THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 27, Issue of July 6, pp. 24978–24984, 2001

*Printed in U.S.A.*

# Interaction of Human Pancreatic Ribonuclease with Human Ribonuclease Inhibitor

## GENERATION OF INHIBITOR-RESISTANT CYTOTOXIC VARIANTS*

Received for publication, March 19, 2001, and in revised form, April 25, 2001
Published, JBC Papers in Press, May 7, 2001, DOI 10.1074/jbc.M102440200

**Deepak Gaur‡, Srividya Swaminathan§, and Janendra K. Batra¶**

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

**Mammalian ribonucleases interact very strongly with the intracellular ribonuclease inhibitor (RI). Eukaryotic cells exposed to mammalian ribonucleases are protected from their cytotoxic action by the intracellular inhibition of ribonucleases by RI. Human pancreatic ribonuclease (HPR) is structurally and functionally very similar to bovine RNase A and interacts with human RI with a high affinity. In the current study, we have investigated the involvement of Lys-7, Gln-11, Asn-71, Asn-88, Gly-89, Ser-90, and Glu-111 in HPR in its interaction with human ribonuclease inhibitor. These contact residues were mutated either individually or in combination to generate mutants K7A, Q11A, N71A, E111A, N88R, G89R, S90R, K7A/E111A, Q11A/E111A, N71A/E111A, K7A/N71A/E111A, Q11A/N71A/E111A, and K7A/Q11A/N71A/E111A. Out of these, eight mutants, K7A, Q11A, N71A, S90R, E111A, Q11A/E111A, N71A/E111A, and K7A/N71A/E111A, showed an ability to evade RI more than the wild type HPR, with the triple mutant K7A/N71A/E111A having the maximum RI resistance. As a result, these variants exhibited higher cytotoxic activity than wild type HPR. The mutation of Gly-89 in HPR produced no change in the sensitivity of HPR for RI, whereas it has been reported that mutating the equivalent residue Gly-88 in RNase A yielded a variant with increased RI resistance and cytotoxicity. Hence, despite its considerable homology with RNase A, HPR shows differences in its interaction with RI. We demonstrate that interaction between human pancreatic ribonuclease and RI can be disrupted by mutating residues that are involved in HPR-RI binding. The inhibitor-resistant cytotoxic HPR mutants should be useful in developing therapeutic molecules.**

Mammalian ribonucleases constitute a ubiquitous superfamily of proteins with a high level of structural and functional divergence. These include a group of homologous proteins isolated from many mammalian, avian, reptilian, and amphibian sources and are collectively known to be part of the RNase A superfamily (1). The current resurgence of interest in RNases is the result of the discovery of RISBASES (RNases with Special Biological Actions), which have been identified to influence tumor cell growth, neurological development, and biological differentiation (2). An important biological function of mammalian RNases may be host defense, as has been observed in the case of the two eosinophil RNases, eosinophil cationic protein and eosinophil-derived neurotoxin, which exhibit antiviral, antibacterial, antiparasitic, and neurotoxic activities (3–7). Also, frog RNase onconase and bovine seminal ribonuclease (BS-RNase)[1] exhibit antitumor activity (8–11).

Human pancreatic ribonuclease (HPR) is secretory in nature and has been considered as a counterpart of bovine pancreatic RNase A (12, 13). Although HPR shares 70% homology with RNase A and possesses similar key structural and catalytic residues, it displays some unique features (14). HPR possesses substantial activity against double-stranded RNA, contains a higher proportion of basic residues, its activity is differentially influenced by ionic strength and divalent ions, and compared with RNase A it has a carboxyl-terminal extension of four residues, EDST (15–17). We have reported earlier that deletion of the carboxyl-terminal EDST extension enhances the RNase activity and thermostability of HPR (18). Although both RNase A and HPR catalyze RNA degradation efficiently, they have not been associated with any special biological action, and hence their physiological role, especially that of HPR, is not clearly defined. RNases have much potential as chemotherapeutics. Onconase is presently undergoing phase III human clinical trials for the treatment of malignant mesothelemia (19) and has also been shown to inhibit human immunodeficiency virus type I replication in chronically infected human cells (20). A majority of pancreatic RNases, with the exception of BS-RNase and onconase, are not cytotoxic. The major apparent reason for this poor cytotoxicity is the neutralization of the ribonucleolytic activity of RNases by the cytosolic RNase inhibitor (RI). Hence, affinity of a RNase for the intracellular RI could play an important role in defining its cytotoxic potency. HPR holds tremendous promise as a therapeutic agent for humans, and compared with other RNases it is likely to be less immunogenic and thus more efficacious. When the RI-sensitive "noncytotoxic" RNases are injected directly into *Xenopus* oocytes, which lack strong inhibitors to mammalian RNases, they display cytotoxic activity comparable to ricin and diphtheria toxin (21). Moreover, the two classes of RNases with anticancer activity, onco-

* This work was supported in part by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India, and Grant SP/SO/D-44/96 from the Department of Science and Technology, Government of India (to J. K. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a senior research fellowship from the Council of Scientific and Industrial Research, India.

§ Present address: Mouse Cancer Genetics Program, Mammalian Genetics Laboratory Bldg. 538, Rm. 133, NCI-Frederick Cancer Research and Development Center, Frederick MD 21702.

¶ To whom correspondence should be addressed. Tel.: 91-11-616-3009 or 616-2281; Fax: 91-11-616-2125 or 610-9433; E-mail: janendra@nii.res.in or jkbatra@yahoo.com.

[1] The abbreviations used are: BS-RNase, bovine seminal ribonuclease; HPR, human pancreatic ribonuclease; RI, ribonuclease inhibitor; hRI, human ribonuclease inhibitor.

TABLE I

*Sequence of primers used for mutagenesis of the putative residues*

The underlined letters in lower case represent the nucleotides mutated.

| Mutant | Primer | Sequence |
|---|---|---|
| K7A | JKB 10 | 5′-ATGGCTAGCAAGGAATCCCGGGCCAAGgctTTCCAGCGG-3′ |
| Q11A | JKB 11 | 5′-ACTGTCTGAGTCCATATGagcCCGCTGGAATTTCTTGGC-3′ |
| N71A | JKB 15 | 5′-GGAGTTGCTCTTGTAGCAagcGCCCTGCCCGTTCTTGCA-3′ |
| E111A | JKB 16 | 5′-TGGCACATATGGGCTCCCagcACAGGCCACAATGATGTG-3′ |
| N88R | JKB 9 | 5′-GTTGGGGTACCTGGAGCCcctTGTCAGGCGGCAGTCTGT-3′ |
| G89R | JKB 8 | 5′-ACAGTTGGGGTACCTGGAcctGTTTGTCAGGCGGCAGTC-3′ |
| S90R | JKB 29 | 5′-TGCACAGTTGGGGTAcctACGGCCGTTTGTCAGGCGGCA-3′ |

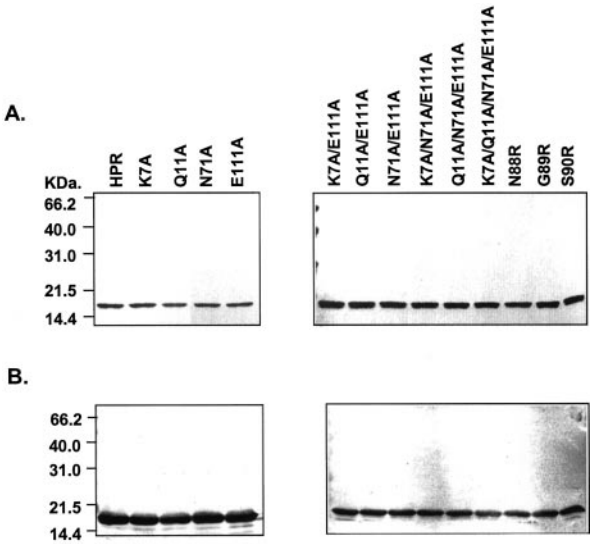


FIG. 1. **SDS-polyacrylamide gel electrophoresis and Western blot analysis of HPR mutants.** The mutants were expressed in BL21(λDE3) cells of *E. coli* and purified from the inclusion bodies by cation exchange and gel filtration chromatography. The recombinant proteins were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis under reducing conditions followed by Coomassie Blue staining (*panel A*). Western blot analysis of the mutants was done using a polyclonal antibody raised against HPR (*panel B*). The different *lanes* in *panel B* correspond to the same proteins in *panel A*.

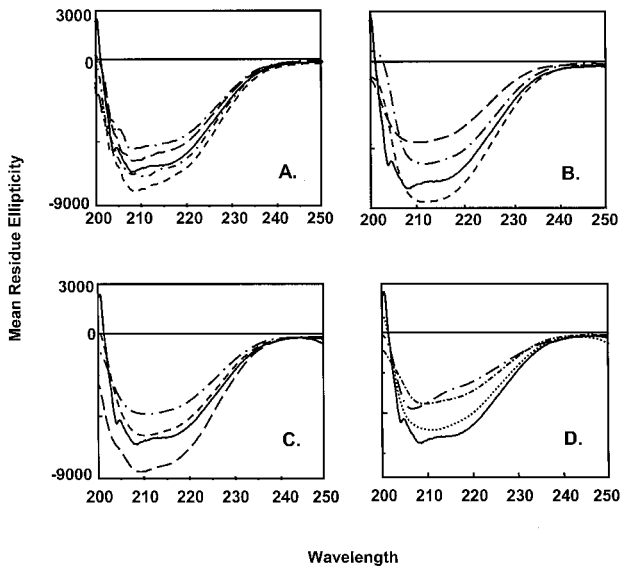


FIG. 2. **CD spectral analysis of HPR and its mutants.** CD spectra were recorded in far UV region (200–250 nm) at 25 °C. The spectra are presented as mean residue ellipticity, expressed in degrees·cm$^2$/dmol × $10^{-3}$. *Panel A*, HPR (——), K7A (– – –), Q11A (– · – · –), N71A (- · - · -), and E111A (- - - - -). *Panel B*, HPR (——), Q11A/E111A (- - - - -), K7A/E111A (– · – · –), and N71A/E111A (– – –). *Panel C*, HPR (——), K7A/N71A/E111A (- - - - -), Q11A/N71A/E111A (– – –), and K7A/Q11A/N71A/E111A (– · – · –). *Panel D*, HPR (——), N88R (- · - · -), G89R (· · · · ·), and S90R (– · – · –).

nase and BS-RNase, are found to be resistant to cytosolic RI protein, and their cytotoxic activities appear to be a consequence of their abilities to escape inactivation by RI. (22, 23). BS-RNase is a naturally occurring homodimer that is stabilized by two intersubunit disulfide bridges. The BS-RNase monomer is highly homologous to RNase A; however, the dimeric form has a much lower affinity for RI than the free monomer (23). Onconase evades RI as a monomer because of a lack of amino acid residues that are responsible for making contact with RI. Only three residues that contact RI in RNase A are conserved in onconase (19, 24). RI is a 50-kDa protein that constitutes 0.01% of the total cytosolic protein and is a highly conserved protein in various mammalian species (25, 26). RI forms a 1:1 noncovalent complex with RNase A; and as seen from the three-dimensional structure of pRI·RNase A complex, one-third of the enzyme, including its active site, sits within the horseshoe-shaped structure of the inhibitor (27). A similar interaction has been observed with angiogenin and human RI (hRI); however, the intermolecular contacts in the RI·RNase complex differ because of the differences in the sequences of the two RNases (28). Recently, the crystal structure of a variant of HPR, having five amino-terminal residues replaced by those in the BS-RNase, has been determined (29). The structure of this HPR variant shares the overall size and characteristic V shape of the other RNases of its family; however, it differs significantly from RNase A in various loop regions (29).

It has been demonstrated recently that RNase A can be engineered as a cytotoxin by mutating the specific contact residue Gly-88, which led to a decrease in its susceptibility to RI inactivation (30). Because the potency of a cytotoxic RNase can be defined in terms of its affinity for the RI, it is possible that HPR could be transformed into a cytotoxin by lowering its sensitivity to RI inactivation. In the current study we have investigated the role of four plausible contact residues in HPR in its interaction with the hRI with an aim to generate cytotoxic HPR variants. We have generated variants of HPR which have enhanced resistance toward inactivation by the hRI and are more cytotoxic.

## EXPERIMENTAL PROCEDURES

*Construction of HPR Mutants*—HPR is a protein consisting of 128 amino acid residues. pHPR, a plasmid containing the 384- base pair HPR gene, cloned downstream of a T7 promoter (18), was used as template to mutate the target residues Lys-7, Gln-11, Asn-71, and Glu-111 to Ala. Similarly, the residues Asn-88, Gly-89, and Ser-90 were mutated to arginine. Except for K7A, all of the mutations were carried out by oligonucleotide-mediated site-directed mutagenesis (31). Uracil containing DNA template was prepared by infecting CJ236 strain of *Escherichia coli* with the recombinant phage and growing it in the presence of uridine and chloramphenicol (31). Mutagenesis was performed using DNA primers JKB 8, JKB 9, JKB 10, JKB 11, JKB 15, JKB 16, and JKB 29 containing the mutations G89R, N88R, K7A, Q11A, N71A, E111A, and S90R, respectively. Sequences of various primers used are shown in Table I. Primer extension products were

TABLE II
*Catalytic activity of HPR mutants*

Substrates poly(C), yeast tRNA, and cCMP were incubated separately with different concentrations of the wild type HPR or its mutants for 1 h at 37 °C. The undigested large molecular weight RNA was precipitated with perchloric acid and uranyl acetate on ice and removed by centrifugation. The acid-soluble product was quantitated by measuring the absorbance at 260 nm. Each dinucleotide substrate was incubated with HPR and its mutants at 25 °C. The change in absorbance at 284 nm was monitored spectrophotometrically. The specific activity has been expressed as Δ*A*/min/mg protein. The percent activity compared with the HPR activity is shown in parentheses. ND, not determinable.

| Protein | Δ*A*/min/mg protein | | | | | |
|---|---|---|---|---|---|---|
| | Poly(C) | Yeast tRNA | cCMP | CpA | UpA | UpG |
| HPR | 227,000 (100) | 16,300 (100) | 0.37 (100) | 0.140 | 0.138 | ND |
| K7A | 241,000 (106) | 12,800 (78) | 0.45 (121) | 0.148 | 0.130 | ND |
| Q11A | 186,000 (82) | 7,950 (48) | 0.29 (78) | 0.130 | 0.020 | ND |
| N71A | 313,000 (140) | 7,600 (46) | 0.67 (181) | 0.120 | 0.050 | 0.280 |
| E111A | 508,000 (224) | 8,800 (53) | 0.54 (145) | 0.130 | 0.077 | 0.070 |

transformed into *E. coli* strain DH5α by standard methods. The mutant K7A was constructed by polymerase chain reaction using pHPR as the template, JKB 10 as the forward primer, and a universal *Eco*RI reverse primer. The primers were designed such that the amplified HPR fragment carrying the mutation K7A had recognition sites for *Nhe*I at the 5′-end and *Eco*RI at the 3′-end. The amplified fragment was digested with *Nhe*I and *Eco*RI, purified by gel electrophoresis, and cloned into a T7 promoter based-*E. coli* expression vector pVEX11 restricted with the same enzymes. pVEX11 is a pUC-based vector that has a phage T7 promoter, multiple cloning sites, and a T7 transcription terminator.

For constructing the double mutants K7A/E111A, Q11A/E111A, and N71A/E111A, mutants K7A, Q11A, N71A, and E111A were digested with *Nhe*I (present at the 5′-end) and *Kpn*I (present at position 292 in the HPR gene), and the 290-base pair fragment released from the mutants was ligated with the *Nhe*I-*Kpn*I vector fragment obtained from the E111A mutant. The triple mutant K7A/N71A/E111A was prepared by mutating the Lys-7 to Ala by polymerase chain reaction as mentioned above, using the double mutant N71A/E111A as the template. The triple mutant Q11A/N71A/E111A was created by oligonucleotide-mediated site-directed mutagenesis using the double mutant N71A/E111A as the template and primer JKB 11. For the construction of the quadruple mutant K7A/Q11A/N71A/ E111A, the triple mutant Q11A/N71A/E111A was used as the template, and the K7A mutation was introduced by polymerase chain reaction. All mutations were confirmed by DNA sequencing using the dideoxy chain termination method (32).

*Expression and Purification of the Recombinant Proteins*—HPR has been overexpressed earlier in *E. coli* and purified from the inclusion bodies to obtain functionally active enzyme (18). The HPR mutant proteins were prepared similarly from the cultures of *E. coli* strain BL21(λDE3), transformed with appropriate plasmid, and grown in superbroth containing 100 μg/ml ampicillin (18). All HPR mutants were found to accumulate in cytoplasmic inclusion bodies that were processed further as described (33). The solubilization of the inclusion body pellet was achieved in 6 M guanidium HCl. Renaturation of the solubilized protein was done by diluting the protein in a refolding buffer containing L-arginine and oxidized glutathione. The renatured protein, after dialysis, was loaded on an S-Sepharose cation exchange column. The protein was eluted with a gradient of 0–1 M NaCl and purified further by gel filtration chromatography (18).

*Structural Characterization by Circular Dichroism*—CD-spectra of purified proteins were recorded using a Jasco J720 (Easton) dichrograph in the far UV region (190–250 nm). Each protein, 33 μg/ml in 10 mM sodium phosphate, pH 7.0, was used in a cell with a 1-cm optical path to record the spectra. The spectra were acquired at a scan speed of 50 nm/min with a sensitivity of 50 mdeg and response time of 1 s. The spectra measured were an average of 10 accumulations, and the results are presented as mean residual ellipticity values.

*Assay of Ribonucleolytic Activity of HPR and Mutants*—The ribonucleolytic activity of various mutants was assayed on substrates poly(C), poly(U), yeast tRNA, and poly(A·U) as described by Bond (34). Each substrate (40 μg) was incubated separately with different concentrations of the wild type HPR or its mutants in 100 mM Tris-HCl, pH 7.5, for 1 h at 37 °C. The undigested large molecular weight RNA was precipitated with perchloric acid and uranyl acetate on ice and removed by centrifugation at 15,000 × *g* for 10 min. The acid-soluble product was quantitated by measuring the absorbance at 260 nm.

The hydrolytic activity of HPR and its mutants on cyclic CMP was assayed according to the method of Crook *et al.* (35). In a reaction buffer consisting of 0.2 M Tris-HCl, pH 7.5, and 0.02 M EDTA, 0.1 mg/ml cyclic CMP was mixed with 40 μg/ml of the enzyme, and the reaction was monitored spectrophotometrically at 284 nm at 25 °C.

TABLE III
*Catalytic activity of the HPR mutants on poly(C)*

The poly(C) substrate was incubated with different concentrations of the wild type HPR and its mutants for 1 h at 37 °C. The undigested large molecular weight RNA was precipitated with perchloric acid and uranyl acetate on ice and removed by centrifugation. The acid-soluble product was quantitated by measuring the absorbance at 260 nm. The specific activity has been expressed as Δ*A*/min/mg protein. The percent activity compared with the HPR activity is shown in parentheses.

| Protein | Δ*A*/min/mg protein |
|---|---|
| HPR | 227,000 (100) |
| K7A/E111A | 292,000 (128) |
| Q11A/E111A | 80,000 (35) |
| N71A/E111A | 688,000 (303) |
| K7A/N71A/E111A | 262,000 (115) |
| Q11A/N71A/E111A | 48,000 (21) |
| K7A/Q11A/N71A/E111A | 48,000 (21) |
| N88R | 237,000 (104) |
| G89R | 220,000 (97) |
| S90R | 232,500 (102) |

The RNase activity of various proteins on dinucleotide substrates CpA, UpA, and UpG was measured by using the procedure of Witzel and Barnard (36). The appropriate substrate, 50 μM in 100 mM Tris-HCl buffer, pH 7.0, was incubated with HPR or its mutants (final concentration 5 μM) at 25 °C. The change in absorbance at 284 nm was monitored spectrophotometrically.

*RI Binding Assays*—The HPR mutants were screened for ribonucleolytic activity in the presence of hRI by using an agarose gel-based assay (30). Briefly, in a total volume of 10 μl, 10 ng of enzyme was mixed with 4 μg of total rat liver RNA and 20 units of recombinant hRI in 100 mM Tris-HCl, pH 7.5, containing10 mM dithiothreitol. The mixture was incubated for 10 min at 37 °C; the reaction was stopped by the addition of 2 μl of gel loading buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM EDTA, glycerol (30% v/v), xylene cyanol FF (0.25% w/v), and bromphenol blue (0.25% w/v) and subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide.

The RNase activity of the mutants, in the presence of RI, was studied quantitatively by assaying their activity on the most preferred RNA homopolymer substrate, poly(C), in an assay described above (34).

The inhibition constants ($K_i$) for the RI-HPR mutant interactions were determined by measuring the steady-state rate of poly(C) cleavage in the presence of RI. Reactions were performed in 100 mM Tris-HCl, pH 7.5, containing 2.8 nM enzyme and 50–300 μM poly(C). RI concentrations in the range 300–700 pM were used, and the initial velocity data were used to prepare Lineweaver-Burk plots, from which $K_i$ was calculated.

*Cytotoxicity Assays*—The cytotoxicity assays were performed on five different cell lines: U373MG (human glioblastoma), J774A.1 (mouse monocyte-macrophage), K562 (human erythroleukemia), A431 (human epidermoid carcinoma), and A549 (human lung carcinoma). Cytotoxicity was evaluated by measuring [$^3$H]leucine incorporation into newly synthesized protein. Cells were incubated with RNases for 40 h, followed by a 3-h pulse with 0.75 μCi/well [$^3$H]leucine. The cells were then harvested onto glass fiber filters using a cell harvester. The filters were dried, and counts were taken using a liquid scintillation counter. The $ID_{50}$ values represent the concentration of the RNase which inhibited the cellular protein synthesis by 50%.

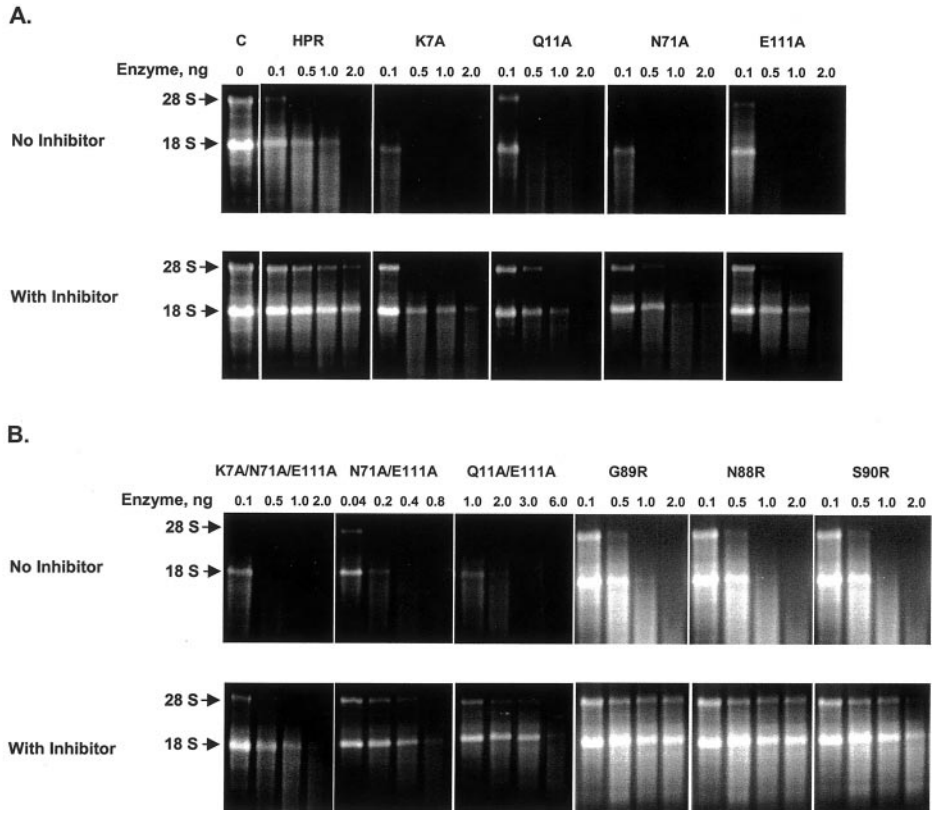


FIG. 3. **Agarose gel-based assay of HPR inhibition by RI.** The extent of RI inactivation of HPR and its mutants was analyzed by visualizing the RNase-catalyzed degradation of the 28 S and 18 S rRNA in the presence and absence of RI (20 units/rxn). *Panel A*, effect of RI on the activity of HPR and single mutants K7A, Q11A, N71A, and E111A. *Panel B*, RNase activity of K7A/N71A/E111A, N71A/E111A, and Q11A/E111A and the arginine mutants N88R, G89R, and S90R in the absence and presence of RI.

## RESULTS

### *Design of HPR Mutants*

The aim of the study was to investigate whether the affinity of HPR for RI could be reduced by mutating specific contact residues, presumably involved in the binding of HPR to RI. We selected target residues in HPR based on two criteria. First, the residue must be involved in binding of HPR with RI by either forming a hydrogen bond or van der Waal contact with RI, as defined by the crystal structure of the RI·RNase complex. Second, the target residue must not be involved in the active site of HPR.

On the basis of homology studies with the residues involved in the RI binding of several RNases, especially RNase A, we selected four target residues in HPR: Lys-7, Gln-11, Asn-71, and Glu-111. We replaced these residues in HPR with alanine to yield mutants K7A, Q11A, N71A, and E111A. Alanine was chosen because it eliminates the side chain beyond the $\beta$-carbon without altering the main conformation. We combined the single mutations to form three double mutants, K7A/E111A, Q11A/E111A, and N71A/E111A; two triple mutants, K7A/N71A/E111A and Q11A/N71A/E111A; and a quadruplet mutant, K7A/Q11A/N71A/E111A.

In RNase A mutation of Gly-88 to Arg has been shown to decrease its sensitivity to RI inactivation and consequently increase the cytotoxic potency by many fold (30). Gly-88 in RNase A is homologous to Gly-89 in HPR. In the current study we individually mutated Gly-89 and also Asn-88 and Ser-90 to Arg, yielding three single mutants, N88R, G89R, and S90R.

### *Expression and Purification of HPR Mutants*

The mutants were expressed in *E. coli*, and the overexpressed proteins, isolated from the inclusion bodies, were purified to homogeneity through a two-step purification scheme comprised of cation exchange and gel filtration chromatography. The purified HPR mutants migrated on SDS-polyacrylamide gel electrophoresis as single bands corresponding to their expected molecular weights (Fig. 1*A*). A polyclonal antibody against HPR reacted with all 13 mutant proteins equally well as shown by the Western blots (Fig. 1*B*). Typical final yields of the purified recombinant proteins were in the range of 10–20 mg/liter of culture.

### *Structural Characterization of the Proteins by Circular Dichroism*

Structural characterization was carried out by CD spectral analysis to study the effect of mutations on the overall conformation of HPR. As shown in Fig. 2, the recombinant HPR appears to be folded compactly with an $\alpha+\beta$ conformation. The CD spectra of the mutant proteins K7A, Q11A, N71A, and E111A (Fig. 2*A*); K7A/E111A, Q11A/E111A, and N71A/E111A (Fig. 2*B*); K7A/N71A/E111A, Q11A/N71A/E111A, and K7A/Q11A/N71A/E111A (Fig. 2*C*); and N88R and G89R (Fig. 2*D*) indicated a modest alteration; however, the overall structure appears to be similar to that of the wild type protein. The conformation of S90R (Fig. 2*D*) was found to be altered compared with the wild type HPR, showing a significant decrease in the $\alpha$-helical content of the mutant.

### *Enzymatic Activity of HPR and Its Mutants*

The RNase activity of the seven single alanine mutants was assayed on three different RNA substrates: poly(C), yeast tRNA, and cyclic CMP. Pancreatic RNases have a preference for pyrimidine-rich RNA substrates. On the single-stranded, pyrimidine homopolymer substrate, poly(C), the mutants K7A, Q11A, N71A, and E111A displayed a similar or higher activity compared with wild type HPR (Table II). On yeast tRNA, the mutant K7A showed 78% activity compared with the wild type enzyme, whereas the mutants Q11A, N71A, and E111A displayed about 50% lower activity (Table II). The hydrolytic activity of HPR and its mutants was studied by spectrophotometrically monitoring the breakdown of the cyclic CMP to 3′-monophosphate. The mutants K7A, Q11A, N71A, and

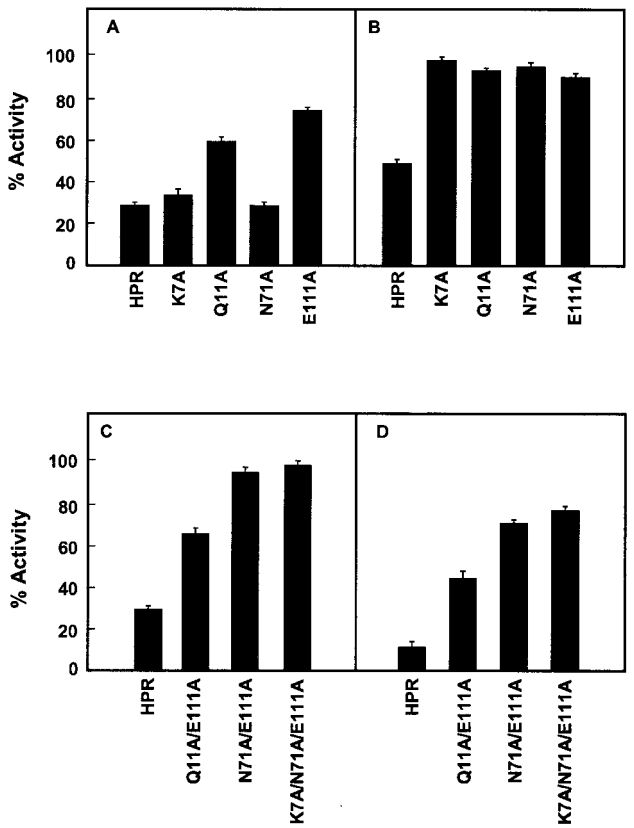


FIG. 4. **RNase activity of HPR mutants on poly(C) in the presence of RI.** The activity of the HPR mutants on the most favored RNA substrate poly(C) in the presence of RI was measured and has been plotted in terms of percent activity compared with the RNase activity of the proteins in the absence of RI. The values presented are means with S.E. from four independent experiments. *Panel A*, activity of mutants K7A, Q11A, N71A, and E111A; 0.8 ng of enzyme and 0.25 unit of RI. *Panel B*, activity of mutants K7A, Q11A, N71A, and E111A; 1.6 ng of enzyme and 0.25 unit of RI. *Panel C*, activity of mutants Q11A/E111A, N71A/E111A, and K7A/N71A/E111A; 0.8 ng of enzyme and 0.25 unit of RI. *Panel D*, activity of mutants Q11A/E111A, N71A/E111A, and K7A/N71A/E111A; 0.8 ng of enzyme and 0.50 unit of RI.

E111A were found to possess hydrolytic activity similar to that of the wild type HPR (Table II).

The mutants K7A, Q11A, N71A, and E111A exhibited activity similar to the wild type enzyme on the most favored dinucleotide substrate CpA (Table II). On UpA, the mutant K7A showed activity similar to that of HPR, but there was a significant loss in activity of the mutants Q11A, N71A, and E111A (Table II). On the dinucleotide substrate UpG, like HPR, mutants K7A and Q11A also were found to be inactive, whereas mutants N71A and E111A displayed RNase activity, with N71A being more active (Table II).

On poly(C), compared with HPR the mutant N71A/E111A was found to have a 3-fold higher activity (Table III). The activity of the mutants N88R, G89R, S90R, K7A/E111A, and K7A/N71A/E111A was found to be similar to that of the wild type HPR on poly(C), whereas the mutants Q11A/E111A, Q11A/N71A/E111A, and K7A/Q11A/N71A/E111A displayed very poor RNase activity (Table III).

### *Interaction of HPR Mutants with RI*

*Agarose Gel-based Assay*—The agarose gel-based assay is a visual qualitative assay in which the extent of RNA degradation observed in the gel is an indication of the effect of RI on HPR activity. In the absence of RI, a progressive increase in total rat liver RNA degradation was observed with increasing amount of HPR (Fig. 3*A*). The RNase shows a preference for the

TABLE IV
*Inhibition constant ($K_i$) values for RI interaction of HPR and its mutants*

The inhibition constants ($K_i$) were determined by measuring the steady-state rate of poly(C) cleavage in the presence of RI. Respective enzymes were incubated with different concentrations of the RNA substrate poly(C) and RI. Initial velocity data were used to prepare Lineweaver-Burk plots, from which the $K_i$ was calculated.

| Protein | $K_i$ |
|---|---|
| | *pM* |
| HPR | 20 |
| K7A | 166 |
| Q11A | 233 |
| N71A | 100 |
| E111A | 366 |
| Q11A/E111A | 212 |
| N71A/E111A | 364 |
| K7A/N71A/E111A | 500 |

28 S rRNA. The mutants K7A, Q11A, N71A, and E111A also displayed RNase activity similar to wild type HPR (Fig. 3*A*). In the presence of RI (20 units), the activity of HPR was significantly inhibited, indicating its high sensitivity to RI (Fig. 3*A*). The single alanine mutants, however, were able to degrade the RNA substrate even in the presence of RI at all enzyme concentrations used, except at a very low concentration of 0.1 ng, implying a decreased inhibitory effect of RI on these mutants (Fig. 3*A*).

The double mutants Q11A/E111A and N71A/E111A and the triple mutant K7A/N71A/E111A also showed a higher resistance to RI, and they were able to degrade rRNA to a greater extent than HPR in the presence of inhibitor (Fig. 3*B*). Because the mutant N71A/E111A was enzymatically 2.5-fold more active, and Q11A/E111A 3-fold less active, in the agarose gel-based assay 2.5 times lower amount of N71A/E111A and 3 times higher amounts of Q11A/E111A were also included to equalize their enzymatic activity with HPR (Fig. 3*B*).

The extent of RNA degradation observed with N88R, G89R, and S90R in the absence or presence of RI was similar to that observed for HPR, implying that these residues are not crucial for HPR-RI binding (Fig. 3*B*).

*Assay of Enzymatic Activity of the Mutants in the Presence of RI*—The RNase activity of the mutants, in the presence of RI, was quantitated by assaying their activity on the most preferred RNA homopolymer substrate, poly(C) (Fig. 4). For these studies mutants K7A, Q11A, N71A, E111A, Q11A/E111A, N71A/E111A, and K7A/N71A/E111A displaying greater resistance to RI were taken. As shown in Fig. 4*A*, at a fixed enzyme concentration of 0.8 ng and RI concentration of 0.25 unit, HPR, K7A, and N71A showed 30% activity, whereas the mutants Q11A and E111A exhibited 60–80% activity. However, when the enzyme concentration was increased to 1.6 ng, all the four mutants showed 100% activity even in the presence of 0.25 unit of RI, whereas HPR exhibited only 50% activity (Fig. 4*B*). These results demonstrate that the four mutants K7A, Q11A, N71A, and E111A are less sensitive to RI than HPR, and among these the mutants Q11A and E111A appear to be more resistant to RI than K7A and N71A (Fig. 4*A*).

Similarly, the mutants Q11A/E111A, N71A/E111A, and K7A/N71A/E111A exhibited 65–95% activity at an enzyme concentration of 0.8 ng and RI concentration of 0.25 unit/rxn, whereas wild type HPR showed only 30% activity (Fig. 4*C*). On further increasing the RI concentration to 0.5 unit/rxn, the three mutants still showed 50–70% activity compared with only 10% of HPR (Fig. 4*D*).

*Inhibition Constants*—The inhibition constants ($K_i$) for the RI-HPR mutant interactions were determined by measuring the steady-state rate of poly(C) cleavage in the presence of RI.

TABLE V
*Cytotoxicity of HPR mutants on various cell line*

Cytotoxicity was evaluated by measuring [$^3$H]leucine incorporation into newly synthesized protein. Results from the cytotoxicity assays are expressed in the form of $ID_{50}$ values. The $ID_{50}$ values represent the concentration of the ribonuclease producing a 50% inhibition of cellular protein synthesis.

| Protein | $ID_{50}$ | | | | |
|---|---|---|---|---|---|
| | U373MG | J774A.1 | K562 | A431 | A549 |
| | | | *μg/ml* | | |
| HPR | >200 | 80 | 150 | 160 | >200 |
| K7A | 120 | 46 | 75 | 62 | 150 |
| Q11A | 98 | 41 | 55 | 60 | 84 |
| N71A | 155 | 57 | 75 | 62 | 84 |
| E111A | 84 | 26 | 54 | 49 | 40 |
| Q11A/E111A | >160 | 42 | 105 | 80 | >160 |
| N71A/E111A | 150 | 52 | 80 | 75 | 150 |
| K7A/N71A/E111A | 20 | 18 | 80 | 57 | 80 |
| Q11A/N71A/E111A | >200 | 68 | 160 | >200 | >200 |
| N88R | >200 | 80 | >200 | >200 | >200 |
| G89R | >200 | 70 | >200 | 175 | >200 |
| S90R | 145 | 38 | 100 | 125 | 120 |

Initial velocity data were used to prepare Lineweaver-Burk plots, from which $K_i$ was calculated. As shown in Table IV, the $K_i$ values for the mutants were significantly higher than that of HPR. The $K_i$ value of the triple mutant K7A/N71A/E111A was 25-fold higher than that of HPR, followed by the mutants N71A/E111A and E111A, which had an 18-fold higher value. Both Q11A and Q11A/E111A had a 10-fold higher $K_i$ value than HPR, whereas those for K7A and N71A were 8- and 5-fold higher, respectively.

### *Cytotoxic Activity of HPR Mutants*

To study the effect of mutations on the interaction of HPR with the intracellular RI, cytotoxic activity of HPR and its mutants was assayed on five different cell lines, U373MG, J774.A1, K562, A431, and A549. All mutants except Q11A/N71A/E111A, N88R, and G89R displayed a higher cytotoxic activity than the wild type HPR, as depicted in their lower $ID_{50}$ values (Table V). The mutants K7A/N71A/E111A, E111A, and Q11A displayed the maximum cytotoxic activity. Out of the five cell lines used in the study, U373MG and J774A.1 were the most sensitive to these mutants. The triple mutant K7A/N71A/E111A was found to be the most potent. It exhibited at least a 10-fold higher cytotoxic activity compared with HPR on U373MG cell line, a 4-fold higher activity on J774A.1, and 2–3-fold higher activity on K562, A431, and A549. Similarly, the cytotoxic activity of the mutants E111A and Q11A varied from at least 2- to 3-fold more than that of HPR (Table V). The mutants N71A/E111A, Q11A/E111A, K7A, N71A, and S90R were found not to be as potent; however, they exhibited up to a 2-fold higher cytotoxic activity than HPR depending on the cell line. The mutants Q11A/N71A/E111A, N88R, and G89R showed cytotoxic activity similar to that of HPR on all cell lines studied (Table V).

## DISCUSSION

An important prerequisite for a RNase to act as a cytotoxic molecule is its ability to escape inactivation by RI, present in the cytosol of mammalian cells, which functions to preserve the integrity of cellular RNA (22, 25, 37). Onconase, although a much weaker RNase than RNase A, is highly cytotoxic because it evades RI exceptionally well (19, 24). In RNase A replacing Gly-88 with arginine or aspartic acid has been shown to result in $10^3$-$10^4$ fold higher resistance to hRI, and the mutants exhibit a potent toxic effect on K562 cells (30).

In this study, with an aim to investigate the HPR-hRI interaction and to generate a cytotoxic HPR mutant(s), we have mutated, either individually or in combination, seven residues in HPR which are presumably involved in its interaction with RI. The individual mutations of the four residues Lys-7, Gln-11, Asn-71, and Glu-111 were not detrimental to the activity of HPR on poly(C); however, these mutants had reduced activity on yeast tRNA. These residues appear to be involved in substrate binding in HPR, similar to that in RNase A. In RNase A, Lys-7 is present in the phosphate binding subsite (38–41), whereas Asn-71 and Glu-111 are present in the base binding subsite (41–43). The primary role of Gln-11, a conserved residue that donates a hydrogen bond to the reactive phosphoryl group of the bound substrate, is to prevent the nonproductive binding of the substrate (44). Using dinucleotide monophosphates as substrates, Witzel and Barnard (36) showed that the rate constant of RNase A is higher when the base at 5′ position is a purine, the order being A > G > C > U (36). The mutations N71A and E111A produced a change in the substrate specificity of HPR. The four individual mutations were combined further to prepare mutants K7A/E111A, Q11A/E111A, N71A/E111A, K7A/N71A/E111A, Q11A/N71A/E111A, and K7A/Q11A/N71A/E111A. However, only K7A/E111A, N71A/E111A, and K7A/N71A/E111A had full enzymatic activity. An interesting inference from these inactive mutants is that the presence of either Gln-11 or Glu-111 in HPR appears to be absolutely essential for the full ribonucleolytic activity of the enzyme, and simultaneous mutation of both these residues is detrimental to the activity of HPR.

The mutation of Lys-7, Gln-11, Asn-71, and Glu-111 to Ala resulted in a decrease in the sensitivity of HPR to RI inactivation. There was a further augmentation in resistance to RI on combining these individual mutations, as seen in the case of the active double mutant N71A/E111A and triple mutant K7A/N71A/E111A. The triple mutant K7A/N71A/E111A showing the maximum RI resistance had a $K_i$ value 25-fold greater than that of HPR. The greater ability of the mutants to escape RI inactivation was also reflected in their improved cytotoxic activity on a variety of cell lines. The triple mutant K7A/N71A/E111A was found to be most potent, displaying a minimum 10-fold higher cell killing ability than HPR on the glioma cell line U373MG. The double mutant Q11A/E111A, despite being 60% less active than HPR, was found to be almost 2-fold more cytotoxic than the wild type enzyme. The 10-fold higher RI resistance of the double mutant Q11A/E111A, compared with HPR, appears to be responsible for its enhanced cytotoxic activity. A similar result was observed in RNase A by Bretscher *et al.* (45). They found a double mutant K41R/G88R of RNase A to be enzymatically less active than the single G88R mutant but more cytotoxic. The double mutant showed a very low affinity for RI, which apparently accounts for the enhanced

cytotoxicity. In contrast, another double mutant K41A/G88R of RNase A, which has the same affinity for RI but is a much weaker RNase compared with K41R/G88R mutant, is not cytotoxic. These data suggest that for a variant of RNase A to be cytotoxic, it is necessary to maintain sufficient ribonucleolytic activity (45). In the current study equivalent mutations in HPR did not produce similar results, and the mutations of Asn-88 and Gly-89 to Arg had no effect on the interaction of HPR with RI. Only the mutant S90R displayed a higher cytotoxicity than HPR. Pous *et al.* (29) have also reported similar results with a N88R/G89R double mutant of HPR. Our study clearly demonstrates that even though RNase A and HPR share a very close homology, with the key structural and catalytic residues identified in the bovine analog retained in the human enzyme, the observations with RNase A cannot be fully extended in HPR.

Recently the crystal structure of a variant of HPR has been determined (29). The variant has 5 residues in the first 20 residues in its amino terminus replaced by the equivalent residues in the BS-RNase. The structure exhibits three helices ($\alpha$, $\alpha2$, and $\alpha3$) and seven $\beta$ strands ($\beta1$–$\beta7$). Strand $\beta1$ is positioned between the helices $\alpha2$ and $\alpha3$, and the rest of the strands are located in sequence after $\alpha3$. Strands $\beta3+\beta4$ and $\beta5+\beta6$ run antiparallel and form a twisted $\beta$ sheet defining the V-shaped cleft where the active site is located. The core structure of the HPR variant is very similar to that of RNase A; however, it differs in the loop regions. The active site cleft shows an architecture similar to that of RNase A with essential amino acids occupying the equivalent positions. However, remarkable differences are found at loops $\beta4\beta5$ (residues 90 and 91), and $\alpha2\beta1$ (residues 37 and 38). The loop $\beta2\beta3$ in the HPR variant also has a different conformation compared with that in RNase A (residue 67). From the three-dimensional structure of pRI·RNase A and angiogenin·hRI complexes it is apparent that the contact surface mainly involves $\alpha2\beta1$, $\beta2\beta3$, and $\beta4\beta5$ in RNase A and loops $\alpha2\beta1$ and $\beta4\beta5$ in angiogenin, apart from the residues belonging to their respective active sites (24, 27, 28). The loop comprising amino acids 87–89 has been shown to be highly exposed in HPR structure with a different conformation; accordingly, it is proposed that regions $\alpha2\beta1$ (residues 33–43) and $\beta2\beta3$ (residues 64–71) might be involved significantly in the interaction of HPR with RI (29). Our results also support this proposal as we have observed an increased resistance in HPR variants containing Asn-71 mutation, which lies in the $\beta2\beta3$ region. Based on the crystal structures of the RI·RNase A complex and hot spot mutagenesis in hRI, residues Lys-7 and Gln-11 of RNase A interact with Ser-460; Asn-71 with Tyr-437; and Glu-111 with Tyr-437, Trp-438, Ser-439, and Glu-460 of the inhibitor (46–48). In this study with HPR we also observed increased resistance in HPR mutants where these residues were mutated.

In conclusion, we have demonstrated that residues Lys-7, Gln-11, Asn-71 and Glu-111 in HPR are involved in its interaction with the hRI, and mutants with these residues replaced by alanine have higher resistance toward inactivation by RI. Further investigation of contact residues might prove useful in developing much more potent cytotoxic variants of HPR.

REFERENCES


1. Beintema, J. J., Breukelman, H. J., Carsana, A., and Furia, A. (1997) in *Ribonucleases*: *Structures and Functions* (D'Alessio, G., and Riordan, J. F., eds) pp. 245–269, Academic Press, New York
2. D'Alessio, G. (1993) *Trends Cell Biol.* **3,** 106–109
3. Domachowske, J. B., and Rosenberg, H. F. (1997) *J. Leukocyte Biol.* **62,** 363–368
4. Lehrer, R. I., Szklarek, D., Barton, A., Ganz, T., Hamann, K. J., and Gleich, G. (1989) *J. Immunol.* **142,** 4428–4434
5. Rosenberg, H. F. (1995) *J. Biol. Chem.* **270,** 7876–7881
6. Durack, D. T., Ackerman, S. J., Loegering, D. A., and Gleich, G. J. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 5165–5169
7. Newton, D. L., Walbridge, S., Mikulski, S. M., Ardelt, W., Shogen, K., Ackerman, S. J., Rybak, S. M., and Youle, R. J. (1994) *J. Neurosci.* **14,** 538–544
8. Nitta, K., Ozaki, K., Ishikawa, M., Furusawa, S., Hosono, M., Kawauchi, H., Sasaki, K., Takayanagi, Y., Tsuiki, S., and Hakomori, S. (1994) *Cancer Res.* **54,** 920–927
9. Mikulski, S. M., Grossman, A. M., Carter, P. W., Shogen, K., and Costanzi, J. J. (1993) *Int. J. Oncol.* **3,** 57–64
10. Vescia, S., Tramontano, D., Augusti-Tocco, G., and D'Alessio, G. (1980) *Cancer Res.* **40,** 3740–3744
11. Laccetti, P., Portella, G., Mastronicola, M. R., Russo, A., Piccoli, R., D'Alessio, G., and Vecchio, G. (1992) *Cancer Res.* **52,** 4582–4586
12. Beintema, J. J., Wietzes, P., Weickmann, J., and Glitz, J. J. (1984) *Anal. Biochem.* **136,** 48–64
13. Seno, M., Futami, J., Kosaka, M., Seno, S., and Yamada, H. (1994) *Biochim. Biophys. Acta* **1218,** 466–468
14. Weickmann, J. L., Elson, M., and Glitz, D. G. (1981) *Biochemistry* **20,** 1272–1278
15. Bardon', A., Sierakowska, H., and Shugar, D. (1976) *Biochim. Biophys. Acta* **438,** 461–473
16. Sorrentino, S., and Libonati, M. (1994) *Arch. Biochem. Biophys.* **312,** 340–348
17. Sorrentino, S., Glitz, D. G., Hamann, K. J., Loegering, D. A., Checkel, J. L., and Gleich, G. J. (1992) *J. Biol. Chem.* **267,** 14859–14865
18. Bal, H. P., and Batra, J. K. (1997) *Eur. J. Biochem.* **245,** 465–469
19. Ardelt, W., Mikulski, S. M., and Shogen, K. (1991) *J. Biol. Chem.* **266,** 245–251
20. Saxena, S. K., Gravell, M., Wu, Y.-N., Mikulski, S. M., Shogen, K., Ardelt, W., and Youle, R. J. (1996) *J. Biol. Chem.* **271,** 20783–20788
21. Saxena, S. K., Rybak, S. M., Winkler, G., Meade, H. M., McGray, P., Youle, R. J., and Ackerman, E. J. (1991) *J. Biol. Chem.* **266,** 21208–21214
22. Wu, Y. N., Mikulski, S. M., Ardelt, W., Rybak, S. M., and Youle, R. J. (1993) *J. Biol. Chem.* **268,** 10686–10693
23. Murthy, B. S., and Sirdeshmukh, R. (1992) *Biochem. J.* **281,** 343–348
24. Kobe, B., and Diesenhofer, J. (1995) *Nature* **374,** 183–186
25. Lee, F. S., and Vallee, B. L. (1993) *Prog. Nucleic Acids Res.* **44,** 1–30
26. Hofsteenge, J. (1997) in *Ribonucleases*: *Structures and Functions* (D'Alessio, G., and Riordan, J. F., eds) pp. 621–658, Academic Press, New York
27. Kobe, B., and Diesenhofer, J. (1996) *J. Mol. Biol.* **264,** 1028–1043
28. Papageorgiou, A. C., Shapiro, R., and Acharya, K. R. (1997) *EMBO J.* **16,** 5162–5177
29. Pous, J., Canals, A., Terzyan, S. S., Guasch, A., Benito, A., Ribo, M., Vilanova, M., and Coll, M. (2000) *J. Mol. Biol.* **303,** 49–59
30. Leland, P. A., Wayne Schultz, L., Kim, B. M., and Raines, R. T. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10407–10412
31. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382
32. Sanger, F., Niklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
33. Buchner, J., Pastan, I., and Brinkmann, U. (1992) *Anal. Biochem.* **205,** 263–270
34. Bond, M. D. (1988) *Anal. Biochem.* **173,** 166–173
35. Crook, E. M., Mathias, A. P., and Rabin B. R. (1960) *Biochem. J.* **74,** 234–238
36. Witzel, H., and Barnard, E. A. (1962) *Biochem. Biophys. Res. Commun.* **7,** 295–299
37. Kawanomoto, M., Motojima, K., Sasaki, M., Hattori, H., and Goto, S. (1992) *Biochim. Biophys. Acta* **1129,** 335–338
38. Richardson, R. M., Pares, X., and Cuchillo, C. M. (1990) *Biochem. J.* **267,** 593–599
39. Boque, L., Coll, M. G., Vilanova, M., Cuchillo, C. M., and Fita, I. (1994) *J. Biol. Chem.* **269,** 19707–19712
40. Boix, E., Nogues, M. V., Schein, C. H., Benner, S. A., and Cuchillo, C. M. (1994) *J. Biol. Chem.* **269,** 2529–2534
41. Fontecilla-Camps, J. C., de Liorens, R., le Du, M. H., and Cuchillo, C. M. (1994) *J. Biol. Chem.* **269,** 21526–21531
42. Zegers, I., Maes, D., Dao-Thi, M., Poortmans, F., Palmer, R., and Wyns, L. (1994) *Protein Sci.* **3,** 2322–2339
43. Pavlovsky, A. G., Borisova, S. N., Borisov, V. V., Antonov, I. V., and Karpeisky, M. Y. (1978) *FEBS Lett.* **92,** 258–262
44. delCardayre, S. B., Ribo, M., Yokel, E. M., Quirk, D. J., Rutter, W. J., and Raines, R. T. (1995) *Protein Eng.* **8,** 261–273
45. Bretscher, L. E., Abel, R. L., and Raines, R. T. (2000) *J. Biol. Chem.* **275,** 9893–9896
46. Shapiro, R., Ruiz-Gutierrez, M., and Chen, C.-Z. (2000) *J. Mol. Biol.* **362,** 487–519
47. Chen, C.-Z., and Shapiro, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1761–1766
48. Chen, C.-Z., and Shapiro, R. (1999) *Biochemistry* **38,** 9273–9285