 60:1 (w/w). RIP concentrations were 0.2 to 0.6 mg/mL in the reaction, and the reactions were terminated by the addition of PMSF to 3 mM final concentration.

The molecular mass of the β fragment of native αβ maize RIP was determined by electro-spray ionization MS performed at the Harvard Microchemistry Facility by Dr. William S. Lane. Samples of the β fragment from three different preparations of αβ RIP purified from maize kernels were prepared by reversed-phase HPLC. The N-terminal sequences of the β fragments were confirmed as being the same as those previously reported (Walsh et al., 1991).

Immunoblot Analysis

Seeds of maize (*Z. mays* L.) and maize relatives were ground in a mortar and pestle to a fine powder and extracted for 4 h at 4°C with 4 volumes of 25 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl, 25 μg/mL leupeptin, 25 μg/mL antipain, and 1 mM sodium

EDTA. Extracts were adjusted to 1 mg/mL protein and 5 μL was separated using 17 to 27% SDS-PAGE gels from Integrated Separation Systems (Natick, MA). The gels were electroblotted onto a polyvinylidene fluoride membrane, and the blots were developed using rabbit antisera against the purified α and β fragments of maize RIP (Walsh et al., 1991). Bands were visualized using alkaline phosphatase-conjugated goat antirabbit antibody, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate following the manufacturer's protocol (Bio-Rad, Richmond, CA).

Depurination Assays

Maize ribosomes were prepared from seedlings 72 h after germination, essentially according to the method of Jackson and Larkins (1976). Depurination assays were performed as described previously by Hartley et al. (1991). This involved incubating isolated ribosomes with and without RIP at 30°C in 25 mM Tris-Cl, pH 7.6, containing 25 mM KCl, 5 mM $MgCl_2$, and 1 mM ATP. The RNA was extracted with phenol/chloroform, and 3-μg aliquots were treated with aniline. Aniline-treated and untreated samples were then run on agarose/formamide gels.

In Vitro Protein Synthesis Assays

In vitro protein synthesis assays using rabbit reticulocyte lysate were performed as described previously (Walsh et al., 1991).

RESULTS

Pro-/αβ RIPs in Other Plant Species

We previously identified a unique zymogen form of type-1 RIP from maize that undergoes extensive proteolytic processing to produce an activated αβ form of RIP (Walsh et al., 1991). However, RIPs characterized from the endosperms of other Gramineae species such as wheat and barley are typical type-1 RIPs with no evidence for unusual precursors or αβ forms (Roberts and Stewart, 1979; Asano et al., 1984). To establish whether related plant species may have RIPs similar to those of maize, extracts of seeds from members of the subfamily Panicoideae, of which maize is a member, were analyzed by immunoblotting using antisera against the α and β fragments of maize RIP (Walsh et al., 1991). The following species and subspecies were tested (in approximate order of relatedness to *Z. mays*: *Z. mays parviglumis* (three accessions), *Z. luxurians*, *Z. mays mexicana*, *T. dactyloides*, *S. bicolor*, and *C. lachryma-jobi*. Figure 2 shows that there was immunoreactivity with all of the extracts except the most distantly related species, *C. lachryma-jobi*. Moreover, the pattern of immunoreactivity observed in the extracts was similar, consisting of a band at 32 to 34 kD corresponding to maize pro-RIP and bands at around 16 and 11 kD corresponding to the α and β fragments of the activated form. Therefore, it appears that seeds of many members of the Panicoideae contain RIPs in a pro-/αβ form, i.e. they contain b-32 homologs. This is also supported by Southern blot analysis of DNA from these species using a maize pro-RIP cDNA as a probe (K. Armstrong and N. Cowen, personal communication).

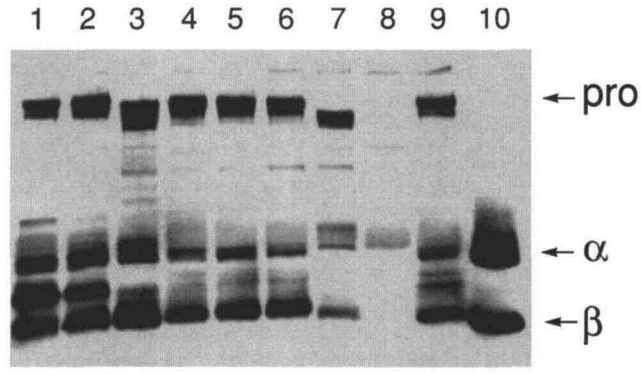

Figure 2. Immunoblot analysis of protein extracts from seeds of *Z. mays* relatives. Each lane contained 5 μg of protein except lane 10, which contained 25 ng of purified maize αβ RIP. Blots were probed with rabbit antisera against purified α and β fragments of maize αβ RIP. The extracts are numbered as follows: lane 1, teosinte (day neutral tunicate); lane 2, teosinte (day neutral); lane 3, *T. dactyloides*; lane 4, *Z. mays mexicana*; lane 5, *Z. mays parviglumis*; lane 6, *Z. luxurians*; lane 7, *S. bicolor* (KFS-1); lane 8, *C. lachryma-jobi*; lane 9, *Z. mays* (Pioneer 3110); and lane 10, purified maize αβRIP.

N-Glycosidase Activity of Maize pro-RIP and αβ RIP

We have shown that there is a significant difference in the RIP activity of maize pro-RIP and αβ RIP in rabbit reticulocyte cell-free protein synthesis assays (Walsh et al., 1991). Other studies with RIPs have shown that many type-1 RIPs not only inactivate heterologous eukaryotic and prokaryotic ribosomes, but also the source plant's own ribosomes (Taylor and Irvin, 1990; Ferreras et al., 1993; Rojo et al., 1993; Bonness et al., 1994). In these cases, the deleterious action of the RIP may be avoided by compartmentalization of the RIP via the secretory system. However, maize RIP is a cytoplasmic, not a secreted protein (DiFonzo et al., 1986). Considering these data, we investigated whether maize ribosomes were susceptible to either form of the RIP by monitoring the effect of pro-RIP and αβ RIP on maize ribosomal RNA. When rRNA is specifically depurinated by the N-glycosidase activity of a RIP, the phosphodiester backbone is rendered susceptible to cleavage by aniline at the site of adenine removal (Endo et al., 1987; Endo and Tsurugi, 1987). This results in the appearance of a small, approximately 300-nucleotide fragment by agarose/formamide gel analysis (the "aniline fragment"), which is diagnostic of RIP action.

Using this type of analysis, we found that maize pro-RIP had no significant effect on isolated maize ribosomes at a concentration of 3.0 μM, corresponding to a pro-RIP:ribosome molar ratio of approximately 8:1. The lack of released aniline fragment is shown in Figure 3, lane 8. These data are in agreement with those of Bass et al. (1992), who tested the susceptibility of maize ribosomes to preparations of b-32. However, these workers did not distinguish between inactive pro- and activated αβ forms of maize RIP. To test the susceptibility of maize ribosomes to activated αβ RIP,

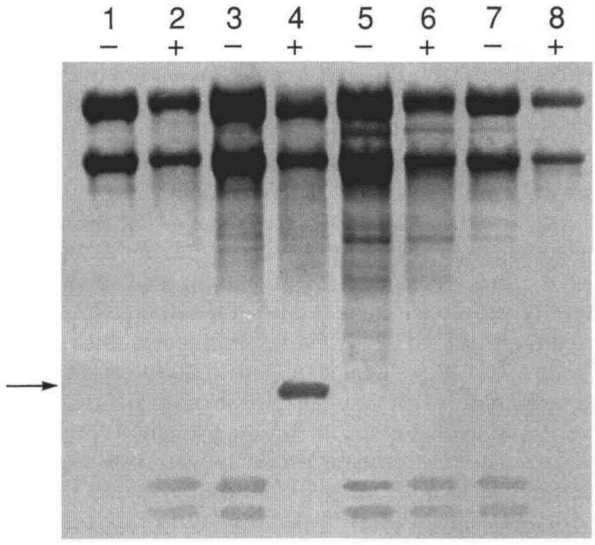

Figure 3. Effect of maize pro-RIP, αβ RIP, and pokeweed antiviral protein on RNA from maize ribosomes. Isolated maize ribosomes (30 μg) were treated with RIP as described in "Materials and Methods." After phenol/chloroform extraction, rRNA was electrophoresed in an agarose/formamide gel and the bands were visualized with ethidium bromide. RNA samples treated with aniline are marked +, those not treated with aniline are marked −. Lanes 1 and 2, Control (no RIP); lanes 3 and 4, 17 nM pokeweed antiviral protein; lanes 5 and 6, 1.8 μM maize αβ RIP; lanes 7 and 8, 3.0 μM maize pro-RIP. The position of the fragment diagnostic for RIP-catalyzed depurination is shown by the arrow.

maize ribosomes were treated with 1.8 μM αβ RIP (αβ RIP:ribosome molar ratio of 4.8:1). Under these conditions, only a very slight trace of the aniline fragment was detected (not visible in the gel shown in Fig. 3, lane 6). Treatment of yeast ribosomes with maize αβ RIP resulted in the release of the aniline fragment, demonstrating that maize RIP is capable of producing an aniline fragment from sensitive ribosomes (data not shown).

In contrast, treatment of maize ribosomes with the RIP from pokeweed (also known as pokeweed antiviral protein) at a 180-fold lower concentration (17 nM; RIP:ribosome molar ratio of 0.044:1) resulted in the release of the aniline fragment, seen in Figure 3, lane 4. Therefore, maize ribosomes are relatively insensitive to both the pro- and αβ forms of maize RIP, but are very sensitive to the action of the heterologous pokeweed RIP (which also has the ability to depurinate pokeweed ribosomes; Taylor and Irvin, 1990; Bonness et al., 1994).

Expression of Maize Pro-RIP in *E. coli*

The T7 expression system described by Studier et al. (1990) was used for expression of maize RIP in *E. coli*. The system relies on the presence of the T7 RNA polymerase for expression of the introduced gene. This positively regulated system allows genetic manipulations to be performed in standard laboratory strains with minimal leaky expression. This was of initial concern because several type-1 RIPs have been reported to have activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a).

Our maize RIP cDNA contained two Met codons near the 5′ end (Walsh et al., 1991). Because we originally isolated the cDNA as a gene fusion from a λ gt11 library, and the N terminus of the naturally occurring pro-RIP was blocked, we had no direct indication as to which Met codon initiated translation. Comparative sequence analysis of the initiator codon context in several maize genes indicated that the 5′ ATG was the more probable start site. This choice was subsequently confirmed by inspection of genomic sequences of b-32 reported by Hartings et al. (1990). The maize pro-RIP cDNA was engineered for expression in *E. coli* using PCR technology. Translation stops in all reading frames immediately upstream of a Shine-Dalgarno sequence were added to the cDNA using appropriate primers. The engineered cDNA was ligated into the expression vector pGEMEX-1 to create plasmid pDE600. When pDE600 was introduced into expression strains containing the T7 RNA polymerase, large amounts of both the vector-encoded gene 10 and maize pro-RIP polypeptides were produced. However, most recombinant material was recovered as insoluble aggregates in the pelleted fraction of bacterial lysates. Removal of the gene 10 coding region from pDE600 to create plasmid pDE601 resulted in both increased production of pro-RIP and in recovery of large amounts of soluble, recombinant protein.

The recombinant pro-RIP was purified to homogeneity and tested for RIP activity in a rabbit reticulocyte lysate protein synthesis assay (Fig. 4). The pro-RIP purified from *E. coli* had a very low level of inhibitory activity on protein synthesis (IC_{50} = 600 nM) relative to native, active αβ RIP (IC_{50} = 0.065 nM). After treatment with subtilisin Carlsberg, the pro-RIP was converted into a potent inhibitor of protein synthesis with an IC_{50} = 0.09 nM, corresponding to

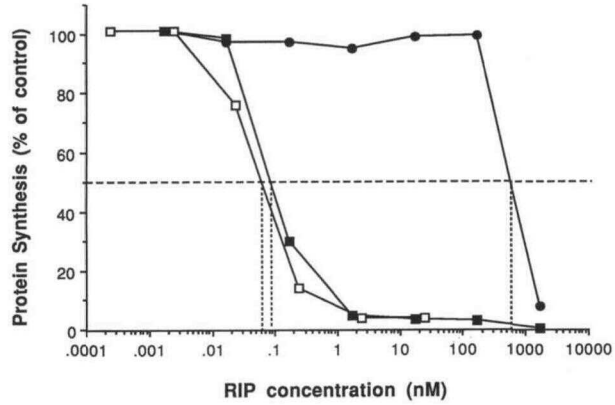

Figure 4. Inhibition of protein synthesis in a rabbit reticulocyte lysate by recombinant maize pro-RIP before and after treatment with subtilisin Carlsberg. The amount of [U-^{14}C]Leu incorporation into protein precipitated by TCA in the presence of varying concentrations of purified, recombinant maize pro-RIP was measured. The recombinant pro-RIP was either untreated (●) or treated with subtilisin (■) as described in "Materials and Methods." The activity of native αβ RIP in the same experiment is also shown for comparison (□). The vertical lines denote the IC_{50} values for each curve.

an approximate 6700-fold increase in activity (Fig. 4). Analysis by SDS-PAGE shows that the recombinant pro-RIP was processed into a two-fragment form that appears very similar to naturally occurring, active $\alpha\beta$ maize RIP (Fig. 5, A and B, lanes 3). These data provide direct evidence that the cDNA we have isolated encodes a polypeptide of low intrinsic RIP activity that can be proteolytically activated to yield a potent RIP.

Determination of Native Pro-RIP C-Terminal Processing

We have previously identified three regions of the inactive pro-RIP that are processed to yield the active $\alpha\beta$ form of maize RIP (Walsh et al., 1991). These consist of a 16-amino acid N-terminal segment, a 25-amino acid insertion in the center of the polypeptide chain, and a segment of unknown length at the C terminus. The extent of C-terminal processing was somewhat ambiguous because of our previous inability to obtain unequivocal sequence data from the C terminus of the β fragment. We have now determined the precise extent of C-terminal processing by an alternative technique. We accurately established the molecular mass of the β fragment of maize $\alpha\beta$ RIP as 11,020 ($\pm$20) D by electro-spray ionization MS (Chait and Kent, 1992) performed on three different samples of β fragment. These were prepared by reversed-phase HPLC from native $\alpha\beta$ RIP purified from maize kernels. Using this value, in combination with the N-terminal sequence of the β fragment and the deduced amino acid sequence of the pro-RIP, the C terminus of the naturally occurring β fragment can be established as Ala^{288}. This gives a predicted molecular mass for the β fragment of 11,074 D, in close agreement with the measured value of 11,020 D. Thus, 14 residues (1,336 D) are processed from the C terminus of maize pro-RIP during activation. The processed regions of maize pro-RIP are therefore residues 1 to 16, 162 to 186, and 289 to 301.

Expression of Modified Maize RIP Derivatives in *E. coli*

Genetic deletions of maize pro-RIP corresponding to each of the naturally processed regions were made and expressed in *E. coli*. This allowed us to investigate the contribution that each of these regions makes in suppressing the activity of maize RIP. The predicted protein sequences for the modified RIP genes are shown in Figure 6. Because of the initial ambiguity regarding the exact C-terminal residue of the β fragment (Walsh et al., 1991), two C-terminal truncation constructions were made. RIP-ΔNIC has seven amino acids deleted from the C terminus of the pro-RIP, resulting in a C terminus that is six amino acids longer than the naturally processed β fragment. The seven deleted residues include all of the charged amino acids naturally processed from the pro-RIP. RIP-ΔNICSN1 is a derivative with five additional C-terminal residues deleted. In both cases, Ala^{292} was changed to a Gly to generate a unique *Bam*HI restriction site. All of the maize RIP derivatives could be expressed as soluble proteins at high levels in *E. coli*. The recombinant products were purified to homogeneity as established by SDS-PAGE analysis shown in Figure 5A. The purified proteins were tested for activity before and after treatment with subtilisin.

Table I shows the IC_{50} values for protein synthesis inhibition in rabbit reticulocyte lysates that were determined for each recombinant RIP derivative. Polypeptides that contain the 25-amino acid insertion (RIP-ΔN, pro-RIP) have low levels of RIP activity, 2000- to 5000-fold lower than $\alpha\beta$ RIP. The level of activity may be even lower, since any slight activation of pro-RIP by minor amounts of contaminating proteinase will result in apparent inhibition of protein synthesis. For example, an IC_{50} value of 600 nM could be accounted for by the presence of about 0.01% of the protein being in the activated form. In contrast to RIP-ΔN and pro-RIP, those recombinant proteins that have the 25-amino acid insertion genetically removed (RIP-ΔNIC, RIP-ΔNICSN1, RIP-ΔNI, RIP-ΔI), are only 2- to 14-fold less active than native $\alpha\beta$ RIP (IC_{50} values of 0.1–1.0 nM). These data clearly identify the 25-amino acid insertion as the primary inactivating element of maize pro-RIP. The presence of the N- and C-terminal segments in proteins that have had the insertion removed (RIP-ΔI, RIP-ΔNI) results in only slightly lower RIP activity (5- to 7-fold) than the fully activated forms, indicating that these segments are not major inactivating elements in the pro-RIP. Although a RIP with only the C-terminal segment deleted was not

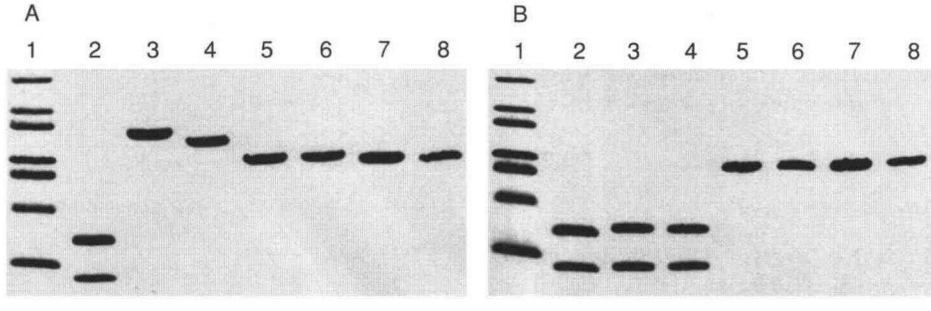

Figure 5. SDS-PAGE of recombinant RIP derivatives before and after treatment with subtilisin Carlsberg. Maize RIP derivatives were expressed in *E. coli* and purified and 0.5 μg were electrophoresed in Phast Gel Homogeneous 20 gels using a Pharmacia PhastSystem. Gels were stained with Coomassie blue. A, Untreated maize RIPs; B, RIPs after treatment with subtilisin Carlsberg as described in "Materials and Methods." Lanes 1, Molecular mass standards (from the top: 66, 45, 36, 29, 24, 20, and 14 kD); lanes 2, maize $\alpha\beta$ RIP purified from maize kernels; lanes 3, recombinant maize pro-RIP; lanes 4, RIP-ΔN; lanes 5, RIP-ΔI; lanes 6, RIP-ΔNI; lanes 7, RIP-ΔNIC; lanes 8, RIP-ΔNICSN1.

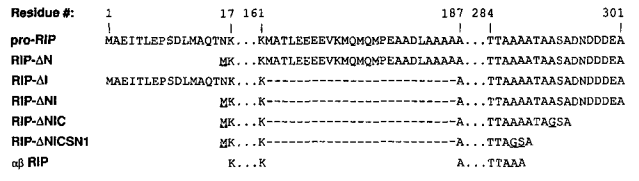

Figure 6. Amino acid sequence differences in maize RIP constructions expressed in *E. coli*. The sequence alignments show the differences between various RIP constructions expressed in *E. coli*. Amino acids that have been changed from the original pro-RIP sequence are underlined. Residue numbers are based on the pro-RIP sequence as previously described (Walsh et al., 1991). Dashed lines denote deleted residues with the presence of a contiguous polypeptide chain. RIP-ΔN has the N-terminal leader deleted, RIP-ΔI has the insertion region deleted, RIP-ΔNI has deletions of both the N-terminal leader and insertion, RIP-ΔNIC has the leader, insertion, and a portion of the C-terminal extension deleted, RIP-ΔNICSN1 is equivalent to RIP-ΔNIC but with an additional C-terminal deletion.

constructed, the relative effect of the C-terminal segment on activity can be inferred by comparing the activities of RIP-ΔI and RIP-ΔNI before and after protease treatment.

The difference in specific activity between the pro-RIP and the most active recombinant form (RIP-ΔNIC) represents a 4,200-fold increase in activity. Those derivatives that have had the insertion deleted represent new forms of maize RIP in which the α and β polypeptides are covalently fused to generate a single polypeptide that retains RIP activity. The specific activity of the most highly modified protein, RIP-ΔNIC, is only marginally less than that of the native αβ form of maize RIP. The potent activity of these fused polypeptides also demonstrates that removal of the insertion is the critical activating factor. Nicking of the polypeptide chain to create separate α and β fragments is not required for activation.

The recombinant RIPs and pro-RIP were treated with the protease subtilisin Carlsberg to investigate proteolytic activation of these forms of maize RIP. In each case processing resulted in higher RIP activity, and the IC_{50} values after protease treatment were approximately equivalent (0.05–0.16 nM; Table I). RIP-ΔNIC activity was unaffected by subtilisin Carlsberg as expected because almost all of the processed regions had been genetically deleted. SDS-PAGE of the RIP derivatives after protease treatment shows that the pro-RIP and RIP-ΔN were processed into two-chain forms of the RIP with α and β polypeptides marginally larger than those of the native form. Cleavage into the two-chain form is a result of proteolytic excision of the internal insertion. Interestingly, polypeptides in which the insertion was deleted genetically and thus were fusions of the α and β fragments (RIP-ΔI, RIP-ΔNI, RIP-ΔNIC) were not susceptible to proteolytic cleavage at the fusion site. Thus, the genetic fusion of the α and β fragments eliminated sensitivity to proteolysis around the internal prosegment region.

DISCUSSION

Maize RIP is the only known RIP that is synthesized as a precursor that undergoes proteolytic processing to a distinctive two-fragment αβ form. This prompted us to survey several related species for proteins with analogous properties. By immunoblot analysis of seed extracts we have found that pro-RIP (or b-32) homologs are not unique to maize but are found in other members of the Panicoideae. The most distant relative of maize that contained cross-reactive pro- and αβ forms of RIP was sorghum. It will be of interest to ascertain whether pro-/αβ RIPs are associated exclusively with Panicoideae or are also found in other plant species. Such a screening project may require alternatives to the conventional technique of monitoring RIP activity in crude extracts, since inactive pro-forms will be overlooked. Sequence analysis of other Panicoideae-type RIPs will provide useful insights into the molecular evolution of both the Panicoideae and RIPs in general.

The question of why these Panicoideae RIPs are expressed as a precursor form is intriguing. Maize RIP is located in the cytoplasm (DiFonzo et al., 1986), in contrast to the more prevalent secreted forms of type-1 RIPs, e.g. trichosanthin (Chow et al., 1990), dianthin (Legname et al., 1991), saporin (Fordham-Skelton et al., 1991), *Mirabilis* antiviral protein (Kataoka et al., 1991), α-momorcharin (Ho et al., 1991), gelonin (Nolan et al., 1993), and pokeweed antiviral protein (Lin et al., 1991). Secretion of these RIPs may provide a mechanism to protect the plant's own ribosomes from the potent deleterious enzymatic action of the RIP. If

Table I. *IC_{50} values for the inhibition of protein synthesis by recombinant maize RIP derivatives before and after treatment with subtilisin Carlsberg*

RIP Derivative	Molecular Mass[a]	Calculated pI[a]	IC_{50}	IC_{50} after Treatment with Subtilisin	Fold Increase in Activity after Treatment with Subtilisin
			nM	nM	
proRIP	33,327	5.99	600	0.09	6,700
RIP-ΔN	31,713	7.31	100	0.05	2,000
RIP-ΔI	30,637	8.50	0.98	0.13	7.5
RIP-ΔNI	29,021	9.06	0.69	0.16	4.3
RIP-ΔNIC	28,233	9.50	0.14	0.14	1
RIP-ΔNICSN1	27,848	9.50	0.14	N.D.[b]	N.D.
αβ RIP	27,573	9.50	0.07	0.07	1

[a] Calculated from the deduced amino acid sequence of each derivative. [b] N.D., Not determined.

maize RIP was active against its own ribosomes, then an inactive pro-form would be essential for cytoplasmic accumulation. Previous work examining the activity of maize RIP on maize ribosomes did not distinguish between inactive pro-RIP and activated $\alpha\beta$ forms of the protein (Bass et al., 1992), and therefore did not fully address this question. The data presented here clearly demonstrate that isolated maize ribosomes are quite resistant to both the pro- and activated $\alpha\beta$ forms of maize RIP, whereas they are readily depurinated by a heterologous RIP from pokeweed.

The insensitivity of maize ribosomes to activated RIP is further demonstrated by the fact that we have expressed both maize pro-RIP and RIP-ΔNIC genes under the control of a constitutive cauliflower mosaic virus 35S promoter in stable transgenic maize callus tissues at levels of approximately 0.01% total protein without any apparent deleterious effect. Similar experiments in tobacco differ: we have recovered many transgenic plants stably expressing the inactive pro-RIP, whereas we did not recover transgenic plants expressing the activated RIP-ΔNIC gene (C. Poirier, A. Morgan, T. Hey, and T. Walsh, unpublished results). In vitro aniline release assays showed that ribosomes from tobacco were insensitive to treatment with pro-RIP at concentrations up to 1.5 μM, but were sensitive to equivalent levels of $\alpha\beta$ RIP (data not shown). Although these in vitro and transgenic experiments may not accurately reflect in vivo conditions during RIP accumulation within endosperm cells, it is apparent that an inactive pro-form of RIP is not essential for the protection of maize ribosomes. Consistent with this conclusion is the observation that nonsecreted RIPs accumulate in wheat and barley endosperms without the need for inactive precursors (Leah et al., 1991; Habuka et al., 1993). If the pro-form of maize RIP is not required for protection of the plant's ribosomes, then the role of the acidic pro-segments of maize RIP may not be restricted to modulation of enzyme activity. For example, they may neutralize the highly basic $\alpha\beta$ RIP (pI = 9.5) to facilitate cytoplasmic accumulation, or their proteolysis during germination may release scarce amino acids such as Met, which is 9 mol % of the pro-segments compared with 2 mol % of the $\alpha\beta$ RIP.

It is becoming clear that the specificity and potency of RIPs from different sources vary (Barbieri et al., 1993). Many have broad activity against prokaryotic and eukaryotic ribosomes (e.g. pokeweed antiviral protein, dianthin, saporin, *Mirabilis* antiviral protein), whereas some have no or limited activity against prokaryotic and plant ribosomes (e.g. gelonin, cereal RIPs). Other factors influence the susceptibility of ribosomes to inactivation by certain RIPs, e.g. the requirement for ATP or other cofactors (Coleman and Roberts, 1981; Sperti et al., 1991; Carnicelli et al., 1992; Brigotti et al., 1993), and these may be linked to the specificity of RIPs. The amount and precise timing of RIP accumulation, particularly in seeds, may also be involved in determining the susceptibility of cellular protein synthesis to the action of a RIP in vivo. A unique RIP, JIP60, has recently been described from barley (Reinbothe et al., 1994). It is induced by methyl jasmonate and is reported to have differential activity against host ribosomes, depending on the stress condition of the plant, although the mechanisms involved in the selectivity have not been elucidated. Appreciation of the details of RIP action will be required to interpret results of experiments involving transgenic expression of RIPs.

Maize pro-RIP is converted to the active form by proteolysis, which removes peptide segments from the N and C termini and also from the center of the polypeptide (Walsh et al., 1991). Several other type-1 RIPs undergo processing at either the N or C termini, although none have been reported to have internal processing and none of the processed regions has been shown to inactivate the RIP (Chow et al., 1990; Benatti et al., 1991; Ho et al., 1991; Kataoka et al., 1991, 1992; Legname et al., 1991). N-terminal processing has generally been associated with presequences that specify translocation into the ER, whereas C-terminal processing has been associated with vacuolar targeting (Benatti et al., 1991; Legname et al., 1991). Maize RIP is cytosolic (DiFonzo et al., 1986) and processing is therefore unlikely to be associated with organellar targeting; rather, it appears to be directly related to RIP activity. To understand the contribution of the processed segments on the ability of maize RIP to inhibit protein synthesis, a series of genetic constructions was made that selectively deleted, either separately or in combination, the N-terminal, C-terminal, or internal processed segments. The deletion mutants were then expressed at high levels in *E. coli*, purified, and tested for RIP activity.

Because some RIPs have significant activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a), heterologous expression can be problematic. In the case of *Mirabilis* antiviral protein (a RIP), reasonable levels of expression were obtained only by using a temperature-sensitive expression system and secretion of the protein via the *ompA* signal sequence (Habuka et al., 1990). Recently, however, successful bacterial overexpression of some type-1 RIPs has been reported (Habuka et al., 1993; Nolan et al., 1993). We have found that all forms of maize RIP can be expressed at very high levels in *E. coli* and so appear to have little or no effect on *E. coli* ribosomes.

The recombinant maize pro-RIP produced in *E. coli* is fully functional, since it can be readily converted by treatment with the nonspecific protease subtilisin Carlsberg into an $\alpha\beta$ RIP form that has activity comparable to that of native $\alpha\beta$ RIP purified from maize kernels. The results of our deletion experiments clearly identify the 25-amino acid insertion as the major inactivating element in the pro-RIP. Removal of the insertion accounts for an increase in activity of at least 5000-fold. In contrast, the removal of the N- and C-terminal segments increases activity only slightly (5- to 10-fold) in the absence of the insertion. This is in contrast to the majority of other zymogen forms of enzymes, particularly proteinases, in which removal of the N-terminal propeptide results in activation (Neurath, 1989). The RIP construction with all three processed regions (RIP-ΔNIC) deleted has an activity close to that of the native $\alpha\beta$ form. The α and β portions of the polypeptide chain are fused in this construction, demonstrating that the polypeptide chain of maize RIP does not have to be cleaved to be active. This

fusion protein (RIP-ΔNIC) is also quite stable to proteolysis and is therefore analogous to typical type-1 RIPs from other plant species.

Alignment of the amino acid sequences of maize RIP and ricin A chain indicates that the maize RIP insertion is at a position equivalent to Thr^{156} of ricin A (Walsh et al., 1991). This places the insertion in a surface loop in ricin A allowing access to processing proteases, and at a position not directly associated with the active site cleft of the enzyme. The loop where the insertion occurs connects helices D and E. Helix E runs through the core of the ricin A molecule, the distal end of which contains Glu^{177} and Arg^{180}, which have been strongly implicated in catalysis (Frankel et al., 1990; Katzin et al., 1991). The presence of the insertion may disrupt the conformation of helix E and therefore the position of the active site residues, such that it renders the enzyme catalytically inert. A mutation in ricin A that is not in the active site region has also been shown to significantly reduce the catalytic activity (Gould et al., 1991). However, the interaction of RIPs with ribosomes may not be limited to active site/ribosomal RNA contacts, and the involvement of ribosomal proteins and/or other translation factors may be critical (Sperti et al., 1991; Ippoliti et al., 1992; Brigotti et al., 1993). The various RIP derivatives that we have prepared will be useful in probing these details.

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