A Single Amino Acid Substitution in Ribonucleolytic Toxin Restrictocin Abolishes Its Specific Substrate Recognition Activity†

Surendra K. Nayak and Janendra K. Batra*

Immunochemistry Laboratory, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110067, India

Received May 19, 1997; Revised Manuscript Received August 18, 1997⊗

ABSTRACT: Restrictocin is a small basic protein produced by the fungus *Aspergillus restrictus*. It potently inhibits protein synthesis in eukaryotic cells by specifically cleaving a single phosphodiester bond in 28S rRNA. A histidine residue at position 49 in restrictocin has been implicated in its active site. A mutant of restrictocin in which the histidine at position 49 was changed to an alanine was constructed by site-directed mutagenesis, and the protein was expressed in *Escherichia coli*. The mutant and the wild type proteins were found to be structurally identical. Unlike restrictocin, the restrictocin H49A mutant did not cleave the ribosomal RNA specifically at the target phosphodiester bond; instead, it extensively degraded the RNA substrate with altered specificity. The mutant exhibited a high ribonuclease activity compared to restrictocin on yeast tRNA, and poly(U) and poly(C). The mutant also poorly inhibited protein synthesis in eukaryotic cells as well as in a cell free system. We therefore propose that histidine 49 of restrictocin is not involved *per se* in the enzymatic activity; however, it does play a crucial role in the specific recognition of the target sequence by restrictocin.

Restrictocin is a fungal ribonucleolytic toxin produced by *Aspergillus restrictus* and belongs to the group termed ribotoxins that includes α-sarcin and mitogillin (*1*). The ribotoxins are potent inhibitors of eukaryotic protein synthesis (*2*). They act as highly specific ribonucleases cleaving a single phosphodiester bond at the 3′ end of 28S rRNA in a 15-nucleotide stem and loop domain (*3*). This region of 28S rRNA, known as the sarcin/ricin (S/R) domain, named after the two toxins that act on this domain, is critical for the ribosome function and is conserved in almost all living organisms so far tested (*4*). The S/R domain plays a critical role in protein synthesis as an oligonucleotide complementary to this region upon microinjection inhibits protein synthesis in *Xenopus* oocytes (*5*). Cleavage of 28S rRNA by ribotoxins produces a fragment about 400 nucleotides in length which has been termed the α-fragment (*3*). The cleavage of large ribosomal RNA by ribotoxins impairs the EF-1-dependent binding of aminoacyl tRNA and GTP-dependent binding of EF-2 to ribosomes which in turn leads to a total collapse of the translational machinery resulting in cell death (*6*). α-Sarcin has been shown to retain its specificity of cleavage on a 35mer synthetic substrate that mimics the S/R domain and cleaves the synthetic oligonucleotide to produce two fragments which are 21 and 14 nucleotides in length (*7*). The ribotoxins do not have an inherent cell binding ability, and so far, no cell surface receptors for these toxins have been reported; however, α-sarcin has been shown to be toxic to cells artificially permeabilized by viral infection or ionophore treatment (*8*−*10*). There are conflicting reports about the activity of α-sarcin on naked rRNA. Endo et al. (*11*) observed nonspecific degradation of the substrate after every purine, whereas Miller and Bodley (*12*) have reported specific cleavage in the S/R domain of *Escherichia coli* and *Saccharomyces cerevisiae* naked large ribosomal RNAs by α-sarcin. The precise catalytic mechanism for the specific recognition of the target and cleavage by ribotoxins is not known. Ribotoxins share considerable sequence homology with two other fungal RNases, RNase U2 and RNase T1 (*13*, *14*). While RNase T1 and U2 cleave RNA after every guanine base, only fungal ribotoxins demonstrate specific cleavage of a single phosphodiester bond in 28S rRNA in the large ribosomal subunit. The potent toxic activity of ribotoxins has been exploited to generate active immunotoxins targeted at cancer cells (*15*−*20*). Chimaeric toxins have been constructed with restrictocin for specific delivery to tumor cells and to elucidate the intracellular mechanism of action of restrictocin (*21*).

Recently, structures of restrictocin and α-sarcin have been solved by X-ray crystallography (*22*) and NMR (*23*), respectively. The structural core of the protein containing a three-turn α-helix packed against a five-stranded antiparallel β-sheet can be well aligned with that of RNaseT1 (*22*). On the basis of structure, restrictocin has been found to contain catalytic components of RNase T1 and base recognition components of RNase Sa (*22*). On the basis of structure, and homology considerations with RNase T1, amino acid residues Tyr 47, His 49, Glu 95, Arg 120, and His 136 have been implicated in the catalytic site of restrictocin (*22*). Substitution of His 136 in restrictocin, and the corresponding His 137 in α-sarcin, has been shown to result in the inactivation of these ribotoxins, confirming the presence of this residue in their active site (*24*−*26*). Mutation of Glu 95 in restrictocin renders the toxin partially inactive, implying a less critical role for Glu 95 in catalysis (*24*).

To investigate the role of histidine 49 in restrictocin, we have mutated the residue to an alanine and expressed and purified the mutant enzyme from *E. coli*. Replacement of histidine 49 with alanine in restrictocin was found to result in a molecule that had full ribonuclease activity but failed to recognize the 28S rRNA at the specific ribotoxin cleavage

† The work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India.

* To whom correspondence should be addressed. Phone: 91-11-6163009. Fax: 91-11-6162125. E-mail: janendra@nii.ernet.in.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

S0006-2960(97)01177-X CCC: $14.00

site, indicating that histidine 49 in restrictocin plays a crucial role in its specific recognition of the S/R domain in the target rRNA.

EXPERIMENTAL PROCEDURES

Materials. All enzymes, cell culture reagents, and yeast tRNA were purchased from GIBCO-BRL. [^{3}H]Leucine and [α-^{32}P]UTP were obtained from Amersham. Reagents for the *in vitro* translation assay were from Promega and Pharmacia. HeLa cells were obtained from the ATCC. Homopolymers were purchased from Pharmacia. RNA Stat-60 was purchased from TEL-TEST Inc.

Site-Directed Mutagenesis. Mutation was carried out by sequential PCR[1] as described (*27*). pRest, a plasmid containing DNA encoding restrictocin in a T7 promoter-based *E. coli* expression vector, was used as a template to amplify restrictocin DNA in two fragments, each containing a mutation which changes the codon for histidine at position 49 to alanine (*28*). The following four primers were used for PCR: XUP, ACTCACTATAGGGAGACCAC; SK4, GTGAACCAGGCAGGATAGCTGC; SK3, GCAGCTATCCTGCCTGGTTCAC; and JRS3, TGTTAGCAGCCGAATTCAATGAGAACACAG. SK4 and SK3 are complementary primers, both containing mutations which change the codon for histidine 49 to alanine. Primers XUP and JRS3 anneal to the 5′ and 3′ ends of restrictocin DNA, respectively. The amplified restrictocin DNA fragments for the 5′ part (with primers XUP and SK4) and the 3′ part (with primers SK3 and JRS3) were purified, mixed in equal amounts, and used as a template for another PCR that was done using primers XUP and JRS3. The amplified product, containing the 450 bp fragment coding for restrictocin with the mutation H49A, was digested with *Nde*I and *Eco*RI and ligated to pVex11 digested with the same enzymes to give rise to the construct pRestH49A. pVex11 is a T7 promoter-based expression vector with a bacterial and phage F1 origin of replication. *E. coli* strain DH5α was used for all DNA manipulations. Mutation was confirmed by DNA sequencing by Sanger's method (*29*).

Expression and Purification of Recombinant Proteins. Restrictocin and restrictocin H49A were expressed, using plasmids pRest and pRestH49A, respectively, in *E. coli* strain BL21(λDE3) (*28*). *E. coli* cells were transformed with the appropriate plasmid, grown in super broth, and induced with 1 mM IPTG for 90 min. Inclusion bodies were isolated from the total cell pellet as described (*30*). The proteins were isolated from inclusion bodies by denaturation in guanidine hydrochloride and reduction by dithioerythritol (DTE), followed by renaturation in a buffer containing arginine and oxidized glutathione (*30*). The renatured proteins were purified to homogeneity by successive cation exchange and gel filtration chromatography on S-sepharose and TSK 3000 columns as described earlier (*28*).

Characterization of Proteins by Circular Dichroism. The purified proteins were dissolved in 10 mM sodium phosphate buffer at pH 7.0, and their CD spectra were recorded in the far-UV range (200−250 nm) at 25 °C on a JASCO J710 spectropolarimeter as described (*31*). A cell with a 1 cm optical path was used to record spectra of proteins. Spectra were acquired at a scan speed of 50 nm/min 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. Results are presented as mean residue ellipticity values (θ).

RNase Activity on Various RNA Substrates. Poly(A), poly(U), poly(G), and poly(C), 320 μM each, and 13 μM yeast tRNA were separately incubated with different amounts of the wild type toxin or the mutant in 50 mM Tris-HCl at pH 7.3 for 1 h at 40 °C as described earlier (*28*). Large, undigested RNA molecules were precipitated with perchloric acid and uranyl acetate on ice and removed by centrifugation (*32*). The acid soluble product was quantitated by measuring A_{260}. Kinetic constants for various substrates, at concentrations ranging between 20 and 800 μM [poly(U)], 50 and 1600 μM [poly(A)], and 0.8 and 48 μM (yeast tRNA), were determined for restrictocin and restrictocin H49A from double-reciprocal plots of initial reaction velocities.

Specific Ribonucleolytic Activity Assay. The ability of the mutant to specifically cleave the 28S rRNA to produce the α-fragment was assayed using rabbit ribosomes. Rabbit reticulocyte lysate was prepared as described (*33*), and 30 μL of lysate was incubated with different concentrations of restrictocin and the mutant in 40 mM Tris-HCl (pH 7.5) containing 10 mM EDTA at 37 °C for 15 min in a 50 μL reaction volume (*34*). The reaction was stopped by adding SDS to a final concentration of 0.4%, and total RNA was extracted using RNAstat-60 solution. The RNA pellet was dissolved in 0.5% SDS, analyzed on 1.5% agarose gels, and visualized by ethidium bromide staining.

In another assay, activity of restrictocin and restrictocin H49A on a 35mer synthetic substrate mimicking the S/R domain was investigated. The 35mer substrate was transcribed *in vitro* using T7 RNA polymerase and synthetic DNA oligonucleotides containing a sequence corresponding to the S/R domain downstream of the T7 promoter sequence (*7*). The oligoribonucleotide was labeled with [α-^{32}P]UTP and purified by PAGE as described (*7*). The purified oligoribonucleotide dissolved in 10 mM Tris-HCl (pH 7.6) containing 10 mM $MgCl_2$ was heated at 90 °C for 2 min and renatured overnight at 4 °C. An equal amount of renatured substrate (3000 cpm) was incubated with restrictocin and the H49A mutant at various concentrations in 10 mM Tris-HCl (pH 7.6) containing 50 mM KCl and 5 mM $MgCl_2$ in a 10 μL reaction volume at 37 °C for 10 min. The products were analyzed by electrophoresis on a 20% polyacrylamide sequencing gel containing 7 M urea and visualized by autoradiography.

In Vitro Translation Assay. The capacity of restrictocin and the mutant to inhibit protein synthesis was measured using a rabbit reticulocyte lysate-based *in vitro* translation assay system. The rabbit reticulocyte lysate was prepared and the assay carried out as described (*33*). The reaction mixture in a 30 μL volume contained 10 μL of lysate, 1 mM ATP, 0.2 mM GTP, 75 mM KCl, 2 mM magnesium acetate, 3 mM glucose, 10 mM Tris-HCl (pH 7.6), 4 μM amino acid mix without leucine, 0.16 μCi [^{3}H]leucine, 1.33 mg/mL creatine phosphokinase, 2.66 mg/mL creatine phosphate, and the wild type toxin or the mutant at various concentrations. The reaction was carried out at 30 °C for 1 h and stopped by adding 1 N NaOH containing 0.2% H_2O_2

[1] Abbreviations: CD, circular dichroism; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; poly(A), poly(adenylic acid); poly(G), poly(guanylic acid); poly(U), poly(uridylic acid); poly(C), poly(cytidylic acid); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

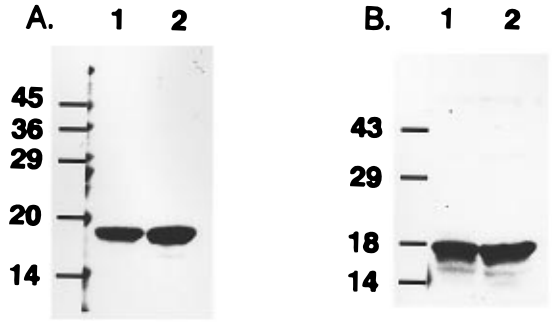


FIGURE 1: SDS−PAGE and Western blot analysis of restrictocin and restrictocin H49A. (A) SDS−PAGE analysis of the purified proteins. Following purification through ion exchange and gel filtration column chromatography, the proteins were analyzed on 12.5% SDS−PAGE and visualized by staining with Coomassie blue. Lanes 1 and 2 show restrictocin and restrictocin H49A, respectively. (B) Western blot analysis of restrictocin (lane 1) and restrictocin H49A (lane 2) using polyclonal antibody raised against restrictocin. Molecular mass markers are shown in kilodaltons.

followed by a further incubation at 37 °C for 10 min. BSA was added to a final concentration of 65 μg/mL, and the nascent polypeptides synthesized were precipitated with 15% trichloroacetic acid on ice and harvested on 26 mm glass fiber filters. The filters after drying were counted in a liquid scintillation counter.

Cytotoxicity Assay. Cytotoxic activity of restrictocin and restrictocin H49A was assayed by measuring protein synthesis in adenovirus-infected HeLa cells in the presence and absence of toxin (*9*). HeLa cells were grown at a density of 2×10^4 cells per well in 96-well culture plates for 12 h at 37 °C. Adenovirus and various dilutions of the toxin were added to the cells and the mixtures incubated for 5 h before labeling with 0.1 μCi [^{3}H]leucine per well. The cells were further incubated at 37 °C for 2 h, followed by harvesting and counting on filtermats using an LKB β-plate counter. Adenovirus was grown in 293 cells and isolated by lysing the cells by freezing and thawing. To titrate the virus, HeLa cells were incubated with various dilutions of adenovirus at 37 °C for 5 h followed by a 2 h pulse with [^{3}H]leucine, and a dilution of the virus that had no effect or a marginal (<20%) effect on protein synthesis of the cells was used for cytotoxicity assays.

RESULTS

We have earlier overexpressed restrictocin in *E. coli* and shown that the recombinant protein is structurally and functionally similar to the native protein produced by the fungal host (*28*). In this study, the same clone of restrictocin was used to mutate histidine at position 49 to an alanine. Alanine was chosen because it eliminates the side chain beyond the β-carbon without altering the main conformation, and being the most abundant amino acid in proteins, it is found in both buried and exposed positions in a variety of secondary structures.

Expression, Purification, and Structural Characterization. Restrictocin and restrictocin H49A were expressed in *E. coli* strain BL21(λDE3). The overexpressed proteins were localized in the insoluble inclusion bodies from which they were isolated and purified to homogeneity by simple chromatography. The purified mutant protein on SDS−PAGE gave a single band at a position identical to that of restrictocin (Figure 1A). A polyclonal antibody against restrictocin reacted with the mutant protein equally well as shown on a Western blot in Figure 1B.

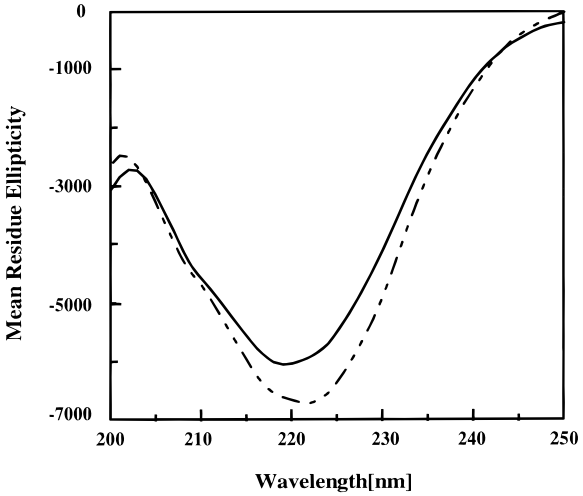


FIGURE 2: CD spectral analysis of restrictocin and restrictocin H49A. CD spectra of restrictocin (—) and restrictocin H49A (— - - —) dissolved in 10 mM sodium phosphate buffer at pH 7.0 were recorded in the far-UV region (200−250 nm) at 25 °C. Data were averaged over 10 scans. The mean residue ellipticity is expressed as deg cm^2 dmol^{-1} $\times$ 10^{-3}.

To investigate if introducing a point mutation in restrictocin affects its structure, proteins were analyzed by CD spectroscopy. The molar ellipticity curves of restrictocin and restrictocin H49A are shown in Figure 2. The CD spectra of the two proteins were found to be very similar, indicating that the mutation did not alter the structure of restrictocin (Figure 2).

Specific Ribonucleolytic Activity. Restrictocin cleaves the 28S rRNA specifically at a single phosphodiester bond, releasing the α-fragment which is 393 nucleotides long in the case of rat ribosomes. In this study, rabbit reticulocyte lysate was treated with the mutant and the toxin at various concentrations to study the generation of the α-fragment. The wild type toxin produced an ~400 base long α-fragment, the appearance of which was dose-dependent as its amount increased with increasing amounts of toxin (Figure 3). In contrast, with the mutant H49A, generation of the α-fragment was not observed (Figure 3). At lower concentrations of the mutant, no activity was observed, while with increasing amounts of protein, extensive degradation of ribosomal RNA was observed (Figure 3).

To confirm that mutation of histidine 49 to alanine abolishes the specificity of restrictocin, the ribonucleolytic activity of wild type and mutant proteins was assayed on a 35mer synthetic oligonucleotide that mimics the site of action of the toxin on 28S rRNA. The oligonucleotide was transcribed *in vitro* and purified as has been described (*7*). The ribotoxins have been shown to recognize this synthetic substrate by cleaving a single phosphodiester bond to produce two oligonucleotide fragments, 21mer and 14mer (*7*). While restrictocin cleaved the substrate specifically in a dose-dependent manner, producing the 21mer and 14mer fragments, the mutant showed no such specific cleavage; instead, it degraded the substrate with altered specificity, resulting in the generation of smaller fragments (Figure 4). Thus, in both the assays, restrictocin H49A did not recognize the S/R domain and instead degraded the RNA substrates extensively with altered specificity.

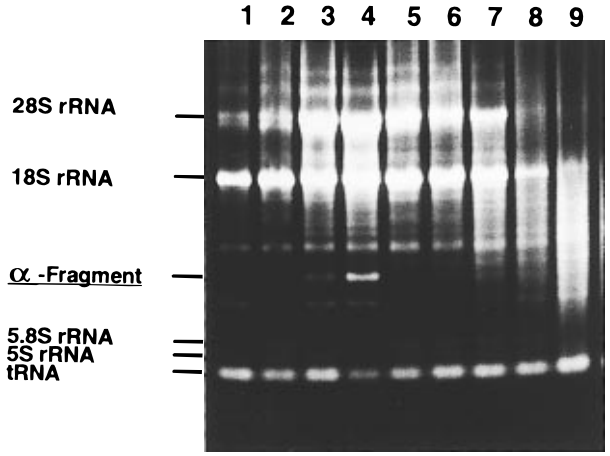


FIGURE 3: Specific ribonuclease activity on 28S rRNA. Rabbit reticulocyte lysate was treated with no toxin (lane 1), restrictocin at 1.0 μg/mL (lane 2), 5.0 μg/mL (lane 3), and 25.0 μg/mL (lane 4), or restrictocin H49A at 1.0 μg/mL (lane 5), 5.0 μg/mL (lane 6), 25.0 μg/mL (lane 7), 50.0 μg/mL (lane 8), and 150.0 μg/mL (lane 9). The total RNA was run on 1.5% agarose gel and visualized by ethidium bromide staining. Positions of different RNA fragments are marked by arrows.

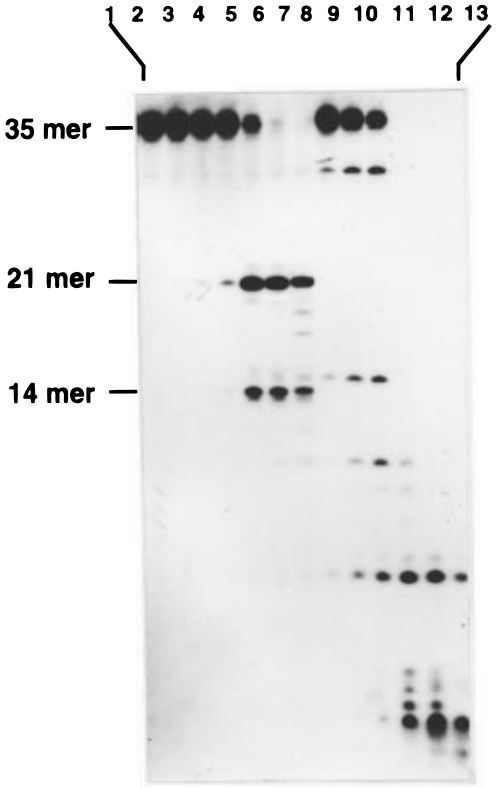


FIGURE 4: Cleavage of the 35mer synthetic RNA substrate. A 35mer RNA oligonucleotide was transcribed *in vitro* and purified. Equal amounts of the oligonucleotide were incubated without toxin (lane 1) and with different concentrations of restrictocin (lanes 2−7) or restrictocin H49A (lanes 8−13). The concentrations of the toxin and mutant used were 5 nM (lanes 2 and 8), 20 nM (lanes 3 and 9), 50 nM (lanes 4 and 10), 500 nM (lanes 5 and 11), 2 μM (lanes 6 and 12), and 8 μM (lanes 7 and 13). The reaction products were separated by PAGE on 20% gels containing 7 M urea and visualized by autoradiography.

Inhibition of Protein Synthesis. The mutant was tested for its ability to inhibit protein synthesis *in vitro* in a rabbit reticulocyte lysate system and its ability compared with that of restrictocin. Rabbit reticulocyte lysate permits translation of endogenous globin mRNA when a suitable energy source and amino acids are supplied to optimize the reaction. Restrictocin potently inhibited the protein synthesis in a dose-dependent manner, whereas restrictocin H49A was significantly less efficient (Figure 5). The amount of toxin needed to inhibit protein synthesis by 50% (ID_{50}) of restrictocin and the mutant were 2 and >1000 ng/mL, respectively, indicating that, compared to restrictocin, the H49A mutant had an inhibitory activity toward protein synthesis that was more than 500 times lower in this assay (Figure 5).

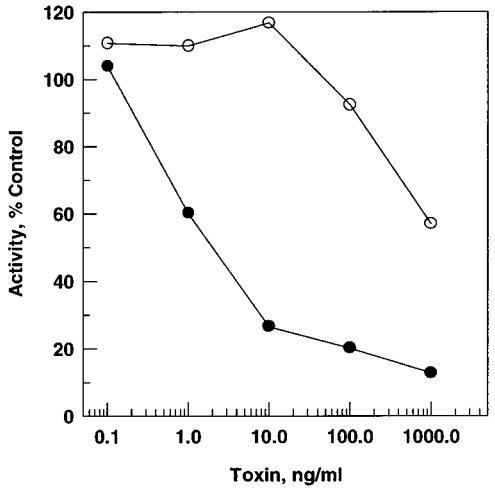


FIGURE 5: Effect of restrictocin and the mutant on *in vitro* protein synthesis. Protein synthesis inhibition was studied using rabbit reticulocyte lysate treated with different concentrations of restrictocin (●) or restrictocin H49A (○). The lysate was incubated with the toxin or the mutant at 30 °C for 1 h. Incorporation of [^{3}H]-leucine was measured in the nascent peptides as a function of toxin concentration and expressed as a percentage of activity over the control reaction where no toxin was added.

Cytotoxic Activity. The mammalian cells are permeabilized to macromolecules when infected with virus particles. Earlier, it has been shown that ribotoxins potently inhibit protein synthesis in cells infected with adenovirus (*9*). This system has been exploited here to compare the ability of the H49A mutant to inhibit protein synthesis with that of restrictocin. As shown in Figure 6, restrictocin potently inhibited protein synthesis in HeLa cells infected with adenovirus, and at 10 μg/mL, almost 100% inhibition was observed. In contrast, with the H49A mutant at 100 μg/mL, only about 25% inhibition was observed (Figure 6). The ID_{50} for restrictocin was calculated to be 0.33 μg/mL, indicating that restrictocin H49A was at least 300-fold less cytotoxic than restrictocin. In the same assay, RNaseA did not show any inhibition of protein synthesis up to 100 μg/mL (data not shown).

Ribonuclease Activity on RNA Substrates. In the assays mentioned above, although restrictocin H49A lost the ability to recognize the target RNA sequence/structure recognized by the native toxin, it appeared to contain a significant amount of ribonuclease activity. To quantitate and compare the ribonuclease activity of restrictocin and restrictocin H49A, a variety of RNA substrates were used. On yeast tRNA, the H49A mutant showed substantially high ribonuclease activity compared to restrictocin (Figure 7A).

Compared to restrictocin, the H49A mutant on RNA homopolymer poly(A) had similar activity, on poly(G) a lower activity, and on poly(U) and poly(C) a much higher activity (Figure 7B). The preference of restrictocin for poly-(A) over poly(G), however, was maintained as reported

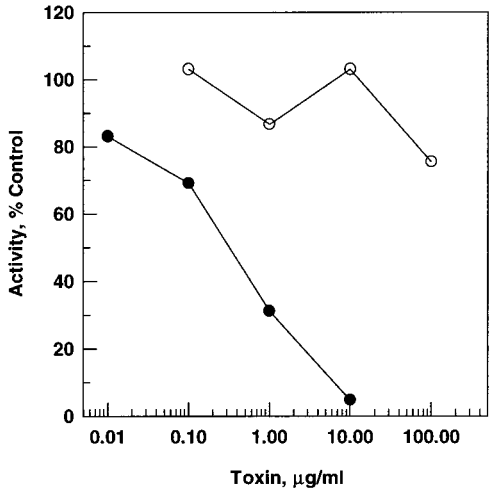


FIGURE 6: Cytotoxicity of restrictocin and restrictocin H49A in adenovirus-infected HeLa cells. HeLa cells were infected with adenovirus and treated with various concentrations of restrictocin (●) or restrictocin H49A (○). Activity of the toxin and the mutant is expressed as a percentage of the control where no toxin was added. The incorporation of [^{3}H]leucine in the newly synthesized proteins with the toxin at increasing concentrations was measured.

earlier (*11*). These results indicate that the H49A mutant displays a preference for cleavage at pyrimidine bases with respect to purine bases.

Table 1 shows the kinetic parameters for restrictocin and restrictocin H49A with various substrates. For poly(U) and yeast tRNA, compared to the native protein, the K_m of the mutant was 7- and 16-fold higher, accompanied by 14- and 30-fold higher k_{cat} values. However, on these substrates, the k_{cat}/K_m values of the two proteins were not drastically different, indicating that the differential enzymatic activity could be due to an enlarged/relaxed active site.

In summary, these results show that restrictocin H49A contains an appreciable amount of ribonuclease activity but has lost the ability to specifically recognize the target sequence in 28S rRNA.

DISCUSSION

We have produced a mutant form of *Aspergillus* ribonucleolytic toxin restrictocin in which the histidine at position 49, implicated in the active site, has been changed to an alanine. The CD spectral analysis of the mutant H49A indicated there was no major alteration in the structure because of the mutation. Surprisingly, when the mutant was tested for α-fragment production from 28S rRNA using rabbit reticulocyte lysate and cleavage of a 35mer *in vitro* transcribed substrate that mimics the site of action of the toxin, specific fragments were not produced, although a substantial amount of ribonuclease activity was observed. Digestion of yeast tRNA also confirmed that the mutant contained a high amount of ribonuclease activity. The mutation caused drastic reduction in the ability of the toxin to inhibit protein synthesis *in vitro* in the rabbit reticulocyte lysate assay system, and in HeLa cells infected with adenovirus. The poor cytotoxic activity of the mutant was comparable to that of RNase A, a pyrimidine specific ribonuclease. These observations have been confirmed with different independent recombinant protein preparations to

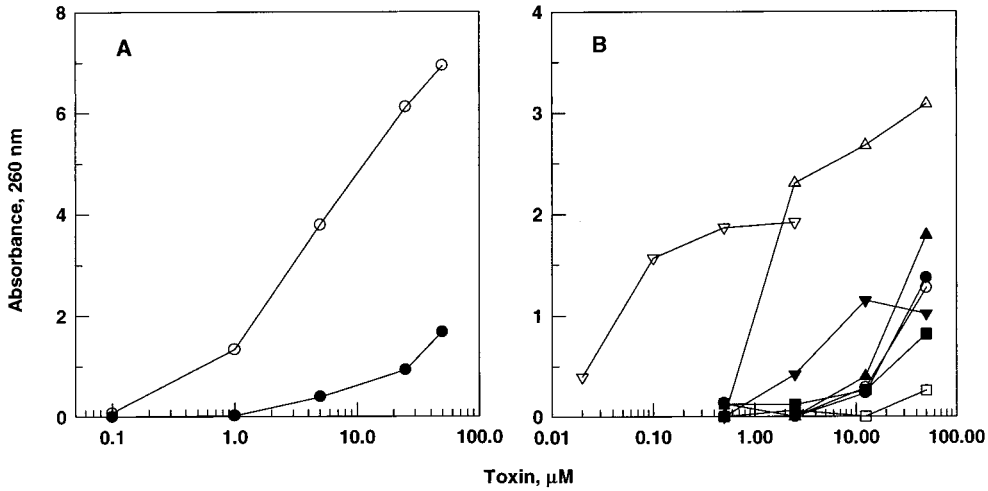


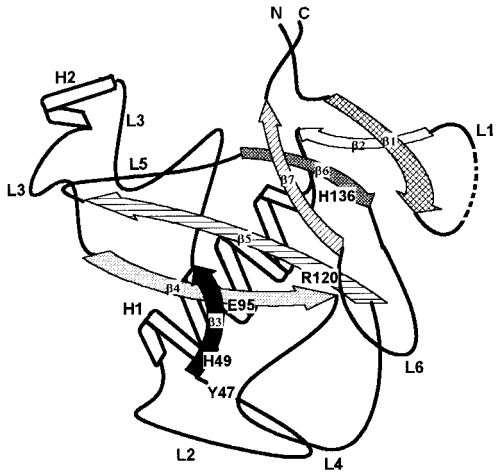
FIGURE 7: Ribonuclease activity on RNA substrates. (A) Yeast tRNA was incubated with different concentrations of restrictocin (●) or restrictocin H49A (○). The acid soluble product after digestion was quantitated in the supernatant by measuring the absorbance at 260 nm. (B) Different concentrations of restrictocin were used to treat poly(A) (●), poly(G) (■), poly(U) (▲), and poly(C) (▼). Similarly, the effect of various concentrations of restrictocin H49A was tested on poly(A) (○), poly(G) (□), poly(U) (△), and poly(C) (▽). In these experiments, two recombinant derivatives of hepatitis B surface antigen similarly produced in *E. coli* were used as controls, and no ribonuclease activity was detected for a protein concentration of up to 18 μM on yeast tRNA and poly(U) substrates.

Table 1: Kinetic Constants for Restrictocin and Restrictocin H49A[a]

	substrate								
	yeast tRNA			poly(U)			poly(A)		
protein	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
restrictocin	1 (0.32)	1	1	1 (38)	1	1	1 (66)	1	1
restrictocin H49A	16 (5.0)	30	1.9	7 (250)	14	2	1.5 (100)	1.9	1.2

[a] K_m and k_{cat} values were determined from initial rate measurements for hydrolysis of various substrates. The K_m and k_{cat} values are normalized to those of the wild type enzyme. The actual K_m values, in micromolar, are given in parentheses.

A.



B.

Ribonuclease	Catalytic residues				
Restrictocin	Tyr47	*His49*	Glu95	Arg120	His 136
Ribonuclease T1	Tyr38	*His40*	Glu58	Arg77	His92
Ribonuclease U2	Tyr39	*His41*	Glu62	Arg85	His101
Ribonuclease Sa	Arg65	*Val35*	Glu54	Arg 69	His85

FIGURE 8: Schematic illustration of the structure of restrictocin. (A) A cartoon drawn, on the basis of the structure of restrictocin described by Yang and Moffat (*22*), showing various structural elements, and the critical residues in the active site of restrictocin: β, β-sheet; H, helix; L, loop; and amino acids in the active site depicted with single-letter codes. Residues assigned to the different structural elements include 1−10 (β1), 11−16 (L1), 17−24 (β2), 25−35 (H1), 36−48 (L2), 49−52 (β3), 53−91 (L3), 73−77 (H2), 92−98 (β4), 99−117 (L4), 118−125 (β5), 126−131 (L5), 132−137 (β6), 138−142 (L6), and 143−147 (β7). (B) Structural alignment of residues involved in catalysis of restrictocin and other ribonucleases having a similar basic core structure. Ribonucleases T1 and Sa are specific for G, whereas RNase U2 prefers A over G.

eliminate the possibility of nonspecific RNase contaminations. The increased ribonuclease activity of restrictocin H49A on pyrimidine substrates appears to be due to an enlarged/relaxed active site which may also account for the lack of specific recognition of the target stem and loop structure. We thus clearly demonstrate that histidine 49 of restrictocin which, on the basis of structural homology, is similar to histidine 40, valine 35, and histidine 41 of RNase T1, Sa, and U2, respectively, is not functionally similar and is involved in the specificity rather than the catalytic activity of restrictocin.

Restrictocin and the other members of the ribotoxin family that include α-sarcin and mitogillin have considerable homology with RNase U2 and RNase T1. The antiparallel β-sheet and an adjacent long α-helix form the structural core of restrictocin (Figure 8A), which represent a common structure motif found in other ribonucleases, including RNase T1, Ms, and Sa and barnase. The main differences are located in the length of the N-terminal β-hairpin and the loops connecting the secondary structure elements which are generally much longer in restrictocin and adopt different conformations than those in RNase T1 (*22*). The crystal structure analysis of restrictocin has indicated that structural elements involved in RNA substrate binding include a landing platform comprised of loops L2 (amino acids 36−48) and a lysine rich loop L4 (amino acids 99−117) and a positively charged ridge in loop domain L3 (amino acids 53−91) (Figure 8A) (*22*). Deletion of K106−K113 in L4 of mitogillin has been shown to result in the loss of substrate specificity (*35*). Thus, it has been proposed that specific recognition between G 4319 and the lysine rich loop may be the key interaction responsible for substrate specificity (*22*). The solution structure of α-sarcin elucidated by NMR spectroscopy has also revealed a large number of potential contacts between the RNA and the long extended loops of the protein (*23*). As shown in Figure 8A, histidine 49 lies right adjacent to the loop L2 (amino acids 36−48), which has been proposed to play a critical role in binding to the RNA substrate (*22*). Alignments based on the crystal structure reveal that ribonucleases with folds similar to restrictocin fall into two groups, a T1 group and a Sa group, which differ in their catalytic mechanism (Figure 8B). Restrictocin seems to combine the catalytic components of the T1 group with the base recognition components of the Sa group. In this regard, as in RNase Sa, His 49 might not be directly involved in bond cleavage (*22*).

On the basis of studies of RNase T1 (*36*−*38*), Yang and Moffat (*22*) have proposed a two-step reaction mechanism for restrictocin catalysis involving a phosphoryl transfer reaction and a hydrolysis reaction. His 49/Glu 95 in restrictocin is thought to serve as the general base and His136 as the general acid, resulting in formation of a 2′−3′ cyclic phosphate intermediate. In the hydrolysis reaction, the roles of the catalytic residues are reversed; His 136 serves as the general base to activate a water molecule, and His 49/Glu 95 serves as the general acid. In RNase T1, His 40 and/or Glu 58 acts as the general base and His 92 as the general acid in the phosphoryl transfer reaction while Tyr 38 and His 92 stabilize the transition state of the phosphate group by hydrogen bonding (*37*−*39*). The role of His 40 in RNase T1 has not been conclusively demonstrated, although it is important for catalysis as changing this amino acid abolishes the enzymatic activity (*37*). While the catalytic residues in RNase Sa, i.e. Glu 54, Arg 69, and His 85, can be appropriately aligned with Glu 95, Arg 120, and His 136 in restrictocin, amino acid residue Val 35 corresponds to His 49 of restrictocin (Figure 8B) (*22*). This suggests that either RNase Sa has a different catalytic mechanism or His 49 might not be directly involved in bond cleavage in restrictocin. In the crystal structure analysis of RNase U2 (*40*), when the Cα atom of His 41 is fitted to the corresponding residue His 40 of RNase T1, it shows a very large positional displacement of 1.16 Å. This large deviation, together with the side chain conformation of His 41, suggests that the role of His 41 in catalysis may be different from that of RNase T1, despite it being conserved in homologous RNases.

In the case of restrictocin, His 136 is probably acting as a general acid, playing a role similar to that of His 92 of RNase T1, since substitution of His 136 has been shown to result in the inactivation of restrictocin (*24*). In an earlier study, Glu 95 was not found to be absolutely critical for the activity of restrictocin (*24*); Yang and Kenealy speculated that His 49 was functioning as a general base, but it does not appear to be doing so in this study. Although the role of Glu 95 has not been conclusively shown, but since its substitution was found to result in partial inactivation of restrictocin, the possibility of Glu 95 acting as a general base still cannot be ruled out. Thus, in restrictocin, Glu 95 and

some other yet unidentified residue may be acting as a general base corresponding to Glu 58 and His 40 in RNase T1. It would be interesting to investigate how histidine 49, which is conserved among various fungal ribonucleases and absent in bacterial RNases (*41*), has come to acquire such a specialized function unique to the ribotoxins.

ACKNOWLEDGMENT

We thank Mr. Dharmendar Rathore for his help with the study and Kevlanand for excellent technical assistance. Thanks are due to Prof. S. K. Basu, Dr. D. M. Salunke, and Dr. R. P. Roy for their valuable suggestions on the manuscript. We are grateful to Mr. Rajneesh Batra for his help with the graphics. Surendra K. Nayak is a Senior Research Fellow of National Institute of Immunology.

REFERENCES

1. Lamy, B., Davies, J., and Schindler, D. (1992) in *Genetically Engineered Toxins* (Frankel, R. E., Ed.) pp 237−258, Marcel Dekker Inc.
2. Schindler, D. G., and Davies, J. E. (1977) *Nucleic Acids Res. 4*, 1097−1110.
3. Endo, Y., and Wool, I. G. (1982) *J. Biol. Chem. 257*, 9054−9060.
4. Chan, Y.-L., Endo, Y., and Wool, I. G. (1983) *J. Biol. Chem. 258*, 12768−12770.
5. Saxena, S. K., and Ackerman, E. J. (1990) *J. Biol. Chem. 265*, 3263−3269.
6. Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L., and Sperti, S. (1989) *Biochem. J. 257*, 723−727.
7. Endo, Y., Chan, Y.-L., Lin, A., Tsurugi, K., and Wool, I. G. (1988) *J. Biol. Chem. 263*, 7917−7920.
8. Turnay, J., Olmo, N., Jimenez, A., Lizarbe, M. A., and Gavilanes, J. G. (1993) *Mol. Cell. Biochem. 122*, 39−47.
9. Fernandez-Puentes, C., and Carrasco, L. (1980) *Cell 20*, 769−775.
10. Alonso, M. A., and Carrasco, L. (1981) *FEBS Lett. 127*, 112−114.
11. Endo, Y., Huber, P. W., and Wool, I. G. (1983) *J. Biol. Chem. 258*, 2662−2667.
12. Miller, S. P., and Bodley, J. W. (1988) *Biochem. Biophys. Res. Commun. 154*, 404−410.
13. Lopez-Otin, C., Barber, D., Fernandez-Luna, J. L., Soriano, F., and Mendez, E. (1984) *Eur. J. Biochem. 143*, 621−634.
14. Martinez del Pozo, A., Gasset, M., Onaderra, M., and Gavilanes, J. G. (1988) *Biochim. Biophys. Acta 953*, 280−288.
15. Orlandi, R., Canevari, S., Conde, F. P., Leoni, F., Mezzanzanica, D., Ripamonti, M., and Colnaghi, M. I. (1988) *Cancer Immunol. Immunother. 26*, 114−120.
16. Conde, F. P., Orlandi, R., Canevari, S., Mezzanzanica, D., Ripamonti, M., Munoz, S. M., Jorge, P., and Colnaghi, M. I. (1989) *Eur. J. Biochem. 178*, 795−802.
17. Wawrzynczak, E. J., Henry, R. V., Cumber, A. J., Parnell, G. D., Derbyshire, E. J., and Ulbrich, N. (1991) *Eur. J. Biochem. 196*, 203−209.
18. Better, M., Bernhard, S. L., Lei, S.-P., Fishwild, D. M., and Carroll, S. F. (1992) *J. Biol. Chem. 267*, 16712−16718.
19. Rathore, D., and Batra, J. K. (1996) *Biochem. Biophys. Res. Commun. 222*, 58−63.
20. Rathore, D., Nayak, S. K., and Batra, J. K. (1997) *Gene 191*, 31−35.
21. Rathore, D., and Batra, J. K. (1997) *Biochem. J. 324*, 815−822.
22. Yang, X., and Moffat, K. (1996) *Structure 4*, 837−852.
23. Campos-Olivas, R., Bruix, M., Santoro, J., Martinez del Pozo, A., Lacadena, J., Gavilanes, J. G., and Rico, M. (1996) *FEBS Lett. 399*, 163−165.
24. Yang, R., and Kenealy, W. R. (1992) *J. Biol. Chem. 267*, 16801−16805.
25. Brandhorst, T., Yang, R., and Kenealy, W. R. (1994) *Protein Expression Purif. 5*, 486−497.
26. Lacadena, J., Mancheno, J. M., Martinez-Ruiz, A., Martinez Del Pozo, A., Gasset, M., Onaderra, M., and Gavilanes, J. G. (1995) *Biochem. J. 309*, 581−586.
27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1993) *Current Protocols in Molecular Biology*, Vol. 1, Supplement 22, pp 8.5.5−8.5.9, Massachusetts General Hospital and Harvard Medical School, Cambridge, MA.
28. Rathore, D., Nayak, S. K., and Batra, J. K. (1996) *FEBS Lett. 392*, 259−262.
29. Sanger, F., Niklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
30. Buchner, J., Pastan, I., and Brinkmann, U. (1992) *Anal. Biochem. 205*, 263−270.
31. Bal, H. P., and Batra, J. K. (1997) *Eur. J. Biochem. 245*, 465−469.
32. Kumagai, H., Igarashi, K., Takayama, T., Watanabe, K., Sugimoto, K., and Hirose, S. (1980) *Biochim. Biophys. Acta 608*, 324−331.
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 3, pp 18.76−18.80, Cold Spring Harbor Laboratory Press, Plainview, NY.
34. Lacadena, J., Martinez del Pozo, A., Barbero, J. L., Mancheno, J. M., Gasset, M., Onaderra, M., Lopez-Otin, C., Ortega, S., Gracia, J., and Gavilanes, J. G. (1994) *Gene 142*, 147−151.
35. Kao, R., and Davies, J. (1995) *Biochem. Cell Biol. 73*, 1151−1159.
36. Heinemann, U., and Saenger, W. (1982) *Nature 299*, 27−31.
37. Nishikawa, S., Morioka, H., Kim, H. J., Fuchimura, K., Tanaka, T., Uesugi, S., Hakoshima, T., Tomita, K., Ohtsuka, E., and Ikehara, M. (1987) *Biochemistry 26*, 8620−8624.
38. Steyaert, J., Hallenga, K., Wyns, L., and Stanssens, P. (1990) *Biochemistry 29*, 9064−9072.
39. Gohda, K., Oka, K., Tomita, K., and Hakoshima, T. (1994) *J. Biol. Chem. 269*, 17531−17536.
40. Noguchi, S., Satow, Y., Uchida, T., Sasaki, C., and Matsuzaki, T. (1995) *Biochemistry 34*, 15583−15591.
41. Hill, C., Dodson, G., Heinemann, U., Saenger, W., Mitsui, Y., Nakamura, K., Borisov, S., Tischenko, G., Polyakov, K., and Pavlovsky, S. (1983) *Trends Biochem. Sci. 8*, 364−369.

BI971177H