The Rana catesbeiana (bullfrog) ribonucleases, which belong to the RNase A superfamily, exert cytotoxicity toward tumor cells. RC-RNase, the most active among frog ribonucleases, has a unique base preference for pyrimidine-guanine rather than pyrimidine-adenine in RNase A. Residues of RC-RNase involved in base specificity and catalytic activity were determined by site-directed mutagenesis, $k_{cat}/K_m$ analysis toward dinucleotides, and cleavage site analysis of RNA substrate. The results show that Pyr-1 (N-terminal pyroglutamate), Lys-9, and Asn-38 along with His-10, Lys-35, and His-103 are involved in catalytic activity, whereas Pyr-1, Thr-39, Thr-70, Lys-95, and Glu-97 are involved in base specificity. The cytotoxicity of RC-RNase is correlated, but not proportional to, its catalytic activity. The crystal structure of the RC-RNase-d(ACGA) complex was determined at 1.80 Å resolution. Residues Lys-9, His-10, Lys-35, and His-103 interacted directly with catalytic phosphate at the P1 site, and Lys-9 was stabilized by hydrogen bonds contributed by Pyr-1, Tyr-28, and Asn-38. Thr-70 acts as a hydrogen bond donor for cytosine through Thr-39 and determines B1 base specificity. Interestingly, Pyr-1, Asp-83, Phe-120, and Ser-123, at the P1 site are involved in the binding of the 5′-ribonucleoside, pyrimidine, whereas two residues, Asn-71 and Glu-111, at the B2 site are involved in the binding of the 3′-ribonucleoside, adenosine (14–18).

A new group of ribonucleases with antitumor activity has been found mainly in frog, i.e. onconase from Rana pipiens (9), RC-RNase, RC-RNase 2 – RC-RNase 6, and RC-RNase L1 from Rana catesbeiana (19), and a sialic acid-binding lectin from Rana japonica (20). These frog ribonucleases are composed of 104–111 amino acid residues, which are similar to mammalian ribonucleases in their amino acid sequence. The conserved amino acid residues for the catalytic activity, i.e. His-12, Lys-41, and His-119 in RNase A, are also found in frog ribonucleases. However, some distinct properties are found mainly in frog ribonucleases rather than in mammalian ribonucleases, i.e. cytotoxicity toward tumor cells, substrate preference for pyrimidine-guanine, the presence of pyroglutamate at the N terminus, a specific location of the fourth disulfide bridge, and resistance to ribonuclease inhibitor from human placenta (4–6).

To investigate the novel properties of frog ribonucleases, we mutated the residue potentially involved in the catalytic activity and substrate specificity of RC-RNase based on the alignment of amino acid sequences in the ribonuclease superfamily (Fig. 1) and the known structures of frog ribonucleases, e.g. onconase (21) and RC-RNase (1KM8). Our results show that the N-terminal pyroglutamate (Pyr-1), Lys-9, and Asn-38 along with the conserved His-10, Lys-35, and His-103 residues are involved in the catalytic activity of RC-RNase, whereas

The abbreviations used are: Pyr-1, N-terminal pyroglutamate; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
residues Thr-39 and Thr-70 are involved in B1 base recognition and Ppyr-1, Lys-95, and Glu-97 are involved in B2 base recognition. The cytotoxicity of RC-RNase is correlated, but not proportional, to its catalytic activity. To elucidate the molecular interaction between frog ribonuclease and possible RNA targets in tumor cells, we grew the co-crystal of RC-RNase and substrate analog d(ACGA) and illustrated the possible hydrogen bonds for the specific recognition of RC-RNase on RNA substrate.

EXPERIMENTAL PROCEDURES

Construction of Expression Vector—The cDNA of wild type RC-RNase was cloned from bullfrog liver using a SMART™ rapid amplification of cDNA ends kit from Clontech (Palo Alto, CA) as described previously (6). Various substitutional and deletional mutants were constructed based on the alignment of amino acid sequence in the ribonuclease family (Fig. 1) and the structure of frog ribonuclease, e.g., onconase (21) and RC-RNase (1KM8). These mutants included RC-Q1A, RC-Q1E, RC-Q1N, RC-Q1S, RC-QNW1, RC-K9A, RC-K9Q, RC-L20A, RC-L20D, RC-L20S, RC-L20Q, RC-N36D, RC-L42A, RC-L42D, RC-L42S, RC-Q1A, RC-Q1E, RC-Q1N, RC-Q1S, RC-QNW1, RC-K9A, RC-K9Q, RC-T39A, RC-T39D, RC-T39S, RC-QNW1, RC-N57A, RC-N59A, RC-RNase 3 (X16546), RC-RNase 6 (AF242556), and RC-RNase L1 (AF288642) are the ribonucleases from R. catesbeiana. Onconase (AF332139) is the ribonuclease from R. pipiens. hRNase A (X07283) is the bovine pancreatic ribonuclease. hRNase 1 (D26129) is the human RNase 4. hRNase 2 (X16546) is the human eosinophil-derived neurotoxin. hRNase 3 (X16545) is the human eosinophil cationic protein. hRNase 4 (D37931) is the human angiogenin. Conserved catalytic residues are boxed, and cysteines for disulfide bridges are shown in gray.

Ribonuclease Activity Assay—Ribonuclease activity was determined by the release of acid-soluble ribonucleotides from the RNA substrate following ribonuclease digestion. The yeast total RNA (120 μg) was incubated with purified ribonuclease in 50 μl of 100 mM Tris-HCl buffer, pH 8.0, at 37 °C for 10 min, and the reaction was terminated by the addition of ice-cold stop solution (7% perchloric acid, 0.1% uranylacetate, 200 μl). The reaction mixtures remained on ice for 30 min before centrifugation (12,000 × g, 4 °C for 20 min). The absorbance of the supernatant was measured at 260 nm. One unit of ribonuclease activity is defined as the amount of enzyme producing one μmol acid-soluble material at 37 °C for 10 min. Ribonuclease activities were also analyzed by zymogram assay on RNA-casting PAGE (22). Briefly, after electrophoresis the gel was washed twice with 25% isopropanol alcohol in 10 mM Tris-HCl, pH 7.5, to remove SDS for protein renaturation. The activity was visible after incubating the gel at room temperature for 30 min in 10 mM Tris-HCl, pH 7.5, and staining with 0.2% toluidine blue O for 1 h.

Base Specificity of Ribonucleases—The specific cleavage sites of ribonucleases were determined by incubation of ribonucleases with 5′-32P-labeled synthetic 18-mer RNA with the known sequence 5′-AAG-GGUAAUCCGACUGAA-3′, followed by denaturing gel electrophoresis and autoradiography (22). The kcat/Km values of ribonucleases toward dinucleotides, e.g., CpG, UpG, UpU, Cpatial, and UpA, were measured by HPLC separation and quantification (23, 24). Briefly, the dinucleotides were digested with ribonucleases at 37 °C for 10 min in buffer containing 100 mM MES, pH 6.0, 50 mM NaCl, and 0.1 mg/ml RNase-free bovine serum albumin. Digested nucleotides were separated by reverse-phase HPLC using 1–5% acetonitrile (depending on products) in 0.1% trifluoroacetic acid on a Vydac C18 column with a Waters automated gradient controller. The kcat/Km values were calculated after quantifi-
Some residues are involved in catalytic activity. Values were obtained from at least two independent experiments.

**Assay of Cytotoxicity by ATP Lite-M Measurement—**HeLa cells (5 × 10⁵) were grown in 100 µl of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in 96-well plates and treated with 2 µm ribonuclease for 48 h. 50 µl of lysis buffer was added to each well and incubated for 2 min before 50 µl of substrate solution was added. The luminescence was measured in a dark adapt plate by Top Count Microplate Scintillation Counter (Packard A Canberra Company) according to the manufacturer’s instructions (ATP Lite-M assay system, Packard BioScience Company, The Netherlands) (6). The cytotoxicity was expressed in percentage of loss in cell viability caused by RC-RNase treatment compared with control cells. The value was determined from the mean of six wells.

**Assay of RNase Activity, Specificity, and Cytotoxicity**—RNase A, onconase (AF332139), cloned in this laboratory, the wild type RC-RNase gene, and the mutated RC-RNase genes were expressed in *E. coli* BL21(DE3). The secreted proteins in the media were purified to homogeneity by phosphocellulose and carboxymethyl column chromatographies (Fig. 2A). Onco-nase and most of the recombinant RC-RNases, except for those with N-terminal mutants, possessed Pyr at their N termini as did that of native frog RC-RNase detected by mass spectrum analysis and Edman degradation after *Pfu* aminopeptidase treatment (6). RC-Q1E had two kinds of product, ended with pyroglutamate or glutamate at the N terminus, which were separated by FPLC Mono S column chromatography using 0.07–0.11 M NaCl salt gradient elution (Amersham Biosciences).

The CD spectra of most RC-RNase mutants were analyzed to rule out the possibility of incorrect folding. There was no significant difference among native RC-RNase, wild type secretory RC-RNase, RC-Q1A, RC-Q9A, RC-N38Q, RC-T39A, RC-T70A, RC-T70D, RC-K95A, RC-E97A, RC-E97D, RC-V102A, RC-V102D, and RC-V102D, some CD spectra of which are shown in Fig. 3. This similarity indicates that the secondary structures of these RC-RNase mutants are similar to that of native RC-RNase.

**Relative Ribonuclease Activity of RC-RNase and Its Variants**—The ribonuclease activities of RC-RNase and its mutants were analyzed using an acid-insoluble method as shown in Table II. The specific activity of RC-RNase toward yeast total RNA was slightly less than that of native RC-RNase (19), whereas the catalytic activity of both RC-RNase mutants were expressed in wild type RC-RNase gene, and the mutated RC-RNase genes, were separated by 13.3% SDS-PAGE and stained with Coomassie Blue. B, zymogram analysis. Ribonucleases (1 ng) were separated by RNA casting 13.3% nonreducing SDS-PAGE and stained by toluidine blue O (19).
(Table II). We found that 5.8% of activity remained in RC-T39A, 41.0% in RC-T39S, 65.8% in RC-T70A, 79.6% in RC-T70S, 21.5% in RC-F104A, and 79.2% in RC-V37A. Similarly, residues adjacent to the hypothesized B2 site were also mutated and assayed for catalytic activity. The RC-K95A (24.2% remained), RC-E97A (11.6%), RC-V102A (19.1%), and RC-V102D (3.4%) had a marked reduction in activity, whereas RC-N57A (48.7%) and RC-N59A (76.2%) did not exhibit marked reduction. These findings suggest that Thr-39, Lys-95, Glu-97, Val-102, and Phe-104 may participate in the catalytic activity of RC-RNase through base recognition.

The relative activity of most RC-RNase mutants was also determined by zymogram as shown in Fig. 2B. Most of the recombinant RC-RNases had the same mobility as native RC-RNase, except for RC-K9A and RC-K95A, which likely have a more compact structure than native RC-RNase. The relative activity of these recombinant ribonucleases shown by zymogram is in good agreement with the activity obtained by the acid-insoluble method (Table II). One exception was RC-K95A, which was less active than native RC-RNase when assayed by the acid-insoluble method, but it was more active than native RC-RNase when assayed by zymogram. This discrepancy in relative activity may result from the difference in molecular weight of the nucleotides released from the gel and that remained in aqueous solution. Because of the broader base specificity of the RC-K95A (see below) and complete digestion of RNA substrate in the gel, the shorter oligonucleotide produced by RC-K95A tends to diffuse from gel. In contrast, the RNA was only partially digested by the ribonuclease to obtain the absorbance of soluble oligonucleotides within the linear range (A260 0.1–0.3).

**Residues Involved in B1 Base Recognition**—The residues adjacent to the predicted B1 site, Val-37, Thr-39, Thr-70, and Phe-104, were mutated and analyzed for base specificity. We found that both CpG and UpG of an 18-mer RNA were cleaved by native and wild type recombinant RC-RNase, whereas CpG was preferentially cleaved by the T39A or T70A mutant (Fig. 4). However, both CpG and UpG were cleaved if Thr-39 or Thr-70 was replaced by a cognate residue in RC-T39S or RC-T70S. Interestingly, UpG of 18-mer RNA was more susceptible than CpG in T70D. The catalytic activity and substrate binding affinities of Thr-39 and Thr-70 mutants toward these dinucleotides CpG or UpG were determined by kcat/Km value analyses (Table III). RC-T70A had a decreased Km for CpG (17%) and increased Km for UpG (21%), whereas RC-T70D had an increased Km for CpG (17%) and decreased Km for UpG (54%). These findings indicate that the hydroxyl group of Thr-70 allows the binding of both cytosine and uracil at B1 site, whereas the carbonyl group of Asp-70 favors the binding of uracil. The base specificity of RC-RNase was not changed significantly in RC-F104A.

**Residues Involved in B2 Base Recognition**—The residues close to the predicted B2 site, Asn-57, Asn-59, Lys-95, Glu-97, and Val-102, were mutated and analyzed for base specificity. The results shown in Table III and Fig. 4A indicate that the base specificities were not significantly changed in N57A and N59A mutants. CpU, along with the original scissile substrate CpG and UpG, was cleaved by RC-E97A. The kcat/Km value of E97A for CpG and UpG decreased to 7 and 6, respectively, whereas their Km values increased 2- and 5-fold, respectively. These results indicate that the binding affinities of E97A for CpG and UpG decrease. When the nearby residue Lys-95 was mutated to Ala, Cpc, Cpa, CpU, UpA, and UpU, along with the original scissile substrate CpG and UpG, were cleaved. The kcat/Km value for CpG and UpG was dramatically decreased to 17 and 36, respectively, whereas the kcat/Km value for UpA increased 1.6-fold compared with that of the wild type RC-RNase. With regard to the substrate binding affinity, the Km of the K95A for UpA decreased to 45%, whereas that for UpG increased to 564%. Similar results were also found in K95G, K9ST, and K95R mutants (data not shown). These results indicate that Lys-95 mutation induced the RC-RNase to change its B2 base specificity from guanine to adenine. Although the base specificity of V102A did not change, the kcat/Km values for five dinucleotides decreased dramatically (5–9% left) (Fig. 4 and Table III).
**N-terminal Pyroglutamate Involved in B2 Base Specificity**—The CpA, CpC, CpU, UpA, UpU as well as the original CpG and UpG of 18-mer RNA were cleaved by Pyr-1 substitutional mutants (RC-Q1A, RC-Q1N, and RC-Q1S) or deletion mutants (dN2 and dQNW1–3), however, only CpG, UpG, and UpU were cleaved by substitution mutants (RC-Q1E, RC-N2D, and RC-K9A) (Fig. 4B). Therefore, it is concluded that Pyr-1 in the right position plays a dual role in both catalytic activity (Table II and Table III) and B2 base specificity (Fig. 4B), and the cognate residue Glu-1 may exert a function similar to that of Pyr-1.

**Residues Involved in Cytotoxicity**—In general, the cytotoxicity of an RC-RNase variant is correlated with its catalytic activity except Lys-9, Asn-38, and Val-102 mutations (Table II). Both RC-N38Q and RC-V102A retained 14.6 and 19.1% activity while still exerting 78.2 and 84.4% cytotoxicity, respectively. In contrast, RC-N38A and RC-V102D lost both catalytic activity (1.0%, 3.4% remained) and cytotoxicity (1.5%, 2.0% remained). Both RC-K9A and RC-K9Q lost most of their catalytic activity (2.2%, 1.9% remained), but they retained differential cytotoxicity (66.7% and 9.2% left, respectively).

**Quality and Statistics of the Structure of the RC-RNase/H18528(ACGA) Complex**—To determine the detail of interactions between RC-RNase and RNA substrate for catalytic reaction and base recognition, the co-crystal of the RC-RNase/H18528(ACGA) complex was grown, and its structure was solved by molecular replacement and refined to 1.80 Å resolution. The final model contained 1928 non-hydrogen, non-solvent atoms and 323 water molecules. The crystallographic R-factor and free R-factor of the final model were 18.9 and 22.9%, respectively. The statistics of refinement are listed in Table I. The crystals belong to the space group P2₁ and have two 1:1 complexes per asymmetric unit. The two independent molecules are related by a noncrystallographic 2-fold axis (Fig. 5).
The structures of all of the residues were defined unambiguously except the weak electron density region at the 5′-end adenosines of d(ACGA). The 3′-end adenosine has two conformational disorders. The average temperature factors for the main chain and side chain atoms are 20.0 and 22.2 Å², respectively. However, there was a difference noted in the average temperature factors between the 3′-end adenosines and the 5′-end adenosine. The average temperature factor of the central d(CG) was only 24.2 Å², which is located clearly in the electron density map.

The Co-crystal Structure of the RC-RNase-d(ACGA) Complex—The overall dimension (backbone to backbone) of the two monomers is about 68 Å × 43 Å × 38 Å (Fig. 5). Interestingly, the base stacking and continuity of the phosphate backbone of the two d(ACGA) molecules are clearly seen in the central area of the diagram. This suggests that a continuous RNA is bound to an RC-RNase. The secondary structures of the RC-RNase-d(ACGA) complex crystal were analyzed using the PROMOTIF program (33). There are three α-helices at α1 (3–10), α2 (19–22), and α4 (45–49), and two 310 helices at α3 (26–28) and α5 (50–52). The four β-strands β4 (37–42), β6 (66–73), β7 (83–90), and β1 (11–12) form sheet A. The β-sheet B contains strand β5 (57–61), β8 (92–97), and β9 (100–107). Two additional short strands, β2 (29–30) and β3 (33–34), form a small sheet. There is one inverse γ-turn at 23–25. There are nine

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**Table III**

Substrate specificities of RC-RNase and its variants

| Enzyme | CpG | UpG | UpU | CpU | UpA |
|--------|-----|-----|-----|-----|-----|
| Wild type | 81 ± 2 (100) | 110 ± 4 (100) | 1.3 ± 0.03 (100) | 0.8 ± 0.02 (100) | 0.4 ± 0.02 (100) |
| P1 site | Q1A | 0.5 ± 0.01 (6) | 1 ± 0.1 (3) | 0.007 ± 0.0002 (1) | 0.007 ± 0.0002 (1) |
| | K9A | 4 ± 0.1 (5) | 12 ± 0.3 (11) | 0.01 ± 0.001 (1) | 0.01 ± 0.0001 (1) |
| B1 site | T39A | 6 ± 0.4 (8) | 0.3 ± 0.01 (0.3) | ND | ND |
| | T39S | 27 ± 0.7 (28) | 39 ± 7 (36) | 0.1 ± 0.03 (11) | ND |
| | T70A | 300 ± 8 (376) | 5 ± 0.3 (5) | 0.01 ± 0.001 (1) | 7 ± 0.08 (888) |
| | T70S | 130 ± 5 (157) | 98 ± 6 (91) | 2 ± 0.03 (163) | 2 ± 0.03 (177) |
| | T70D | 9 ± 0.1 (11) | 62 ± 5 (58) | 0.9 ± 0.02 (71) | 0.1 ± 0.001 (13) |
| | F104A | 7 ± 0.1 (9) | 7 ± 0.6 (6) | 0.05 ± 0.0001 (4) | 0.02 ± 0.0005 (3) |
| B2 site | N57A | 32 ± 2 (40) | 92 ± 9 (86) | 0.4 ± 0.02 (28) | 0.2 ± 0.01 (22) |
| | N59A | 78 ± 7 (96) | 84 ± 6 (78) | 1.2 ± 0.07 (99) | 0.8 ± 0.01 (93) |
| | K95A | 14 ± 1 (17) | 15 ± 1 (36) | 0.1 ± 0.001 (11) | 0.1 ± 0.003 (11) |
| | K97A | 6 ± 0.1 (7) | 6 ± 0.3 (6) | 0.1 ± 0.01 (10) | 0.1 ± 0.02 (16) |
| | V102A | 5 ± 0.2 (6) | 9 ± 0.1 (8) | 0.08 ± 0.004 (6) | 0.04 ± 0.002 (5) |

a The values in parentheses are expressed in percentage compared with that of wild type RNase.

*ND, nondetectable*


**DISCUSSION**

RC-RNase possesses a conserved amino acid sequence and tertiary structure similar to those of ribonuclease in the RNase A superfamily including three catalytic residues, two triple-stranded antiparallel β-sheets, and three α-helices (4, 5, 35). However, the frog ribonucleases in the superfamily possess a distinct base preference and antitumor activity (4, 5). Based on a comparison of the primary structure, the RC-RNase has special residues, e.g., Pyr-1, Lys-9, Thr-70, and Lys-95, which differ from those of mammals. In this report, these residues were mutated and analyzed for catalytic activity, base specificity, and cytotoxicity. The secondary structures of these mutated RC-RNases were similar to native RC-RNase based on the CD spectrum, and thus these residues may be responsible for these novel properties rather than conformational changes. The structure of ribonuclease-oligonucleotide complex has been solved, e.g., RNase A complexed with d(ATAAG), whereas retrobindings were observed in the crystal structure of RNase A complexed with dinucleotide, 2′,5′-UpG, 2′,5′-CpG, or 3′,5′-d(CpG) or in the solution structure of RC-RNase complexed with 2′,5′-CpG or 3′,5′-d(CpG) (17, 35–37). In retrobinding form, the orientation of substrate differs from that in the catalytic binding form. In the case of CpG, sulfate or phosphate occupies the P1 site, and guanine locates in the B1 site, whereas cytosine is not clearly observed. In this report, we grew the RC-RNase-d(ACGA) complex in the catalytic binding form and analyzed the molecular interaction between RC-RNase and the substrate analog.

Regarding base specificity, all members of the RNase A superfamily prefer pyrimidine at the B1 site (38). Some show equal preference for cytosine and uracil, e.g., bovine RNase A, human pancreatic ribonuclease, bullfrog RC-RNase and RC-RNase L1 (19, 36, 39). Others prefer uracil, e.g., human RNase 4, frog onconase, and RC-RNase 2 (19, 40, 41), and the third group prefer cytosine, e.g., human RNase 2, human RNase 5 and frog RC-RNase 4 (19, 23, 42). For RNase A, it is known that Thr-45, Asp-83, Phe-120, and Ser-123 are involved in the B1 base recognition. Thr-45 and Phe-120 are conserved in the
RNase A superfamily and play a role in pyrimidine binding through hydrogen bonds and van der Waals interactions, respectively (43). Although Asp-83 and Ser-123 are not conserved in the RNase A superfamily, they determine the enzyme configuration and B1 base specificity of RNase A through hydrogen bonds to Thr-45 and uracil, respectively (43).

In RC-RNase, Thr-39, Phe-104, Thr-70, and Ile-107 are the equivalent residues of RNase A Thr-45, Phe-120, Asp-83, and Ser-123, respectively. In this study, we found that the wild type RC-RNase has an equal preference for uracil and cytosine at the B1 site, RC-T70A prefers cytosine, and RC-T70D prefers uracil (Table IV and Fig. 4A). T70A has a higher affinity (low \( K_m \), 17%) and RC-T70D a lower affinity (high \( K_m \), 178%) for CpG compared with that of wild type RC-RNase. From x-ray crystallography, we found that the hydroxyl oxygen of Thr-39 may serve as a hydrogen bond donor for cytosine and simultaneously serve as hydrogen bond acceptor from the hydroxyl oxygen of Thr-70; therefore, the Thr-39 may serve as a hydrogen bond acceptor from uracil and donor for Thr-70. In contrast, some ribonucleases possessing Asp at the equivalent Thr-70 residue, e.g. RC-T70D, onconase, RC-RNase 2, and human RNase 4, prefer uracil at the B1 site because the carboxyl group of Asp-70 only serves as a distal hydrogen bond acceptor for uracil but not as a hydrogen bond donor for cytosine. It is concluded that Thr-70 is the key residue that determines B1 base specificity through its dual function of hydrogen bond donor or acceptor.

Although Val-37 in the B1 site of RC-RNase exhibits a dramatic chemical shift in the RC-RNase-d(CG) complex as detected by NMR study (35), RC-V37A and RC-V37K did not reveal significant changes in catalytic activity (79.2 and 91%, respectively) or base specificity (Fig. 4). Our results from x-ray crystallography show that the side chain rotation of Val-37 only provides a better hydrophobic contact with the cytosine at the B1 site without affecting hydrogen bonds involved in the catalytic activity and base specificity.

From the aspect of B2 base specificity, mammalian ribonucleases prefer adenine, whereas frog ribonucleases prefer guanine. In RNase A, residues Asn-71 and Glu-111 contribute to the B2 base specificity and substrate binding through hydrogen bonds (34). However, the Asn-71-equivalent residue and all of the residues corresponding to the loop between Cys-65 and Cys-72 of RNase A are absent in all known frog ribonucleases. In RC-RNase, the possible Asn-71-equivalent residue is Asn-57 or Asn-59, but they are distant from the B2 substrate in the complex and not involved in base recognition (Figs. 4A and 7C). The equivalent residue of RNase A Glu-111 is Glu-97 in RC-RNase, which is conserved in the ribonuclease superfamily.
bound directly to guanine, and involved in B$_1$ base specificity (Figs. 4A and 7C). In this study, we found that a nearby residue, Lys-95, is bound directly to Glu-97 and guanine and is involved in base specificity (Figs. 4A and 7C). In contrast, the equivalent residue of RC-RNase Lys-95 in mammalian ribonucleases is Ala, which preferentially recognizes pyrimidine-adenosine at the B$_2$ site. Therefore, it is concluded that Lys-95 and Glu-97 are the key residues of RC-RNase which determine B$_1$ base specificity for guanine in contrast to Asn-71 and Glu-111 in RNase A which determine for adenine.

The catalytic residues His-10, Lys-35, and His-103 of RC-RNase are conserved in all known members of the RNase A superfamily (Fig. 1). However, Pyr-1 is found mainly in frog ribonucleases and not in most mammalian ribonucleases. In mammals, Pyr-1 is only found in human RNase 4 and RNase 5 (angiogenin) (Fig. 1), but it is structurally flexible and is not involved in catalytic activity (44, 45). In frog ribonucleases, Pyr-1 has been found to be crucial for the catalytic activity and cytotoxicity of onconase through two hydrogen bonds with Lys-9 and Val-102, and guanine in the RC-RNase-d(ACGA) complex (Fig. 7, C and D), and it participates in both catalytic activity and base specificity.

The Asn-44 of RNase A, equivalent to Asn-38 of RC-RNase, is conserved in the ribonuclease superfamily and is involved in the formation of a binding pocket for substrate as predicted by computer modeling (46). In RC-RNase, the N38A mutation caused a marked decrease in catalytic activity (1.0% remained) and cytotoxicity (1.5% remained), whereas the N38Q mutation caused less reduction in both properties (14.6 and 78.2% left, respectively) (Table II). However, their base specificities did not change (data not shown). Similarly, Val-118 of RNase A is also conserved in the RNase A superfamily, and the mutation of its equivalent residue Val-102 in RC-RNase causes differential reduction of catalytic activity and cytotoxicity, e.g. 3.4 and 2% left in RC-V102D, 19.1 and 84.4% left in RC-V102A, respectively (Table II). The base specificities of these two mutants did not change. It is suggested that N38A and V102D mutations may cause severe disruption of hydrogen bonds that are essential for catalysis and cytotoxicity because the secondary structures of these mutants are similar to that of native RC-RNase. RC-K9Q drastically lost most of its catalytic activity and cytotoxicity (1.9 and 9.2% remained, respectively), whereas the RC-K9A only lost its catalytic activity (2.2% remained) but not cytotoxicity (66.7% remained). It is suspected that the side chain of Lys-9 may play an important role in the cytotoxicity in addition to catalytic activity.

In general, the cytotoxicity of RC-RNase is correlated, but not proportional to, the reduction of catalytic activity and is not correlated with base specificity. In comparing the relative catalytic activity of the seven ribonucleases from bullfrog, they exhibit differential catalytic activity that varies from 1 to 10$^5$-fold, but they possess similar cytotoxicities toward HeLa cells (19). The results indicate that cytotoxicity of bullfrog ribonucleases is not proportional to their catalytic activities. The results in Table II show that ~10% of catalytic activity is sufficient to retain ~80% of cytotoxicity. Therefore, it is suggested that minimum catalytic activity is sufficient for cytotoxicity, whereas other residues may be required to bind specific receptors for ribonuclease entry or to bind specific substrates inside the cell (47). Our finding that the mutation K9A, N38A, K95A, and V102D reduce the cytotoxicity of RC-RNase toward HeLa cells, in conjunction with previous study that showed that replacement of an acidic residue with a basic residue enhances the entry of RC-RNase and its cytotoxicity toward murine leukemia P388 cells (48), suggests that positively charged residues are crucial for the cytotoxicity as well as the catalytic activity of RC-RNase.

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