Eur. J. Biochem. **267**, 1777–1783 (2000) © FEBS 2000

Localization of the catalytic activity in restrictocin molecule by deletion mutagenesis

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Restrictocin, produced by the fungus *Aspergillus restrictus*, is a highly specific ribonucleolytic toxin which cleaves a single phosphodiester bond between G4325 and A4326 in the 28S rRNA. It is a nonglycosylated, single-chain, basic protein of 149 amino acids. The putative catalytic site of restrictocin includes Tyr47, His49, Glu95, Arg120 and His136. To map the catalytic activity in the restrictocin molecule, and to study the role of N- and C-terminus in its activity, we have systematically deleted amino-acid residues from both the termini. Three N-terminal deletions removing 8, 15 and 30 amino acids, and three C-terminal deletions lacking 4, 6, and 11 amino acids were constructed. The deletion mutants were expressed in *Escherichia coli*, purified to homogeneity and functionally characterized. Removal of eight N-terminal or four C-terminal amino acids rendered restrictocin partially inactive, whereas any further deletions from either end resulted in the complete inactivation of the toxin. The study demonstrates that intact N- and C-termini are required for the optimum functional activity of restrictocin.

Keywords: ribotoxin; ribonuclease; toxin; protein engineering; ribosome-inactivating proteins.

Restrictocin is a ribosome-inactivating protein toxin, produced by the fungus *Aspergillus restrictus*, which is grouped under a class of protein toxins termed ribotoxins [1]. α-Sarcin and mitogillin are two other members of the ribotoxin family that are produced by different strains of *Aspergillus* and restriction and mitogillin share 85% homology with α-sarcin [2]. The cytotoxic activity of ribotoxins is due to their ability to inhibit eukaryotic protein synthesis, as a result of their ribonucleolytic effect on the 28S ribosomal RNA [3,4]. The ribotoxins act as highly specific ribonucleases cleaving a single phosphodiester bond between G4325 and A4326 in the 28S rRNA [3,5–7], in a 12 nucleotide purine-rich stretch, which is conserved in large subunit ribosomal RNA of almost all living species [8]. The cleavage of the 28S rRNA releases a 400 nucleotide long fragment from the 3′ end, known as the α-fragment [5]. Once the 28S rRNA is cleaved by these toxins, EF-1 dependent binding of aminoacyl tRNA and the GTP-dependent binding of EF-2 to the ribosomal site is impaired [2,3,9,10] which causes the total collapse of the translation machinery leading to the death of the cell. The ribotoxins have great potential to be utilized in the construction of immunotoxins for targeted therapy of various diseases. Immunoconjugates and recombinant chimeric toxin have been made with restrictocin that have been found to be active on various tumor cell lines [11–15].

Restrictocin is a nonglycosylated, single-chain, basic protein of 149 amino acids [1]. It has four cysteine residues at positions 5, 75, 131 and 147 that are involved in the formation of two disulfide bridges between residues 5–147 and 75–131 [1]. We have recently reported that none of the four cysteine residues is directly involved in restrictocin catalysis, and presence of any one of the two disulfide bonds is absolutely essential and sufficient to maintain the enzymatically active conformation of restrictocin, however, for maintaining the unique stability displayed by the native toxin, both disulfide bonds are required [16].

The structural core in restrictocin closely resembles that in RNase T1, a guanine-specific ribonuclease produced by *Aspergillus oryzae*, suggesting that they have similar catalytic mechanisms, however, restrictocin contains long connecting loops which have been proposed to be important for the unique substrate specificity of this protein [17]. The docking model of the NMR structure of a 29-mer RNA substrate analog with restrictocin crystal structure has indicated that structural elements involved in RNA substrate binding include a landing platform comprising of loops L2 and a lysine rich loop L4, and a positively charged ridge in loop domain L3 [17]. It has been proposed that specific interaction between G4319 and the lysine-rich loop may be responsible for the substrate specificity of restrictocin [17]. Deletion of K106-K113 in L4 of mitogillin has been shown to result in the loss of substrate specificity [18]. Kao and Davies [19] on the basis of deletion mutagenesis in mitogillin have postulated that the specificity of ribotoxins is 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. Based on structural and sequence homology studies Tyr47, His49, Glu95, Phe96, Pro97, Arg120 and His136 have been proposed to be in the active site of restrictocin [17,20]. We have shown that His49 is involved in the target recognition activity of restrictocin [21]. Earlier, mutation of His136 in restrictocin and His137 in α-sarcin have been shown to completely inactivate the toxins [22,23], whereas mutation of Glu95 in restrictocin rendered the toxin partially inactive [22]. Kao *et al*. [24] have carried out chemical mutagenesis in mitogillin, and shown that His49, Glu95, Arg120 and His136 are important for catalysis by the toxin.

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(Received 30 July 1999, revised 15 November 1999, accepted 24 January 2000)

In the construction of chimeric toxins, a small toxin is desirable as it provides better folding of the chimeric molecule and results in their better penetration into the target cells, and lower antigenicity in the foreign host. To obtain the smallest biologically active derivative of a protein, it is important to map and localize the functional activity in the molecule. In the current study, an attempt has been made to localize region(s)/domain(s) in restrictocin essential for its catalytic activity. In order to study the involvement of N- and C-termini in restrictocin catalysis, systematic deletions of amino-acid residues from both the termini were carried out. Three N-terminal deletions removing 8, 15 and 30 amino acids and three deletion mutants from the C-terminus lacking 4, 6, and 11 amino acids were constructed. The deletion mutants were expressed and purified from *E. coli* and functionally characterized. The study demonstrates that intact N- and C-termini are required for the optimum functional activity of restrictocin.

EXPERIMENTAL PROCEDURES

Construction of deletion mutants of restrictocin

The plasmid pRest, containing restrictocin gene in a T7 promoter-based *E. coli* expression vector was used as the template to amplify the desired region of restrictocin by PCR while inserting initiation codon and termination codon at appropriate locations to construct the deletion mutants [25]. For deleting 8, 15, and 30 amino acids from N-terminus, fragment of restrictocin gene was amplified using 5′ primers from the appropriate region containing *Nde*I restriction site and ATG initiation codon placed upstream of the codons for amino-acid residues 9, 16, and 31, respectively, by PCR [26]. The 5′ primers SD-8, SD-15, and SD-30 were used to construct the deletion mutants Δ1–8, Δ1–15 and Δ1–30, respectively (Table 1). The common 3′ primer, JRS-3 contained *Eco*RI site after the stop codon and annealed to the 3′ end of restrictocin. For the construction of C-terminal deletion mutants, Δ146–149, Δ144–149, and Δ139–149 restrictocin gene was similarly amplified with stop codon and *Eco*RI site placed down stream of codons for amino-acid residues 145, 143, and 138 by PCR using primers S3′D4, S3′D6, and S3′D11, respectively, along with a common 5′ primer, XUP that anneals to the sequences upstream to restrictocin gene in the vector (Table 1). The purified amplified fragments were digested with *Nde*I and *Eco*RI and ligated to pVex11 digested with the same enzymes. pVex11 is a pET-derived T7 promoter-based expression vector with a bacterial and phage F1 origin of replication, kindly provided to us by V.K. Chaudhary (University of Delhi, India). The mutations were confirmed by DNA sequencing using the dideoxy chain termination method [27].

Isolation and purification of the proteins from the inclusion bodies

Restrictocin and the various mutants were expressed in BL21 (λDE3) strain of *E. coli*. Bacterial cells were transformed with the desired construct and grown in Super broth (pH 7.2) [16], containing 100 $\mu g \cdot mL^{-1}$ ampicillin, at 37 °C with shaking. At a D_{600} of 2.0, the cells were induced with 1 mM isopropyl thio-β-D-galactoside, and harvested 2 h later. The recombinant proteins were purified from the inclusion bodies using the procedure described by Buchner *et al*. [28]. The total cell pellet was resuspended in 50 mM Tris/HCl pH 7.4 containing 20 mM EDTA and incubated at room temperature for 1 h after adding 0.2 $mg \cdot mL^{-1}$ lysozyme. At the end of the incubation, NaCl and Triton X-100 were added to a final concentration of 0.4 M and 2%, respectively, and the suspension was further incubated at room temperature for 30 min. The mixture was centrifuged at 13 000 ***g*** and the pellet was repeatedly washed in 50 mM Tris/HCl pH 7.4 containing 20 mM EDTA. The pellet was dissolved in 6 M guanidine hydrochloride and incubated for 2 h at room temperature followed by centrifugation at 50 000 ***g*** at 4 °C, for 30 min. The concentration of denatured protein in the supernatant was adjusted to 10 $mg \cdot mL^{-1}$ with 6 M guanidine hydrochloride, and it was reduced by adding 65 mM dithioerythritol and incubating at room temperature for 2 h. Renaturation was carried out by diluting the denatured protein, 100-fold, in a buffer consisting of 0.1 M Tris/HCl pH 8.0, 0.5 M L-arginine-HCl, 0.9 mM GSSG, and 0.2 mM EDTA. After incubating at 10 °C for 48 h, the renatured material was dialyzed against 20 mM Mes, pH 5.0, containing 100 mM urea, loaded on a S-Sepharose column, and eluted with a NaCl gradient 0–1 M in 20 mM Mes buffer (pH 5.0) using an FPLC system. Relevant fractions were pooled, concentrated and purified to homogeneity by gel filtration chromatography on a TSK 3000 column (Pharmacia LKB) in NaCl/P_i.

Table 1. Primers used for the construction of deletion mutants of restrictocin. The letters in small case represent the nucleotides mutated and the underlined sequence denotes the restriction sites created in the primer.

Primer	Sequence
XUP	5′-ACTCACTATAGGGAGACCAC-3′
JRS-3	5′-TGTTAGCAGCCGAATTCAATGAGAACACAG-3′
SD-8	5′-ACATGCATCcAtatgCAGCTGAATCCC-3′
SD-15	5′-AATCCCAAGcatAtgAAATGGGAAGAC-3′
SD-30	5′-CAAGCCAAAcatatgAGCAACTCCCAC-3′
S3′D4	5′-CTAATGAGgAattcaTCTCAAGTCTCC-3′
S3′D6	5′-AGAACACAGaaTtcAGTCTCCCTGATT-3′
S3′D11	5′-CAAGTCTCCCTGaaTtCaCCGCTGATGGGC-3′

CD-spectral analysis of restrictocin and its mutants

To analyse the secondary structure, restrictocin or the mutants were diluted to 67 $\mu g \cdot mL^{-1}$ in 10 mM sodium phosphate buffer (pH 7.0), and CD spectra were recorded in the far-UV range (200–250 nm) at room temperature, using a Jasco J710 spectropolarimeter. A cell with a 1-cm optical path was used to record the spectra at a scan speed of 50 $nm \cdot min^{-1}$ with a sensitivity of 50 mdeg and a response time of 1 s. The sample compartment was purged with nitrogen, and spectra were averaged over 10 scans. The results are presented as mean residue ellipticity.

Specific ribonucleolytic activity assay

To evaluate the ability of restrictocin and its mutants to specifically cleave the 28S rRNA and produce the characteristic 400 nucleotide long α-fragment, rabbit reticulocyte lysate was treated with different concentrations of the toxin or the mutants as described earlier [21]. Total RNA was isolated using the Trizol reagent (Gibco BRL) as recommended by the manufacturer and dissolved in a buffer containing 32% formamide, 4 mM EDTA, 0.04% xylene cyanol, and 0.04% bromophenol blue. The samples were heated at 60 °C for 10 min and

analyzed on 2% agarose gels. The RNA was visualized by ethidium bromide staining.

Assay for *in vitro* protein synthesis

Rabbit reticulocyte lysate was used to assay the ribonucleolytic activity of restrictocin and its mutants by investigating their effect on *in vitro* protein synthesis. Reticulocyte lysate was prepared from rabbit blood [29], and assay was carried out as previously described [30]. Rabbit reticulocyte lysate was treated with different concentrations of various proteins at 30 °C for 1 h and incorporation of [^{3}H]leucine, in the newly synthesized proteins, was quantitated. Activity was expressed as percent of control, where no toxin was added.

Cytotoxic activity of restrictocin and its mutants

To assess the cytotoxic activity of restrictocin and its deletion mutants, HeLa cells permeabilized by adenovirus-infection were used as described [31]. Adenovirus was propagated in HEK 293 cells. The HEK 293 cells were grown to a monolayer in a T25 flask in MEM containing 10% fetal bovine serum. The monolayer was washed twice with MEM to remove the fetal bovine serum and inoculated with adenovirus stock in 2 mL MEM. The cells were incubated at 37 °C for 1 h for infection to occur and after one wash with MEM, were grown in 3–4 mL of MEM until cytopathic effects were visible in the monolayer. The virus was extracted into the medium by lysing the cells by freezing and thawing. The lysed cell suspension was centrifuged at 1500 ***g*** for 10 min at 4 °C. The clear supernatant contained the virus, which was titrated and used for the cytotoxicity assay. To titrate the virus, HeLa cells, grown in 96 well plates as described below, were incubated with various dilutions of adenovirus at 37 °C for 5 h followed by 2 h pulse with [^{3}H]leucine. The cells were harvested onto filtermats and radioactivity measured using a β-plate counter. The minimum dilution of the virus, that had no effect or marginal effect on protein synthesis of the cells, was used for infection in the cytotoxicity assays.

For the cytotoxicity assay, HeLa cells were grown in RPMI 1640 containing 10% fetal bovine serum at a density of 2×10^4 cells per well in 96 well culture plates for 12 h at 37 °C. The medium was replaced with leucine free RPMI containing 1% fetal bovine serum. Adenovirus and different dilution of restrictocin or its mutants in 0.2% HSA were added to the cells and incubated for 5 h. The cells were labeled with 0.1 μCi [^{3}H]leucine per well for 2 h at 37 °C, harvested onto filtermats and counted using Pharmacia LKB β-plate counter to estimate the incorporation of the [^{3}H]leucine in the newly synthesized proteins. Activity was expressed as percentage of control where no toxin was added to the cells.

Stability of proteins in *in vitro* translation assay

Various proteins (5 μg) were incubated with rabbit reticulocyte lysate and the other reaction components for the *in vitro* translation assay for 1 h at 30 °C. At the end of the incubation, restrictocin and its mutants were immunoprecipitated, by incubating at 4 °C for 1 h, with 50 μg of a polyclonal antirestrictocin antibody in NaCl/P_i containing 0.05% Tween 20. *S. aureus* cell suspension (Sigma) was added to the reaction to a final concentration of 1.5% and the mixture was further incubated at 4 °C for 1 h. The precipitated proteins were recovered by centrifugation and the pellets, after washing three times with NaCl/P_i/Tween, were dissolved in the SDS/PAGE gel-loading buffer. The samples were electrophoresed on a 14% polyacrylamide gel, transferred onto the nitrocellulose paper and probed with a polyclonal antirestrictocin antibody.

General methods

The purified proteins were analysed by SDS/PAGE as described by Laemmli using 12.5% gels [32], and visualized by staining the gels with Coomassie brilliant blue. For Western blotting, the proteins were resolved on SDS-polyacrylamide gels, transferred onto the nitrocellulose membrane, and detected using a polyclonal antirestrictocin antibody. Protein concentrations were measured by the Bradford's method [33].

RESULTS

Construction of deletion mutants of restrictocin

To investigate the role of N-terminus in restrictocin catalysis, three deletion mutants were constructed by PCR. Mutations were confirmed by DNA sequencing. A diagramatic representation of these truncated versions of restrictocin is presented in Fig. 1. All the three N-terminal mutants lack Cys5 and hence lack the outer disulfide, however, they have all the known and predicted essential residues intact.

Three C-terminal mutants, Δ146–149, Δ144–149, Δ139–149, were constructed, which, respectively, have 4, 6, and 11 amino acids deleted from the carboxyl end of restrictocin. The deletions and incorporation of stop codon were confirmed by DNA sequencing. All the deletion mutants thus made lack Cys147 and hence the Cys5–Cys147 disulfide is missing in all of them (Fig. 1).

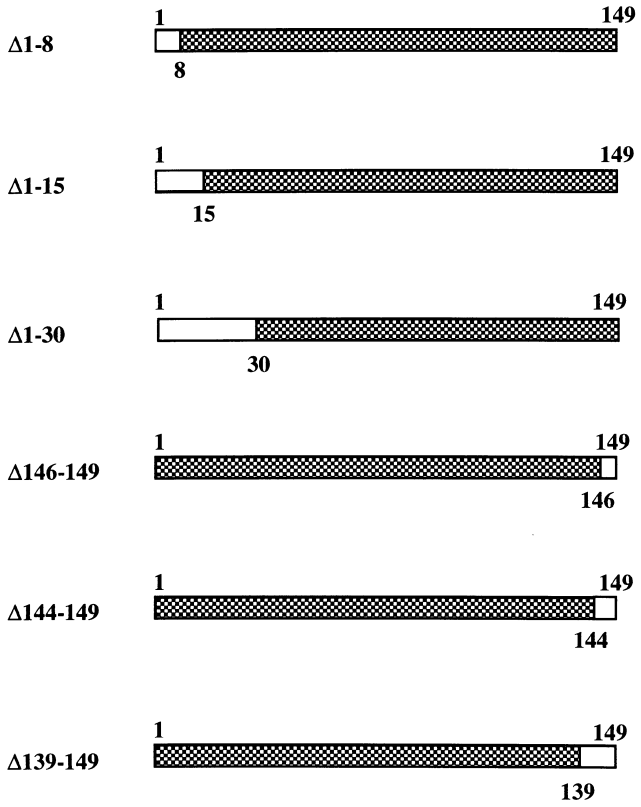

Fig. 1. A diagramatic representation of various deletion mutants of restrictocin.

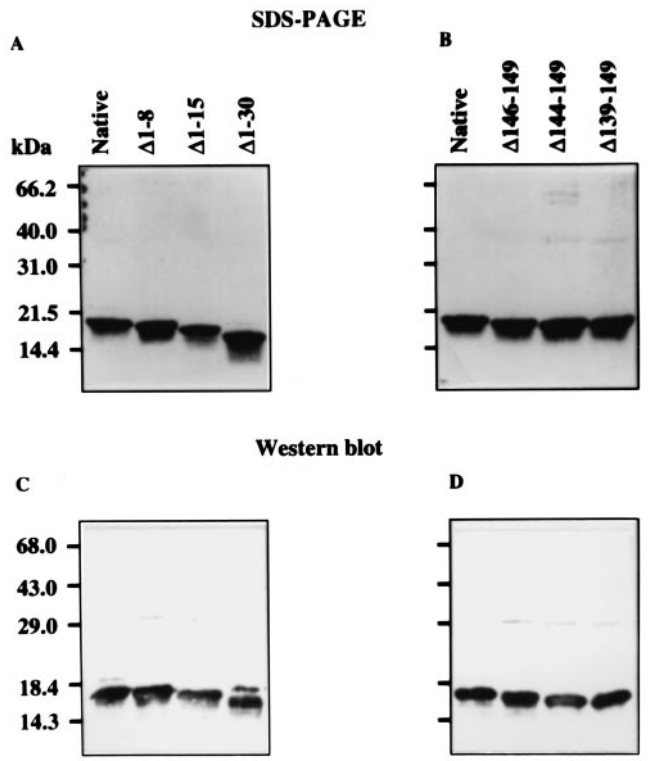

Fig. 2. SDS/PAGE and Western blot analysis of restrictocin and its deletion mutants. SDS/PAGE analysis of the N-terminal (A) and C-terminal (B) deletion mutants of restrictocin. The purified proteins were subjected to SDS/PAGE (12.5%) under reducing condition and stained with Coomassie blue. Western blot analysis of N-terminal (C) and C-terminal (D) deletion mutants using antibody raised against restrictocin. The different lanes in (C) and (D) correspond, respectively, to the same proteins as in (A) and (B).

Expression and purification

The deletion mutants of restrictocin were expressed in BL21(λDE3) strain of *E. coli*, and were localized in the inclusion bodies like restrictocin. The recombinant proteins from the inclusion bodies were denatured in guanidine hydrochloride and renatured in a glutathione-based refolding buffer. The renatured material after dialysis was loaded onto a S-Sepharose cation exchange column. The mutants like the native toxin eluted at 0.6 M sodium chloride from the S-Sepharose column. The relevant fractions were pooled and further purified using a TSK gel filtration column to obtain the monomeric forms of the proteins. The purified preparations of mutants gave single bands on SDS/PAGE (Fig. 2A,B). The size of the mutants was smaller than the native toxin corresponding to the number of amino acids deleted. All the mutants and the native toxin reacted equally well with a polyclonal antibody raised against restrictocin as shown in Western blots (Fig. 2C,D).

Structural characterization of mutants by circular dichroism

The effect of the N- and C-terminal deletions on the secondary structure of restrictocin was studied by far-UV CD spectral analysis of the purified mutants. The mean residue ellipticity curves of the mutants have been compared with that of restrictocin (Fig. 3). There were significant differences observed between the mean residue ellipticity curves of the native toxin and the N-terminal deleted versions of the toxin. There was a substantial decrease in the helix content of the mutants indicating a reduced secondary structure and among the three, Δ1–8 had a relatively more compactly folded structure (Fig. 3A). This indicated that truncation of restrictocin from the N-terminus by even as few as eight residues causes major alterations in the secondary structure of the toxin molecule.

The mean residue ellipticity curves of the C-terminal truncated molecules were not remarkably different than that of restrictocin (Fig. 3B). Both Δ146–149 and Δ144–149 mutants had a α + β conformation similar to restrictocin (Fig. 3B).

Effect of deletions on the specific ribonucleolytic activity of restrictocin

Restrictocin specifically cleaves a single phosphodiester bond between G4325 and A4326 to generate a 400-nucleotide fragment from the 3′ end of the large ribosomal RNA, known as the α-fragment. To evaluate the effect of deletions on the specific ribonucleolytic activity of restrictocin rabbit reticulocyte lysate was treated with restrictocin and its truncated versions, and the total RNA was analyzed on gel to detect the production of the α-fragment. The mutant Δ1–8 was found to be partially active as it produced the α-fragment at a much higher concentration compared to restrictocin (Fig. 4A). Δ1–15 and Δ1–30 were both inactive and did

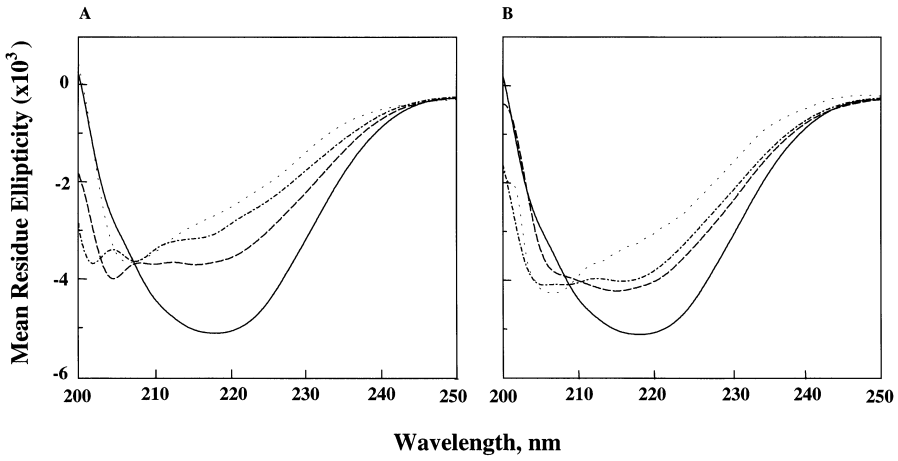

Fig. 3. CD spectral analysis of restrictocin mutants. (A) CD spectra of N-terminal deletion mutants of restrictocin. The CD spectra of restrictocin (—), Δ1–8 (---), Δ1–15 (-·-·-), and Δ1–30 (···) were recorded in the far-UV region (200–250 nm) at 25 °C. The spectra are presented as mean residue ellipticity (MRE), expressed in degree·cm^2·dmol^{-1} × 10^{-3}. (B) CD spectra of C-terminal mutants. The spectra of restrictocin (—), Δ146–149 (---), Δ144–149 (-·-·-), and Δ139–149 (···) were obtained as in (A).

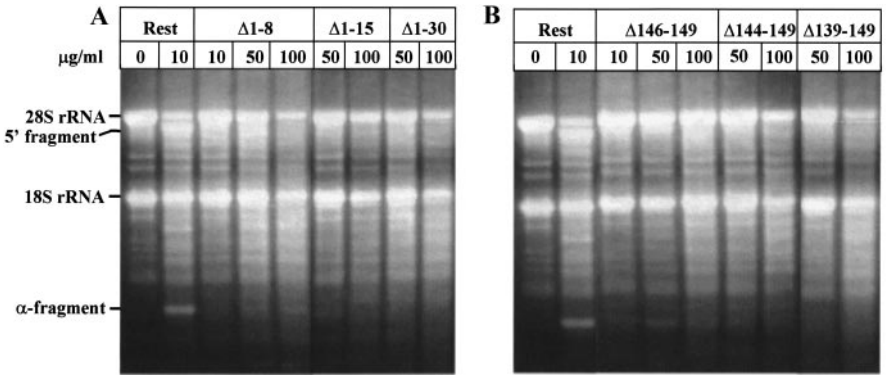

Fig. 4. Specific ribonucleolytic activity of restrictocin and mutants on 28S rRNA. Rabbit reticulocyte lysate was treated with various mutants of restrictocin at the indicated protein concentrations. The reaction was carried out in 40 mM Tris/HCl, pH 7.5, buffer containing 10 mM EDTA at 37 °C for 15 min followed by termination of reaction using 0.4% SDS. Total RNA was extracted and resolved by electrophoresis on a 2% agarose gel.

not produce the α-fragment even at 100 $\mu g \cdot mL^{-1}$ concentration (Fig. 4A). Thus, removal of eight N-terminal amino-acid residues rendered the toxin partially inactive while removal of 15 or more residues made it completely inactive in its ribonucleolytic activity. The C-terminal mutant Δ146–149, with the smallest deletion of four amino-acid residues, was partially active and produced a faint α-fragment at a higher concentration compared to restrictocin (Fig. 4B). The deletion mutants Δ139–149 and Δ144–149 failed to cleave the ribosomal RNA even at 10 times higher concentration than the native toxin (Fig. 4B).

Effect of deletions on *in vitro* protein synthesis inhibitory activity of restrictocin

The ability of the restrictocin mutants to inhibit protein synthesis was tested in a rabbit reticulocyte lysate-based *in vitro* translation assay. The lysate was treated with different concentrations of the native toxin and the deleted variants, and the translation of endogenous globin mRNA was assayed. Inhibition of protein synthesis was measured as decrease in incorporation of [^{3}H]leucine in the nascent polypeptides synthesized. Restrictocin caused a dose-dependent inhibition of protein synthesis with an ID_{50} of 8 $ng \cdot mL^{-1}$ (Table 2). The mutants Δ1–8 and Δ1–15 were partially active with respective ID_{50} values of 49 $ng \cdot mL^{-1}$ and 290 ng mL^{-1}, however, the mutant Δ1–30 was completely inactive (Table 2). The mutant Δ146–149 was about three times less active compared to the native toxin with an ID_{50} of 23 $ng \cdot mL^{-1}$, while the mutants Δ139–149 and Δ144–149 were completely inactive manifesting no inhibition of protein synthesis even upto 1000 $ng \cdot mL^{-1}$ (Table 2).

Table 2. Effect of N- and C-terminal deletions on *in vitro* protein synthesis inhibitory activity, and cytotoxic activity of restrictocin A. Rabbit reticulocyte lysate was treated with various concentrations of deletion mutants of restrictocin at 30 °C for 1 h. B. HeLa cells were infected with adenovirus and treated with different concentrations of the toxin and mutants. Incorporation of [^{3}H]leucine in the newly synthesized proteins was measured. ID_{50} refers to the amount of toxin required to inhibit protein synthesis by 50%.

	ID_{50}			
	Inhibition of *in vitro* translation		Cytotoxic activity	
Protein	($ng \cdot mL^{-1}$)	(pM)	($\mu g \cdot mL^{-1}$)	(nM)
Δ1–8	49	3159	>100	>6447
Δ1–15	290	19674	>100	>6784
Δ1–30	>1000	>76390	>100	>7639
Δ146–149	23	1442	>100	>6269
Δ144–149	>1000	>63570	>100	>6357
Δ139–149	>1000	>65870	>100	>6587
Restrictocin	8	476	2	119

Effect of deletions on the cytotoxic activity of restrictocin on HeLa cells

Restrictocin and its mutants were tested for their cytotoxic activity on HeLa cells permeabilized by infection with adenovirus. The decrease in incorporation of [^{3}H]leucine in the newly synthesized cellular proteins was taken as the measure of the cytotoxicity. None of the N- or C-terminal deletion mutants showed any cytotoxic activity, while the full length toxin showed a dose dependent cytotoxicity in this assay. The deletion mutants were completely inactive, as even with 100 $\mu g \cdot mL^{-1}$ they did not produce 50% inhibition of cellular protein synthesis as compared to the native toxin which had an ID_{50} of 2 $\mu g \cdot mL^{-1}$ (Table 2).

Stability of proteins in *in vitro* translation assay

To investigate if the loss of enzymatic activity observed in the deletion mutants of restrictocin was a reflection of an increased proteolytic degradation, various proteins were incubated with rabbit reticulocyte lysate for 1 h at 30 °C. At the end of the

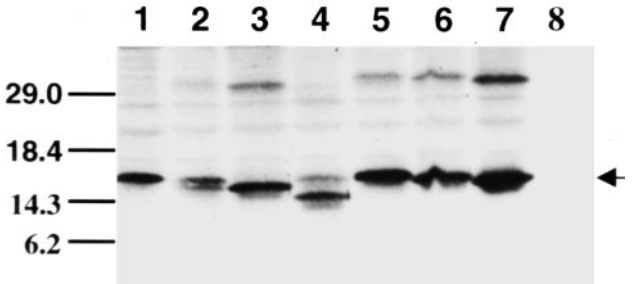

Fig. 5. Effect of rabbit reticulocyte lysate on the integrity of restrictocin and mutants. Restrictocin and its deletion mutants (5 μg) were incubated at 30 °C for 1 h with rabbit reticulocyte lysate followed by immunoprecipitation with an antirestrictocin antibody. The precipitated proteins were separated by SDS/PAGE on a 14% gel and analysed by Western blotting. Lanes 1, restrictocin; 2, Δ1–8; 3, Δ1–15; 4, Δ1–30; 5, Δ146–149; 6, Δ144–149; 7, Δ139–149; 8, none. The arrowhead marks the location of the proteins of interest. Molecular mass markers have been shown in kDa.

incubation the proteins were immunoprecipitated and visualized by Western blotting. As shown in Fig. 5, all mutants were found to be intact and the amounts recovered were comparable to that of the native protein treated similarily.

DISCUSSION

The study shows that small deletions of eight amino acids from the N-terminus or four amino acids from the C-terminus render the toxin partially inactive. Any further deletions from either ends resulted in the total inactivation of the toxin. Δ1–8 and Δ146–149, although less active than the native protein, retained the specific ribonucleolytic activity. The CD-spectral analysis clearly demonstrates major alterations in the structure of Δ1–8, however, the conformation of Δ146–149 was not altered remarkably. Both of these deletion mutants are devoid of the outer disulfide bond because of the respective deletions of Cys5 and Cys147 in Δ1–8 and Δ146–149. However, we have earlier shown that individual mutations of Cys5 and Cys147 in restrictocin do not affect the conformation and functional activity of the protein [16]. Therefore, the loss of activity of Δ1–8 and Δ146–149 does not appear to be an outcome of the absence of Cys5–Cys147 disulfide bond.

The deletions in this study have been made from either N- or C-terminus of restrictocin in order to localize the activity in the molecule and also to investigate the importance of the termini in the function of the toxin. All deletion mutants had all the putative active site residues intact. In the *in vitro* translation assay, various mutant proteins were found to be intact at the end of the incubation, suggesting that the loss of enzymatic activity is not a consequence of relatively increased degradation of these proteins during the assay. The loss of activity observed appears to be a reflection of structural perturbations. The deletions that retain the partial activity of the toxin are those that remove partly β1 (Δ1–8) and β7 (Δ146–149), respectively, from the N- and C-terminal ends of restrictocin. Further deletions from the N-terminus deleting β1 + L1 (Δ1–15) and β1, L1 + β2 (Δ1–30) or from C-terminus totally deleting β7 (Δ144–149) and β7 + L6 (Δ139–149) apparently produce a total collapse of restrictocin structure. Kao and Davies [19] have proposed that there is hydrogen bond formation between amino-acid residues in β1 and β2 with residues in β6 and hence deletion of residues from this region causes loss of catalytic activity of mitogillin. The loss of restrictocin function arising out of the small deletions may possibly be due to the collapse of such hydrogen bonded links that usually contribute to maintaining an active conformation even in the absence of the outer disulfide bond. It is quite evident that the amino-acid sequences at both ends of restrictocin are extremely crucial in maintaining an enzymatically active conformation of the toxin. The C-terminus seems relatively more crucial than the N-terminus as comparatively smaller deletions on the carboxyl end make the toxin partially inactive.

The role of the termini in the structure and function of different ribonucleases has been studied quite extensively. The N-terminal sequence of bovine RNase A known as S-peptide, consisting of 20 amino-acid residues, contributes to the maintenance of the structure of the rest of the molecule called S-protein [34]. The side chain of Phe 8 in S-peptide has been found to contribute to the stabilization of the transition state of the complex between S-peptide and S-protein that gives rise to the stability of RNase S [35]. A deletion of seven amino-acid residues from the N-terminal end of human pancreatic ribonuclease destabilizes the folded structure of the RNase molecule and the truncated molecule was found to retain some ribonuclease as well as RNase inhibitor binding ability [36]. The C-terminal end tetrapeptide sequence EDST has been found not to be essential for the ribonuclease activity of human pancreatic ribonuclease but the deletion of the four amino acids seems to increase the thermal stability of the enzyme [37]. The C-terminal EDST extension in human pancreatic ribonuclease has been proposed to be destabilizing the C-terminal helix and that its deletion may facilitate efficient folding of the molecule [37]. It appears that both the N- and C-termini in restrictocin are contributing towards the folding of the molecule to attain an active conformation.

In conclusion, the current study has shown that intact N- and C-termini are extremely crucial for retaining the enzymatically active conformation of restrictocin. Although the putative active site residues of this unique ribonuclease are quite far from both the termini, and an intact Cys5–Cys147 disulfide bond connecting the two termini is not essential for its activity, yet the two termini seem to play an important role in maintaining its functionally active conformation.

ACKNOWLEDGEMENTS

This work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. S. K. N. is a Senior Research Fellow of National Institute of Immunology, and Shveta is a Junior Research Fellow of the Council of Scientific and Industrial Research, India. We gratefully acknowledge Mr G. Basnotra's help with the graphics.

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