Molecular Dissection of Mitogillin Reveals That the Fungal Ribotoxins Are a Family of Natural Genetically Engineered Ribonucleases

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Mitogillin and the related fungal ribotoxins are highly specific ribonucleases which inactivate the ribosome enzymatically by cleaving the 23–28 S RNA of the large ribosomal subunit at a single phosphodiester bond. The site of cleavage occurs between G4325 and A4326 (rat ribosome numbering) which are present in one of the most conserved sequences (the a-sarcin loop) among the large subunit ribosomal RNAs of all living species. Amino acid sequence comparison of ribotoxins and guanyl/purine ribonucleases have identified domains or residues likely involved in ribonucleolytic activity or cleavage specificity. Fifteen deletion mutants (each 4 to 8 amino acid deletions) in motifs of mitogillin showing little amino acid sequence homology with guanyl/purine ribonucleases were constructed by site-directed mutagenesis. Analyses of the purified mutant proteins identified those regions in fungal ribotoxins contributing to ribosome targeting and modulating the catalytic activity of the toxin; some of the identified motifs are homologous to sequences in ribosomal proteins and elongation factors. This mutational study of mitogillin together with the recently published x-ray structure of restrictocin (a close relative of mitogillin) supports the hypothesis that the specific cleavage properties of ribotoxins are the result of natural genetic engineering in which the ribosomal targeting elements of ribosome-associated proteins were inserted into nonessential regions of T1-like ribonucleases.

Mitogillin and the related Aspergillus fungal ribotoxins restrictocin and a-sarcin, are small basic proteins of ~17 kDa (kilodaltons). Mitogillin differs from restrictocin by only 1 amino acid and has 86% amino acid sequence identity with a-sarcin (1–4). The fungal ribotoxins are a family of highly specific ribonucleases which inactivate the ribosome by cleavage of the 23–28 S RNA of the large ribosomal subunit (5) at a single phosphodiester bond. The site of cleavage occurs between G4325 and A4326 (rat ribosome numbering) in a 14-base sequence (the a-sarcin loop) found in the large subunit ribosomal RNAs (rRNAs) of all living species. This single cleavage completely abolishes the capability of ribosomes to carry out protein synthesis (6–8) by inhibiting elongation factor 1-dependent binding of aminoaeyl-tRNA and GTP-dependent binding of elongation factor 2 to ribosomes. Mitogillin-like ribotoxins are among the most potent inhibitors of translation so far identified and studies have been carried out to investigate their potential as anti-tumor agents or components of immunotoxins (9–14).

The recognition elements of the structure of the a-sarcin loop in 28 S rRNA have been studied extensively (15, 16) and G4319 was proposed to be the identity element for a-sarcin (17). However, very little is known about the ribosome-targeting elements of the protein toxins. Mitogillin and related ribotoxins are known to share amino acid sequence similarity with T1-like ribonucleases (8) and a-sarcin has been shown to behave as a cyclizing ribonuclease like many other ribonucleases (18), but their property to interact specifically with the ribosome and cause a single ribonucleolytic cleavage in the large subunit RNA is unique. Previous studies indicated that the similarities and differences detected in amino acid sequence comparison of ribotoxins and a large family of other guanyl/purine ribonucleases may represent domains or residues key to ribonucleolytic activity and specificity (19, 20). The presence of these "extra" protein domains (some of which are similar to sequences in ribosome-associated proteins) in fungal ribotoxins led to the hypothesis that fungal ribotoxins are a family of naturally engineered toxins with ribosomal targeting elements acquired from different ribosome-associated proteins (19). The prediction that the fungal ribotoxins are T1-like ribonucleases with additional protein domains extended from the catalytic core of the RNase is confirmed by the crystal structure of restrictocin determined by x-ray analysis (21) and the three-dimensional structure of a-sarcin in solution determined by NMR (22). These structural analyses also support the proposal that the ribosomal protein-like region Lys106-Lys113 is the major ribosomal recognition element in mitogillin (19). In the present study we report the characterization and properties of 15 deletion mutants (4 to 8 amino acid deletions) in motifs of mitogillin having little amino acid sequence homology with guanyl/purine ribonucleases and the identification of elements in mitogillin (and related ribotoxins) contributing to its specific cleavage of ribosomes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—An Escherichia coli host (derivative of W3110) and the plasmid pING3522 were used for the production of mitogillin and mutant mitogillin proteins. pING3522 is an inducible secretion vector with the expression of the inserted gene under the control of the Salmonella typhimurium araB promoter; the secretion of recombinant proteins through the cytoplasmic membrane is directed by the Erucina carotovora petB leader sequence (14).

Design and Construction of Deletion Mutants—Polymerase chain reaction (PCR)-mediated site-directed mutagenesis was employed to construct deletion mutants in three regions (Gln8-Thr43, Asp56-Lys88, and Lys106-Lys113) of mitogillin (Fig. 1A). Nucleotide sequences of oligonucleotides used to construct mutants are shown in Table I. PCR-
mediated site-specific mutagenesis is described in Fig. 1B. RK-01 (forward "universal" primer) was mixed with a reverse mutagenic oligo and RK-02 (reverse universal primer) was mixed with a forward mutagenic oligo. PCR was carried out directly on single *E. coli* colonies (containing pING3522 plasmid) in two separate 100-μl reactions (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl₂, 100 pmol of each primer oligonucleotide, and 200 μM of each dNTP). The tubes were heated at 94°C for 5 min before the addition of 2.5 units of *Taq* polymerase, overlaid with paraffin oil and 30 cycles of PCR carried out (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min). The PCR products were electrophoresed on a 1% agarose gel and purified using QIAquick gel extraction kit (QIAGEN). Purified PCR products were mixed and the volume was brought to 100 μl with 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl₂, 200 μM of each dNTP, and 2.5 units of *Taq* polymerase, overlaid with paraffin oil and reincubated at 94°C for 1 min, 55°C for 2 min, and 72°C for 10 min to achieve the 3' extension of the mixed PCR products. PCR amplification of the mutated mitogillin gene was then accomplished by adding 100 pmol of each of oligomers RK-01 and RK-02, and 2.5 units of *Taq* polymerase, overlaid with paraffin oil and reincubated at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, for 30 cycles. The final PCR product was purified by electrophoresis followed by QIAquick extraction. The purified fragment was ligated to *PstI*-HindIII-digested pING3522 and transformed into *E. coli*. The nucleotide sequence of each mutant was verified. Production of mutant mitogillin proteins was detected by SDS-PAGE (0.1% SDS, 15% polyacrylamide) of the induced culture supernatants followed by Western blotting using rabbit antiserum raised against mitogillin.

**Production and Purification of Mutant Mitogillins—** *E. coli* harboring pING3522-derived plasmids containing the mutated mitogillin gene were grown at 37°C to *A*ₚₒₒ₋ₒ = 0.4 in Tryptone broth (containing 10 μg of tetracycline/ml) induced by the addition of 0.1% l-arabinose (Sigma) and grown for 18 h. Secreted proteins were purified by cation exchange chromatography followed by size-exclusion chromatography as described previously (20) except that 50 mM sodium phosphate buffer, pH 7.2, was used for cation exchange chromatography.

**Assay for Nonspecific Ribonucleolytic Activity—** Ribonucleolytic activity of ribonuclease T1, mitogillin, and mutant mitogillins against poly(I)-homopolymer (Sigma) was examined by methods described previously (20). 200 μM poly(I) homopolymer substrate was used in all cleavage assays. In initial rate experiments, 50 μM of each protein was used and the extent of poly(I) homopolymers hydrolysis was less than 10% of the total and MicroCon-100 concentrators (molecular cut-off = 300 nucleotides for single-stranded RNA; purchased from Amicon) were used to separate cleavage products from poly(I) homopolymers. Ribonucleolytic activity against MS-2 phage RNA was determined by incubating RNA (400 ng) with 300 μM purified protein in 10 μl of reaction buffer (15 mM Tris-Cl, pH 7.6, 15 mM NaCl, 50 mM KCl, 2.5 mM EDTA). The reaction was incubated at 37°C for 30 min, stopped by the addition of 1.0 μl of 5% SDS, and separated by electrophoresis (1.2% agarose) as described previously (19); the gel was stained with ethidium bromide to visualize the degradation of the RNA substrate. An activity staining assay (xyloglucan electrophoresis) described by Blank et al. (23) was employed to monitor the presence of contaminating ribonucleases. Briefly, SDS-PAGE of 150 ng of each of the mutant mitogillins was performed under nonreducing condition on a 0.1% SDS, 15% polyacrylamide gel containing 3.0 mg of poly(I) substrate. The gel was treated as described by Blank et al. (20) and incubated in 100 mM Tris-Cl, pH 7.4, at 37°C for 1 h, stained with 0.2% toluidine blue for 10 min at room temperature and washed extensively with distilled water for 1 h. RNAase activity was indicated by the appearance of a clear zone on a dark blue background.

**Specific Cleavage of Rabbit Ribosomes and Synthetic a-Sarcin Loop RNA—** Specific cleavage of eukaryotic ribosomes was done in reaction mixtures with 10 μl of untreated rabbit reticulocyte lysate (Promega), 600 μM toxin protein, 15 mM Tris-Cl, pH 7.6, 15 mM NaCl, 50 mM KCl, and 2.5 mM EDTA, incubated at 37°C for 15 min. Extracted RNA was separated by electrophoresis (1.5% agarose) and RNA species visualized by staining with ethidium bromide. The specific ribonucleolytic activity of the toxin derivatives was followed by the appearance of the a-fragment, a -400 nucleotide RNA species on the gel. A RNA oligonucleotide (a 35-mer) that mimics the a-sarcin domain of the eukaryotic large
Oligonucleotides used for site-directed mutagenesis of the mitogillin gene

| Primer     | Site of mutation | Sequence                                                                 |
|------------|------------------|--------------------------------------------------------------------------|
| RK-01      | Universal, forward | 5’-GCCAATCTCTACTGTTTCTCCAT-3’                                           |
| RK-02      | Universal, reverse  | 5’-GCCGTTAAGATTACTGTTGATA-3’                                            |
| RK-05      | Δlys106,Lys113, forward | 5’-CCAGTGGCATGACTGACAAATGATGTA TGCCACGAGCAG-3’                       |
| RK-06      | Δlys106,Lys113, reverse | 5’-GGTCTGGACACATTTCCTCCATGACTGTTGATA-3’                                 |
| Δ1.1F      | ΔAsp96-Lys98, forward  | 5’-CTGTGTTGCGCTTCCATGACTGTTGATA-3’                                      |
| Δ1.2F      | ΔGly92-Ile96, reverse | 5’-ACAGTTGGATAGGCGAACAGGGTGACATCAATTTGAGG-3’                           |
| Δ1.3F      | Δlys92-Ile96, forward  | 5’-CGTGGTTGCGCTTCCATGACTGTTGATA-3’                                      |
| Δ1.4F      | Δlys92-Ala93, forward  | 5’-CAAGGTTGACCTGCGACTTAGGAGCCACATTAGG-3’                               |
| Δ1.4R      | Δlys92-Ala93, reverse | 5’-CAATTTGCGCTGCTGCTTGAGGACTTAGGAGCCACATTAGG-3’                       |
| Δ1.5F      | ΔArg77-Gln83, forward  | 5’-TTGTCGTTGCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.5R      | ΔArg77-Gln83, reverse | 5’-TGTACGGTTGCGCTTCCATGACTGTTGATA-3’                                   |
| Δ1.6F      | ΔAsn54-lys56, forward  | 5’-CCACCTAGCTCCATGTCGCGACTTAGGAGCCACATTAGG-3’                         |
| Δ1.6R      | ΔAsn54-lys56, reverse | 5’-CCATTTGCGCTGCTGCTTGAGGACTTAGGAGCCACATTAGG-3’                       |
| Δ1.7F      | ΔGln91-Asn93, forward  | 5’-CCACATGTCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.7R      | ΔGln91-Asn93, reverse | 5’-TGTCGTTGCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.8F      | Δlys91-lys93, forward  | 5’-CCACATGTCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.8R      | Δlys91-lys93, reverse | 5’-TGTACGGTTGCGCTTCCATGACTGTTGATA-3’                                   |
| Δ1.9F      | Δlys95, forward  | 5’-CCACATGTCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.9R      | Δlys95, reverse  | 5’-TGTACGGTTGCGCTTCCATGACTGTTGATA-3’                                   |
| Δ1.10F     | Δlys102, forward  | 5’-CCACATGTCGCTTCCATGACTGTTGATA-3’                                    |
| Δ1.10R     | Δlys102, reverse  | 5’-TGTACGGTTGCGCTTCCATGACTGTTGATA-3’                                   |

Production of mutant mitogillin proteins

| Mutant mitogillin | Site of deletion | Production of protein* |
|-------------------|------------------|-----------------------|
| ΔGln9, Asn11      | Gln9 to Asn11    | –                     |
| ΔLys13,Lys16      | Lys13 to Lys16   | +                     |
| ΔLys16,Asp19      | Lys16 to Aas19   | +                     |
| ΔLys20,Leu23      | Lys20 to Leu23   | +                     |
| ΔArg77 to Ala77   | Arg77 to Ala77   | +                     |
| ΔLys83 to Ile86   | Lys83 to Ile86   | +                     |
| ΔLys89 to Ala73   | Lys89 to Ala73   | +                     |
| ΔArg77 to Gln83   | Arg77 to Gln83   | +                     |
| ΔAsn54 to Lys88   | Asn54 to Lys88   | +                     |
| ΔLys102 to Lys113 | Lys102 to Lys113 | +                     |

*+ , production of protein detected; –, production not detected.

Subunit rRNA was prepared with synthetic DNA templates, 77 RNA polymerase, and the four nucleotide triphosphates and purified using denaturing polyacrylamide gel electrophoresis (24, 25). 1.0 µm synthetic RNA 35-mer was combined with 600 nM mitogillin or mutant mitogillin near the ara gene, see Better et al. (14). Cleavage products were separated by denaturing polyacrylamide gel electrophoresis and the gel stained with SYBR-GOLD nucleic acid stain (Molecular Probes).

RESULTS

Mutant Construction and Protein Purification—Fifteen deletion mutants of mitogillin in regions predicted to be functional elements inserted into a T1 ribonuclease core structure were constructed. The mutant genes were completely sequenced to confirm the nature of the deletions; no other alterations were detected. Production of mutant proteins on growth in liquid culture was detected from 10 clones (Table II) by Western blotting with a rabbit antiserum (data not shown). SDS-PAGE followed by silver staining of the purified proteins confirmed their purity (Fig. 4A).

Nonspecific Ribonuclease Activity—RNase activity of mitogillin and all 10 mutant proteins was detected when poly(I) homopolymers (Fig. 2) or MS-2 phage RNA (Fig. 3) were used as substrates. The comparison between the ribonuclease activities of mutant and wild-type mitogillin using poly(I) homopolymer is shown in Fig. 2; initial rates of reaction are tabulated in Table III. The data indicate that deleting Lys13-Lys16 decreases the RNase activity, while the deletion of Asp56-Lys60, Gly59-Ile62, Lys63-Ile66, or Asn84-Lys88 had little effect as judged by the initial rates of ribonuclease reaction. Results obtained from the zymogram electrophoresis assay (Fig. 4B) eliminate the possibility of the presence of contaminating ribonucleases in the preparations.

Specific Ribonuclease Activity—On treatment of rabbit reticulocyte lysates with mitogillin or the deletion variants, a distinctive α-fractoglycogen band was detected in all samples except the ΔLys9-Lys13 deletion mutant (Fig. 5A). Extensive degradation of 28 S RNA in ribosomes by Lys13-Leu23 deletion mutants suggests that this region in the fungal ribotoxin is involved in modulating the activity and specific recognition of the cleavage site in the ribosome. Results obtained from assays using synthetic α-sarcin loop (a 35-mer) as substrate showed that deletions in regions Lys13-Lys16, Lys16-Asp19, or Lys20-Leu23 gave rise to mitogillin variants with elevated ribonuclease activity.
lytic activity while deletion of Lys\textsuperscript{106}-Lys\textsuperscript{113} created a mutant which fails to recognize and cleave the \(\alpha\)-sarcin loop (Fig. 5B).

**DISCUSSION**

Structural comparison between the three-dimensional structures of restrictocin (99% identity with mitogillin) and T1 ribonuclease reveals strong structural similarities with certain domains in the fungal ribotoxins absent from the T1 ribonucleases; the most obvious differences are the \(\beta\)-loop-1-\(\beta\)-2 region, the loop 3 region, and the loop 4 region of restrictocin (Fig. 6). It has been postulated that these domains are “inserted” elements contributing to the specific targeted, cytotoxicity of this class of proteins; preliminary studies on the loop 4 region indicated that this region indeed is the major ribosome-targeting elements of mitogillin (19). We have generated deletions (4–8 amino acid residues) in those regions of mitogillin proposed to be inserted elements. Deletions were preferred over amino acid substitutions for this analysis in order to identify domains responsible for targeting mitogillin to the ribosome and examine the influence of these inserted regions on the general properties of the fungal ribonuclease. Ten of the 15 deletion mutants produce biologically active proteins using the E. coli expression system. It is interesting to note that the five non-expressible mutant mitogillin proteins have deletions located near or extending into elements such as disulfide bridge-forming cysteine residues, proline residues, or helices, which are likely to be susceptible to host protease activity. The majority of the deletions obtained retain the catalytic activity of the ribotoxin which supports the proposal that these domains have roles in ribotoxin function distinct from the RNase activity.

Deletions in loop 4 and in \(\beta\)-1-loop 1-\(\beta\)-2 regions generate mutants with interesting properties in terms of their ribonucleolytic activities. We have shown that the octapeptide Lys\textsuperscript{106} to Lys\textsuperscript{113} in the loop 4 region has sequence similarity to various ribosome-associated proteins (19) and that the heptapeptide Thr\textsuperscript{14} to Lys\textsuperscript{20} in the \(\beta\)-1-loop 1-\(\beta\)-2 region is strongly related to motifs in various elongation factors (Table IV). Although the significance of these relationships has yet to be examined, it is noteworthy that the \(\Delta\text{Lys}^{106-113}\) mutant (previously termed \(\Delta2\) mutant in Ref. 19) has lost the ability to recognize and cleave the \(\alpha\)-sarcin loop (19) (Fig. 5, A and B, this study), and that the ribonucleolytic activity of the deletion mutants in the \(\beta\)-1-loop 1-\(\beta\)-2 region is greatly elevated (20–30-fold higher than that of wild-type mitogillin, Table III). The \(\alpha\)-sarcin loop/restrictocin docking model of Yang and Moffat (21) suggests that the loop 4 region of mitogillin is close proximity to the 28 S rRNA identity element (G\textsuperscript{3119}) of the fungal ribotoxin; our results support the conclusion that elements in the loop 4 domain interact with the “bulged” G\textsuperscript{3119} in the \(\alpha\)-sarcin loop to promote specific recognition and cleavage of the substrate.

However, the \(\beta\)-1-loop 1-\(\beta\)-2 region of mitogillin is relatively distant from the catalytic center of the nuclease; the role of this
domain in attenuating the catalytic activity of the toxin is intriguing. We propose that this is the result of hydrogen bond formation between amino acid residues in $\beta_1$ and $\beta_2$, and residues in $\beta_6$ in which the catalytic residue His$^{136}$ is situated (Fig. 7). It is possible that the $\beta_1$-loop 1-$\beta_2$ domain attenuates the catalytic activity of the toxin by keeping the catalytic residue His$^{136}$ in a configuration suboptimal for nucleolytic activity through hydrogen bonding with $\beta_6$. Upon binding to the ribosome, the interactions between loop 4 and the $\alpha$-sarcin loop trigger a conformational change in the protein which disrupts the interactions between the $\beta_1$-loop 1-$\beta_2$ domain and $\beta_6$, so positioning the catalytic residue His$^{136}$ in an optimal environment for cleavage of phosphodiester bond of the RNA substrate. Evidence in favor of this interpretation comes from the analysis of a N7A mutant of mitogillin (which presumably eliminates the hydrogen bonding between Asn$^7$ and His$^{136}$) which shows greatly elevated ribonuclease activity (Table III). The detailed biochemical properties of the N7A mutant are currently under investigation. Our interpretation also agrees with the findings of Takeda et al. (27) which suggest that the fungal ribotoxins bind to two sites in the RNA substrate; one through the bulged G$^{4319}$ and the other around the GAGA tetraloop cleavage site. An RNA oligomer substrate with the deleted bulged G is unsusceptible to $\alpha$-sarcin cleavage but becomes a strong noncompetitive inhibitor of the wild-type RNA molecule (with the bulged G), which suggests that the association between the RNA recognition element (bulged G$^{4319}$) and the first substrate-binding site in mitogillin (elements in loop 4 of the protein) may trigger a conformational change in the active site of the ribotoxin which permits the efficient cleavage of the GAGA tetraloop. Shapiro (26) demonstrated that the enzymatic potency and specificity of human angiogenin are partly modulated by hydrogen bonding between two distant amino acid residues (Thr$^{144}$ and Thr$^{205}$) that inability to form the hydrogen bond (made possible by a T80A mutation) increases the ribonuclease activity of angiogenin 11–15-fold (26). This raises the possibility that substrate-specific ribonucleases other than fungal ribotoxins may also modulate their specific activity by similar mechanisms.

Deletions in region Asp$^{56}$ to Lys$^{88}$ (loop 3) of mitogillin do not appear to interfere with the ribonuclease activity or the specificity of mitogillin. The dramatic decrease in RNase activity in Arg$^{77}$-Gln$^{83}$ may be due to the deletion of a catalytically important residue(s) or a change in the structure of the toxin affecting its catalytic activity. The latter interpretation is more likely since the active site of mitogillin does not indicate the involvement of any residues in region Arg$^{77}$-Gln$^{83}$ for catalysis (20). However, these residues being adjacent to Cys$^{75}$ which forms a disulfide bond with Cys$^5$ in mitogillin and deletion of Arg$^{77}$-Gln$^{83}$ would presumably induce a change in the microenvironment of the active site of mitogillin and, as a result, reduce RNA cleavage activity. Based on available information we cannot define further the functions of loop 3 of mitogillin. Yang and Moffat (21) suggest that this region may influence mitogillin/cell surface receptor recognition or translocation of the toxin through the lipid bilayer of the cell; mutants constructed in this region would be good candidates to elucidate the functions of this extra loop of the fungal ribotoxins.

In conclusion, we have described the production and proper-
ties of 10 deletion mutants of mitogillin, predicated on the hypothesis that motifs showing little amino acid sequence homology with guanyl/purine ribonucleases are functional domains inserted into T1-like ribonucleases through natural genetic engineering. Characterization of the enzymatic properties of the mutant proteins suggests that elements in loop 4 region of mitogillin are involved in the specific recognition of the toxin to the ribosome and elements in β1-loop 1-β2 region involved in modulating the catalytic activity of the toxin. Our studies also suggest that loop 3 of mitogillin may be involved in functions other than targeting mitogillin to the ribosome. It is evident that these additional elements, which appear to have been...
engineered naturally into T1-like ribonucleases, are not required for RNase activity but have functions specific to cytotoxic activity. The availability of these mutants will aid in further investigation of the biochemical and/or physiological roles of ribotoxin function. An interesting question is whether it will be possible to insert such targeting motifs into other protein sequences to generate other types of ribosome-specific toxins.

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