Single amino acid substitutions affecting the specificity of the fungal ribotoxin mitogillin

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Received 3 December 1999

Edited by Horst Feldmann

Abstract Mitogillin and related fungal ribotoxins are small basic ribonucleolytic proteins that inhibit protein synthesis by specifically hydrolyzing a single phosphodiester bond in the universally conserved α-sarcin/ricin loop (SRL) of large subunit ribosomal RNAs. It was previously shown that mitogillin is a natural derivative of a T1/U2-like ribonuclease with inserted domains that are involved in target selection and specificity. Site-directed mutagenesis was used to substitute single amino acids in the previously identified functional domains Ala1–Tyr24 (B1-L1-B2 domain) and Lys106–Lys113 (L4 region). Examination of the activities of the mutants in the digestion of polyinosinic acid (a ribonuclease substrate) and specific cleavage of the SRL shows that Asn7Ala and Lys111Gln substitutions lead to altered ribonuclease activity and diminished substrate specificity consistent with the proposed functions of these domains.

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Key words: Mitogillin; Restrictocin; Ribotoxin; Specificity; Targeted toxin; α-Sarcin/ricin loop

1. Introduction

Fungal ribotoxins are small basic proteins of ~17 kDa that are highly specific ribonucleases which inactivate ribosomes by cleavage of the 23–28S rRNA of the large ribosomal subunit at a single phosphodiester bond [1,2]. The site of cleavage occurs between G4325 and A4326 (rat ribosome numbering) in the universally conserved α-sarcin/ricin loop (SRL) in the large subunit ribosomal RNAs of all living organisms. This single covalent modification impairs elongation factor 1 (EF-1)-dependent binding of aminoacyl-tRNA and GTP-dependent binding of elongation factor 2 (EF-2) to ribosomes and thus completely abolishes the ability to carry out protein synthesis [3–5].

Mitogillin and related ribotoxins share considerable amino acid sequence similarity with T1-like ribonucleases [5,6] and it has been shown recently that α-sarcin (and presumably other members of the fungal ribotoxins) behaves as a typical cyclizing ribonuclease [7]. However, unlike RNase T1, which cleaves RNA after every guanine base, the fungal ribotoxins make a single cleavage at a specific site and leave both the 3′ and 5′ cleavage products intact.

Wool and co-workers have shown that G4319 is a crucial identity element for α-sarcin action [8–10]; it was suggested that on binding to the SRL the toxin uses G4319 for orientation and cleaves the RNA at a fixed position from the binding site. The structures of synthetic oligoribonucleotides mimicking the SRL have been characterized by X-ray crystallography and NMR spectroscopy [11–13]. However, the functional aspects of the targeting of the ribotoxins to ribosomes is not well characterized, although studies have identified certain mitogillin residues that are required for the ribonucleolytic activity [14] and specificity of this ribotoxin [15,16]. It was proposed that the ribosomal protein-like lysine-rich loop 4 region of mitogillin (and presumably the corresponding regions in all other fungal ribotoxins) is the major ribosome targeting element and that the B1-L1-B2 domain is involved in substrate selection [16].

To investigate further the involvement of the B1-L1-B2 domain in specific substrate selection, we have mutated the asparagine residue at position 7 to alanine which eliminates the hydrogen bonding between Asn7 and the B6-L6-B7 domain (residues Gly132–Cys147, in which the catalytically essential residue His136 is situated). The Asn7Ala mutant mitogillin has elevated ribonucleolytic activity but has reduced selectivity with regard to the specific cleavage site. In addition, alteration of Lys111 in the L4 region (Lys106–Lys113) of mitogillin markedly reduced the specific cleavage of SRL but increased non-specific RNase activity, establishing the role of this residue in substrate recognition.

2. Materials and methods

The use of *Escherichia coli* W3110 for mitogillin production and the manipulation of plasmid pING3522, an L-arabinose-inducible secretion vector with the expression of the inserted gene under the control of the *Salmonella typhimurium ara*B promoter, was described previously [14,17]. PCR-based mutagenesis, mutant mitogillin isolation and purification, and ribonucleolytic analysis were as described earlier [14,16].

3. Results

The Asn7Ala mutant of mitogillin was constructed based on a mutational study of the B1-L1-B2 domain of mitogillin [16] and the analysis of the crystal structure of restrictocin [18]. The Lys111Gln in the L4 region of the ribotoxin was identified by PCR mutagenesis. The mutant proteins were overproduced in *E. coli* and purified to homogeneity (Fig. 1A).

Zymogram electrophoresis/activity staining was used to verify that no contaminating ribonuclease co-purified with the mutant mitogillins. From the zymogram gel (Fig. 1B) it can be seen that the Lys111Gln mutant has slightly higher activity than native mitogillin whereas the Asn7Ala mutant is significantly more active in degrading poly(I) homopolymer (judging from the size and the intensity of the clearing zone). Quanti-

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PII: S0014-5793(99)01753-6

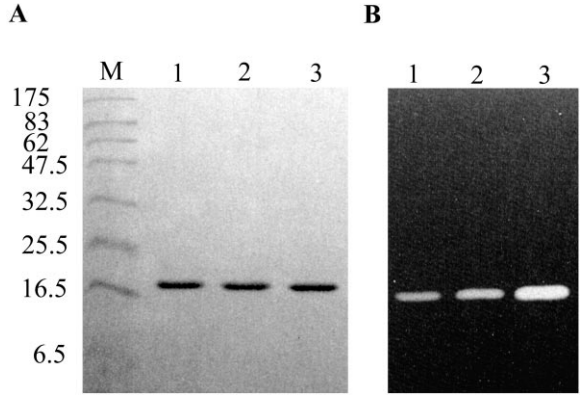

Fig. 1. SDS-PAGE of mutant mitogillins and their RNase activity detected by zymogram electrophoresis. A: SDS-PAGE of mutant mitogillins. 1 μg of each mutant protein was run on a 0.1% SDS/15% polyacrylamide gel under non-reducing conditions. Presence of protein was detected by silver staining. B: Zymogram electrophoresis. SDS-PAGE of 150 ng of each of the mutant mitogillin proteins was performed under non-reducing conditions on a 0.1% SDS/15% polyacrylamide gel containing 0.3 mg poly(I) (Sigma). RNase activity was indicated by the appearance of a clearing band on staining with toluidine blue. Lane 1, mitogillin; lane 2, Lys111Gln mutant; lane 3, Asn7Ala mutant. Shown also are the positions of molecular markers in kDa.

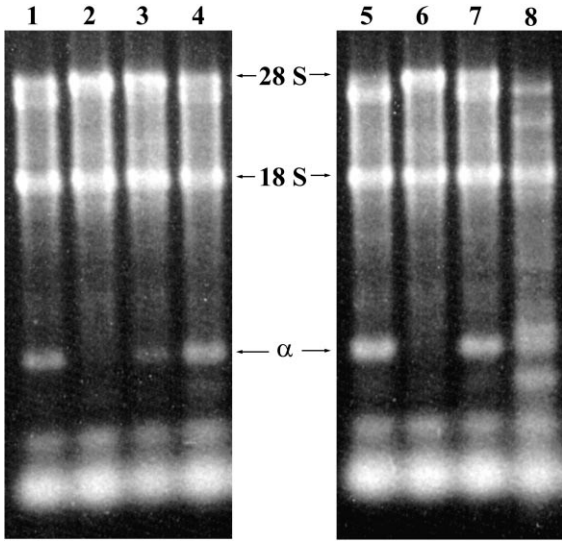

Fig. 2. Specific ribonucleolytic activity (in vitro α-fragment release from rabbit ribosomes) of mitogillin and its variants. 600 nM mitogillin or its variants was used for the assay. Positions of 28S rRNA (28S), 18S rRNA (18S), and α-fragment (α) are indicated. Samples in lanes 1–4 were incubated at 37°C for 0.5 min. Samples in lanes 5–8 were incubated at 37°C for 15 min. Lanes 1 and 5, mitogillin; lanes 2 and 6, no protein; lanes 3 and 7, Lys111Gln mutant; lanes 4 and 8, Asn7Ala mutant.

tative RNase determinations by measuring the degradation products of poly(I) smaller than 60 nucleotides in length confirmed the elevated RNase activity of the mutants (Table 1).

The specific activities of mitogillin and the mutants were compared by their ability to cleave rabbit ribosomes or synthetic 35-mer SRL, which indicated that the Lys111Gln mutant has decreased specific activity (judging from the relative amounts of the α-fragment band released after 30 s incubation, Fig. 2) compared with that of native mitogillin. Increasing the incubation time shows that the Asn7Ala variant cleaves rRNA at many locations in contrast to the native mitogillin which cleaves only the SRL, leaving other RNA species intact (Fig. 2). Results from synthetic SRL cleavage assays show that in contrast to the specific cleavage products of mitogillin, the Asn7Ala mutant cleaves non-specifically and the Lys111Gln mutant fails to produce detectable cleavage products (Fig. 3A). Even at higher concentrations (600 nM), mitogillin maintains specificity, but the Asn7Ala mutant digests the substrate completely and the Lys111Gln mutant gives multiple cleavage products (Fig. 3B,C).

4. Discussion

Previous studies [15,16] indicated that deletion of residues Lys106–Lys113 in the L4 region, a lysine-rich loop of mitogillin that resembles a region of ribosomal protein S12, abolishes the ability of mitogillin to recognize and specifically cleave the SRL, suggesting that the L4 region is the major recognition element of mitogillin. Amino acid substitutions indicate that Lys111 is important for the substrate specificity of mitogillin; the purified Lys111Gln mutant protein exhibits slightly increased non-specific RNase activity (about two-fold increase compared with the native mitogillin) with reduced

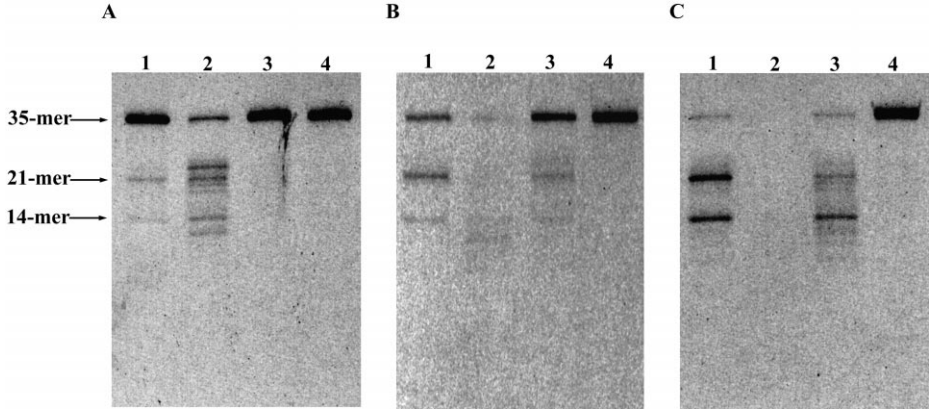

Fig. 3. Synthetic SRL cleavage assay. A: 1.0 μM of synthetic SRL RNA was incubated with 60 nM of mitogillin or its variants at 37°C for 15 min. B: 1.0 μM of SRL was incubated with 600 nM of mitogillin or its variants at 37°C for 0.5 min. C: 1.0 μM of SRL was incubated with 600 nM of mitogillin or its variants at 37°C for 15 min. Positions of 35-mer, 21-mer, and 14-mer are indicated.

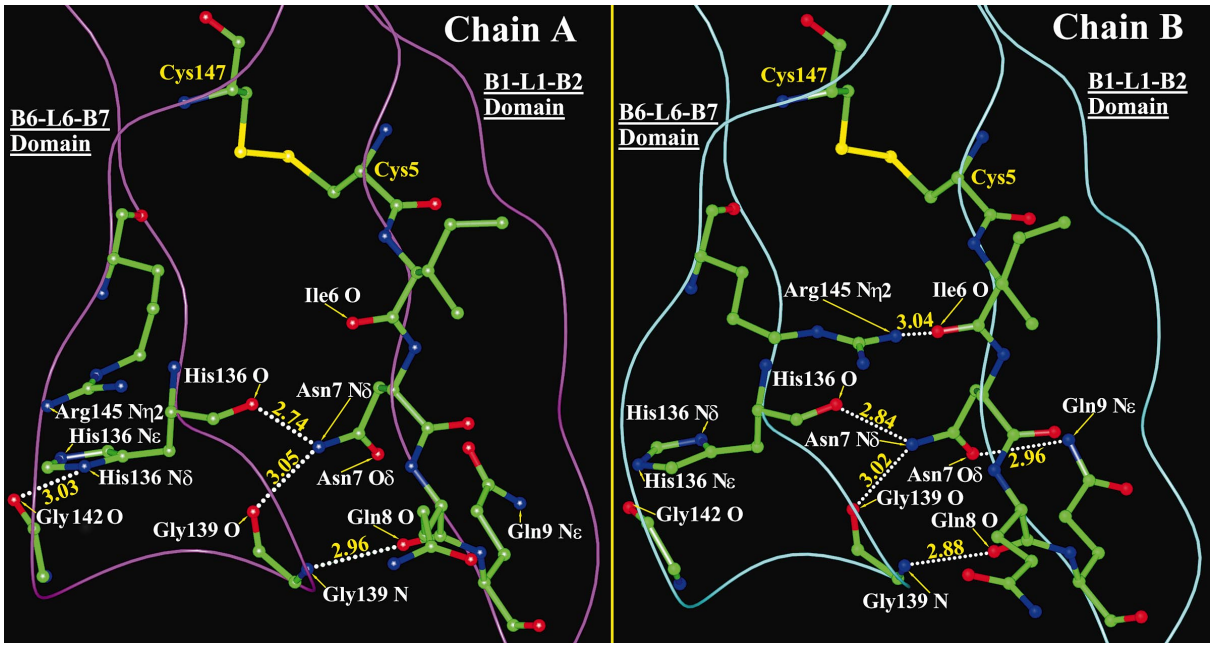

Fig. 4. Comparison of hydrogen bonding between B1-L1-B2 and B6-L6-B7 domains of chain A and chain B of restrictocin. Left panel: Chain A; noted is the hydrogen bond formed between Gly142 O and His136 Nδ. Right panel: Chain B; noted is the flipping of His136 Nδ to a position unfavorable for hydrogen bonding with Gly142 O, and the formation of new hydrogen bonds between Arg145 Nη2 and Ile6 O, and between Asn7 Oδ and Gln9 Nε. Loop 1 residues 11–16 are fully exposed to solvent and are consequently missing in the atomic model. Hydrogen bonds are denoted by dotted lines and bond distances indicated in Å. Shown also is the disulfide bridge (in yellow) formed by residues Cys5 and Cys147. Amino acid residues not relevant in bond formation are omitted for clarity. The coordinates of restrictocin are taken from PDB files 1AQZ [18].

ability to cleave the ribosome specifically at the SRL. We suggest that the lysine residue at position 111 in mitogillin increases recognition of the SRL, but suppresses the toxin's interaction with other RNA substrates, reducing non-specific RNase activity.

We showed earlier [16] that disruption of a region near the N-terminus of mitogillin (the B1-L1-B2 domain) by site-specific deletions resulted in variants with markedly increased ribonucleolytic activity against various substrates including the rabbit ribosome. In the structure of restrictocin [18] hydrogen bonds are observed between amino acid residues in regions B1 and B6 (in which the catalytic residue His136 is situated). Notably, Asn7 Nδ in the B1-L1-B2 domain is hydrogen-bonded to His136 O and to Gly139 O in the B6-L6-B6 domain (Fig. 4). Based on deletion studies [16], the L1-B2-L2 domain of fungal ribotoxins was proposed to modulate the catalytic activity of the protein by formation/disruption of hydrogen bonds with the B6-L6-B7 domain, and that Asn7 plays an important role in this process. An Asn7Ala replacement (the replacement of Asn by Ala eliminates hydrogen bonding between this residue with His136 and Gly139) was prepared and elimination of these pairs of hydrogen bonds between the B1-L1-B2 domain and the B6-L6-B7 domain markedly increases the ribonuclease activity of mitogillin with the selection of the SRL cleavage site becoming less stringent. This suggests that the B1-L1-B2 domain (which is absent in the T1 ribonuclease family) in fungal ribotoxins may enhance the specificity of the enzyme by maintaining the toxin in an 'attenuated' form and the ribotoxin thus becomes less active against other RNA substrates. Upon binding to a suitable substrate (in this case the SRL), a conformational change in the enzyme–substrate complex is induced that involves the breaking/forming of hydrogen bonds and possibly the rearrangement of the structure of the active site. This conformational change poises the His136 residue (in the B6-L6-B7 domain) in a catalytically favorable orientation and cleavage of the substrate proceeds; the enzyme flips back to its attenuated form once the enzyme–product complex is disassociated. It is possible that there is an equilibrium between the two populations (attenuated and active forms) of the toxin; the Asn7Ala mutation shifts the equilibrium in favor of the active form of the enzyme at the expense of reducing substrate specificity.

Table 1
Comparison of the ribonucleolytic activity of mitogillin and variants (Lys111Gln and Asn7Ala) against poly(I)

Protein	Poly(I) degradation (%)
Mitogillin	1.5 ± 0.26
Lys111Gln	3.1 ± 0.22
Asn7Ala	38.8 ± 0.59

200 μM of poly(I) was treated with 200 nM of mitogillin or variants at 37°C for 10 min. Results are expressed as percentage of poly(I) degraded (mean ± S.D. for four independent experiments).

The crystal structure of restrictocin has two molecules in the asymmetric unit (chain A and chain B, PDB files 1AQZ); the overall conformations of the molecules are closely similar but significant differences are observed in the orientation of the His136 residue and the pattern of hydrogen bonds formed between the B1-L1-B2 domain and the B6-L6-B7 domain of restrictocin (Fig. 4). One of the most noticeable differences is that His136 Nε in chain A is hydrogen-bonded to the O3 atom of PO_4 (not shown) while the His136 imidazole ring in chain B is flipped to a different orientation and cannot form an equivalent hydrogen bond with the bound phosphate

anion. This suggests that the Asn7 residue is involved in the rearrangement of the hydrogen bonding network affecting the orientation of His136 and the conformation of the active site residues. Could these restrictocin conformations represent naturally occurring interchangeable forms of the fungal ribotoxins? This question remains to be answered.

These studies show that both the L4 region and the B1-L1-B2 domain of mitogillin (and presumably other members of the fungal ribotoxin family) contribute to the exquisite specificity of the toxin for the SRL. The Lys111 residue in L4 may play a key role in recognition/binding to the SRL, with the Asn7 residue in the B1-L1-B2 domain modulating the catalytic activity of the toxin. This dual control mechanism for specificity (which may be concerted or sequential) explains why the fungal ribotoxins are poor general ribonucleases but are extremely specific and potent inhibitors of protein synthesis.

Acknowledgements: We thank Drs. S. Mosimann and N. Strynadka for helpful discussions. This work was supported by Natural Sciences and Engineering Research Council of Canada.

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