The Flexible and Clustered Lysine Residues of Human Ribonuclease 7 Are Critical for Membrane Permeability and Antimicrobial Activity*

Yu-Chie Huang †‡§, Yu-Min Lin †, Ting-Wei Chang ‡, Shih-Jung Wu †, Yan-Shin Lee †, Margaret Dah-Tsyr Chang †, Chinpan Chen †, Shih-Hsiung Wu †**, and You-Di Liao †‡

From the †Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan, ‡Institute of Biochemical Sciences, National Taiwan University, Taipei 106 Taiwan, ‡Department of Life Science, National Tsing-Hua University, Hsin-Chu 300, Taiwan, §Institute of Pharmacology, National Yang-Ming University, Taipei 112, Taiwan, and **Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan

The ubiquitous ribonucleases (RNases) play important roles in RNA metabolism, angiogenesis, neurotoxicity, and antitumor or antimicrobial activity. Only the antimicrobial RNases possess high positively charged residues, although their mechanisms of action remain unclear. Here, we report on the role of cationic residues of human RNase7 (hRNase7) in its antimicrobial activity. It exerted antimicrobial activity against bacteria and yeast, even at 4 °C. The bacterial membrane became permeable to the DNA-binding dye SYTOX® Green in only a few minutes after bactericidal RNase treatment. NMR studies showed that the 22 positively charged residues (Lys118 and Arg4) are distributed into three clusters on the surface of hRNase7. The first cluster, K3, K3, K111, K112, was located at the flexible coil near the N terminus, whereas the other two, K32, K35, and K96, K97, K100, were located on rigid secondary structures. Mutagenesis studies showed that the flexible cluster K3, K3, K111, K112, rather than the catalytic residues His15, Lys38, and His123 or other clusters such as K32, K35, and K96, K97, K100, is critical for the bactericidal activity. We suggest that the hRNase7 binds to bacterial membrane and renders the membrane permeable through the flexible and clustered Lys residues K3, K3, K111, K112. The conformation of hRNase7 can be adapted for pore formation or disruption of bacterial membrane even at 4 °C.

*This work was supported by Academia Sinica, Taiwan, and the National Science Council of the Republic of China (NSC 92-2311-B-001-086). 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.

Ribonucleases (RNases) are found widely among living organisms, and they play an important role in the metabolism of RNA (1). Recently, novel biological functions other than the intrinsic ribonucleolytic activity have been demonstrated for several members of the bovine RNaseA superfamily. In the human RNase family, eosinophil-derived neurotoxin (RNase2) and eosinophil cationic protein (RNase3) have neurotoxic (2) as well as antiparasitic activity (3). Angiogenin (RNase5), which induces blood vessel formation, is also an RNase (4). In the frog RNase family, most of the members have antimicrobial activity (5). For the above-mentioned functions, the catalytic activities of RNases are essential.

Some members of RNase superfamily, such as human RNases 3, 5, and 7 (hRNase3, hRNase5, and hRNase7), mouse angiogenins 1 and 4 (mAng1 and mAng4), as well as chicken RNaseA2, also have antimicrobial activities (6–10). The mechanisms for these antibacterial activities remain unclear, although some distinct properties exist in these bactericidal RNases. First of all, the calculated isoelectric points of bactericidal RNases are higher than those of non-bactericidal RNases. Second, the net positive charges of the bactericidal RNases are higher than those of non-bactericidal RNases (Table 1). There is no evidence, however, that the high positive charge or high pl value is critical for the antimicrobial activity. In this study, we have presented evidence that one flexible Lys cluster K3, K3, K111, K112, rather than the catalytic residues His15, Lys38, His123 or other Lys clusters such as K32, K35, and K96, K97, K100, is critical for the bactericidal activity of hRNase7. The functional role of these Lys residues (K3, K3, K111, K112) on the bactericidal activity of hRNase7 will be discussed.

**EXPERIMENTAL PROCEDURES**

Preparation and Enzymatic Assay of RNases—The coding region of the human RNase7 (hRNase7) gene was obtained from the genomic DNA of HeLa cells by PCR. The NdeI and BamHI restriction sites were hung on the 5′ and 3′ end of the gene fragment, respectively, and subcloned into the T7 RNA polymerase-driven expression vector pET22b (Novagen) (11). Site-directed mutagenesis was made by PCR as previously described (12). The uniformly 15N- or 13C-labeled hRNase7 with an extra Met at the N terminus was produced in *Escherichia coli* BL21(DE3). The recombinant hRNase7 from inclusion bodies were refolded and purified as previously described (11). The ribonucleolytic activity of recombinant hRNase7 was
analyzed by the zymogram assay on RNA-casting PAGE as previously described (13).

**Antimicrobial Activity Assay**—The bacteria were cultured in Nutrient broth and plated on Nutrient agar (Difco 0001) for *Pseudomonas aeruginosa* Migula (American Type Culture Collection (ATCC) 27853), tryptic soy broth/agar (Difco 0369) for *Staphylococcus aureus* subspecies Aureus Rosenbach (ATCC 6538P), and Luria-Bertani broth/agar for *E. coli* DH5α. The yeast *Candida albicans* (Robin) Berkhout (ATCC 14053) was cultured and plated in/on yeast malt broth/agar, and *Pichia pastoris* X-33 was cultured and plated in/on yeast extract-peptone-dextrose broth/agar. The microbes were grown overnight, washed, and diluted 1:10 in 10 mM sodium phosphate, pH 7.4. 45 μl of the microbes (5–10 × 10⁵ colony-forming units (cfu)) was mixed with various concentrations of RNase/oligopeptide (5 μl), which was dissolved in 20 mM Hepes, pH 7.4, 50 mM NaCl, and incubated at 37 °C for 3 h. Serial dilution of the remaining cfu (14).

**Binding of RNase to Bacteria**—Small aliquots of RNases (2 μg in 5 μl) were incubated with 45 μl (5 × 10⁶ cfu) of sodium phosphate-washed *P. aeruginosa* at 37 °C for 30 min. The RNases bound onto bacteria were spun down, washed five times with 10 mM sodium phosphate, and analyzed by SDS-PAGE and silver staining.

**Assays of the Permeability of Bacterial Membrane**—The overnight culture of *P. aeruginosa* (10⁵ cfu) was washed and resuspended in 100 μl of water and incubated with 1 μM SYTOX Green (Molecular Probes) in a dark 96-well plate for 15 min in the dark. After the addition of RNase, the increase of fluorescence due to the binding of the dye to the intracellular DNA was measured in the same microplate reader using 485- and 520-nm filters for excitation and emission wavelengths, respectively (15).

**Nomenclature of Mutated hRNase7 and Oligopeptide**—Some of the hRNase7 mutants are designated as follows: K1A (substitution of Lys1 by Ala); H15A (substitution of His15 by Ala); K32N/K35Q (substitution of Lys32 by Asn and Lys35 by Gln); K32N/R97A/K100T (substitution of Lys32 by Asn and Lys97 by Ala, and Lys100 by Thr); K111Q/K112Q (substitution of Lys111 by Gln and Lys112 by Gln); Δ1KPKG (deletion of N-terminal Met and Lys1-Pro2-Lys3-Gly4 residues from the recombinant hRNase7; and (R₃S₁)₃, (12-mer oligopeptide containing the triple-repeated Arg-Arg-Arg-Ser sequence).

**Circular Dichroism (CD) Experiments**—CD experiments were carried out using an Aviv CD 202 spectrometer (Lake-wood, NJ) using a 1-mm path length cuvette with 20 μM hRNase7 in 20 mM sodium phosphate. The CD spectra at different temperatures and pH values were recorded from 190 to 260 nm using a wavelength step of 0.5 nm. Equilibrium thermal denaturing experiments were performed using protein samples dissolved in 20 mM phosphate buffer, pH 3.5, by measuring the change of molar ellipticity at 201 nm. Data were collected as a function of temperature with a scan rate of 2 °C/min and allowing 3 min to reach equilibrium over the range of 4–95 °C. Equilibrium unfolding induced by guanidine HCl was monitored by CD as described previously (11). The curves were fitted and analyzed using SigmaPlot version 8.02 (SPSS Inc.).

**NMR Spectroscopy**—All NMR experiments were performed on Bruker AVANCE 600 and 800 spectrometers equipped with triple (H, ¹³C, and ¹⁵N) resonance probes, including a shielded z-gradient. The RNase samples (0.6 mM in 0.35 ml) were prepared in 50 mM phosphate buffer in 90% H₂O/10% D₂O or 99.9% D₂O at pH 3.5, 310 K and kept in a Shigemi NMR tube. All heteronuclear NMR experiments were carried out as described previously (16). Sequence-specific assignment of the backbone atoms of hRNase7 was achieved by the independent connectivity analysis of CBCA(CO)NH, HNCA, HNCO, HN(CA)CO, and (C=O)NH. The ¹H resonances were assigned using TOCSY-HSQC, HAHB(CO)NH, and HCHC-TOCSY. Combined information from two-dimensional ¹H-¹⁵N HSQC and three-dimensional NOESY-HSQC experiments yielded assignments for side chain amide resonances of the Asn and Gln residues. Aromatic resonances were assigned using TOCSY-HSQC, NOESY, and TOCSY data. Linear prediction was used in the ¹³C and ¹⁵N dimensions to improve the digital resolution. All spectra were processed using the NMRPipe software package (17) and analyzed using NMRView version 5.0 (18).

**NMR Restraints and Tertiary Structure Calculation of hRNase7**—The dihedral angle information was predicted by the TALOS program (19). The hydrogen bonding information was obtained from D₂O exchange monitored by two-dimen-
Residues Responsible for Antimicrobial Activity of hRNase7

**RESULTS**

**Antimicrobial Activity of RNase Superfamily Proteins**—The hRNase7 was more effective (0.1 μM for 102-fold reduction) in cfu compared with that of buffer only against bacteria (105 cfu \(P. \) aeruginosa) than bullfrog RC-RNase6 (5 μM), whereas the active RNA-degrading RNases (bovine RNaseA and bullfrog RC-RNase) and all other bullfrog oocytic RNases were not bactericidal at 80 μM. The 12-mer Arg-rich positively charged oligopeptide (R3S1)3, however, possessed similar bactericidal activity (0.2 μM) as that of hRNase7 (0.1 μM) (Fig. 1, A and B).

The antimicrobial spectrum of hRNase7 was also examined, and the yeast \(P. \) pastoris X-33 (0.03 μM for 102-fold reduction in cfu compared with that of buffer only) was the most sensitive among all of the microbes tested and thereafter in order the Gram-negative bacterium \(P. \) aeruginosa (0.1 μM), whereas the Gram-positive \(S. \) aureus (1 μM) and Gram-negative \(E. \) coli bacteria (10 μM) and yeast \(C. \) albicans (> 20 μM) were not sensitive (Fig. 1, B and C).

**Binding and Permeability to Bacterial Membrane**—The cfu of susceptible bacteria was reduced 102-fold in only a few min-
utes after RNase addition (data not shown). The bactericidal RNases (hRNase7 and RC-RNase6) were bound to the susceptible bacterium *P. aeruginosa* (Fig. 2, lanes 2 and 11). In contrast, the non-bactericidal bovine RNaseA and bullfrog RC-RNase did not bind to the bacteria (Fig. 2, lanes 5 and 8).

The membrane of *P. aeruginosa* became permeable to the DNA-binding dye SYTOX Green in only a few minutes after the addition of 2.5 μM hRNase7 or bullfrog RC-RNase6 to the bacteria (107 cfu in 100 μl). However, the non-bactericidal bovine RNaseA and bullfrog RC-RNase had no effect under the same conditions, which was in good agreement with the antimicrobial assay (Figs. 1A and 3). The bactericidal activity of hRNase7 was only slightly reduced at 4 °C (0.3 μM for 102-fold reduction in cfu compared with that of 0.1 μM at 37 °C), whereas that of indolicidin, a 13-mer bactericidal oligopeptide from bovine neutrophils, was almost abolished (Fig. 4). These results suggest that the bactericidal activity of hRNase7 is not energy-dependent, whereas that of indolicidin is energy-dependent.

**Conformational and Structural Stability of hRNase7**—To elucidate the mechanism for the antimicrobial activity of hRNase7, the structure of the recombinant protein was analyzed by CD spectra and NMR studies. The important features are summarized and discussed below. First, the conformation of hRNase7 was pH-independent over the range of 3.5–9.5. The *Tm* value of hRNase7 was 66.4 °C. The chemical stability was determined with a *Cm* value at 3.27 M guanidine-HCl. These *Tm* and *Cm* values indicate that hRNase7 is a stable protein in structure, similar to other RNaseA superfamily members.

**Structure of hRNase7**—The 800-MHz two-dimensional 1H,15N HSQC spectrum of the hRNase7 was obtained in which cross-peaks clearly dispersed (Fig. 5). For the determination of tertiary structures, a set of 1661 restraints were collected for simulated annealing calculations. Among these restraints, 1447 were interproton distances, 94 were hydrogen bonds, 116 were torsional angles, and 4 were disulfide bond restraints (Table 2). The 15 structures of the lowest total energy were chosen to represent the ensemble of NMR structures (Fig. 6A). These structures were consistent with both experimental data and standard covalent geometry and displayed no violations >0.3 Å for distance restraints and no violations >3° for torsional angles. Superposition of each structure with the mean structure yielded an average root mean square deviation of 0.34 ± 0.09 Å for the backbone atoms and 1.06 ± 0.08 Å for the heavy atoms in residues 7–128. Analysis of the ensemble using PROCHECK-NMR revealed that 64.4% of the residues lay in the most favored regions and 33.9% of the residues lay in allowed regions in the Ramachandran *ϕ*, *ψ* dihedral-angle plot (Table 2).

The solution structure of hRNase7 displayed an α + β folding topology, which was composed of three α-helices and two antiparallel β-sheets, typical for the RNaseA superfamily. The structure was stabilized by four disulfide bridges (C23–C81, C55–C106, C37–C91, and C62–C69). Most of the hydrogen bonds were located in the α-helix and β-sheet regions. Most positively
charged residues were distributed into three clusters (Fig. 6B). The first was composed of Lys1, Lys3, Lys111, and Lys112; the second, Lys28, Lys32, Lys35, Arg36, and Lys38; and the third, Lys82, Lys94, Lys96, Arg97, and Lys100. On the other hand, the negatively charged residues were randomly distributed over hRNase7 (Fig. 6C).

**TABLE 2**

| Constraints used                                                                 |              |
|----------------------------------------------------------------------------------|--------------|
| NOE distance restraints                                                          | 211          |
| Intraresidue ([i–j] = 0)                                                          | 363          |
| Sequential ([i–j] = 1)                                                            | 335          |
| Medium range ([i–j] < 5)                                                          | 538          |
| Long range ([i–j] ≥ 5)                                                            | 1447         |
| Total NOE distance restraints                                                     |              |
| Hydrogen bonds                                                                    | 47 × 2       |
| Disulfide bonds                                                                   | 4            |
| Dihedral angles                                                                   | 116          |

**Statistics for the final X-PLOR structures**

| Number of structures in the final set | 15           |
| X-PLOR energy (kcal mol⁻¹)            | 131.3 ± 4.0  |
| E_NOE                                | 3.3 ± 0.4    |
| E_NOE + E_angle + E_improper          | 231.1 ± 6.0  |
| E_VDW                                | 322.0 ± 6.4  |
| Mean global root mean square deviation (Å) |          |
| Backbone atoms (N, C², C³) (residues 7–128) | 0.34 ± 0.09 |
| Backbone atoms (N, C², C³) (secondary structure) | 0.24 ± 0.06 |
| Heavy atoms (residues 7–128)          | 1.06 ± 0.08  |
| Heavy atoms (secondary structure)     | 0.95 ± 0.10  |
| Ramachandran plot (%)                 | 64.4         |
| Residues in most favored regions     | 26.9         |
| Residues in generously allowed regions | 7.0         |
| Residues in disallowed regions       | 1.7          |

**Residues Responsible for Bactericidal Activity of hRNase7**—Because cationic residues were abundant in bactericidal RNases as demonstrated in Table 1, we thus proceeded to determine the role of the RNA catalytic activity or cationic residue in the bactericidal activity. First of all, we found that the catalytic activity-deficient mutants of hRNase7 (H15A, K38A, and H123A) still conferred the same level of antimicrobial activity as the wild-type hRNase7 (Fig. 7, A and B; and Fig. 8A). The result shows that RNA catalytic activity is not essential for the antibacterial activity of RNases.

Deletion of the N-terminal Met and four residues (Δ¹PKPG⁴) from recombinant hRNase7 markedly reduced the bactericidal activity but did not alter the RNA catalytic activities (Fig. 7, C and D; and Fig. 8B). Further analysis showed that the K3A mutant exerted less bactericidal activity than the K1A mutant (Fig. 7, C and D; and Fig. 8C). However, the substitution...
charged clusters (K32N/K35Q and K96A/R97A/K100T) altered neither the catalytic nor the bactericidal activity (Fig. 7, C and D; and Fig. 8B). These results show that the flexible Lys1,Lys3, as well as Lys111,Lys112 residues in the first cationic cluster are critical for the bactericidal activity.

Structural Comparison of Wild-type and K3A Mutated hRNase7—The structure of K3A-hRNase7 was nearly identical to that of wild-type hRNase7, as there was no difference in the cross-peaks of two-dimensional N15-H1-HSQC and three-dimensional N15-NOESY and HSQC NMR spectra between them, except residues Gly4, Leu124, and Ala3, although K3A-hRNase7 had less antimicrobial activity than wild-type hRNase7 (Fig. 8C).

DISCUSSION

The structures of bactericidal RNases hRNase3 and hRNase7 are similar to those of RNaseA superfamily proteins with three \( \alpha \)-helices and two triple-stranded antiparallel \( \beta \)-sheets. Among these RNases, only the bactericidal RNases contained abundant positively charged residues on the enzyme surface, but the non-bactericidal RNases did not (Table 1). The abundance of the cationic residues Lys and Arg is also found in most antimicrobial peptides. These peptides possess amphipathic structures that are composed of clustered cationic and hydrophobic residues on each side of the structure, although they have diverse primary sequences and different secondary structures (23, 24). The cationic residues may facilitate their interaction with the negatively charged components on the microbial surface, and the hydrophobic residues may permit their incorporation into microbial membrane (25).

In this study, we have found that three clusters of cationic residues are located on the surface of hRNase7. The first cluster consists of Lys1,Lys3, Lys111,Lys112 residues, the second and third clusters contain Lys32,Lys35 and Lys96,Arg97,Lys100, respectively. Only cationic residues in the first cluster, K1,K3,K111,K112, are

![Residues Responsible for Antimicrobial Activity of hRNase7](https://example.com/figure6.png)

FIGURE 6. Structure of hRNase7. A, NMR solution structures of hRNase7. Left, the ensemble of 15 NMR solution structures is displayed. The \( \alpha \)-helical structures are shown in red, \( \beta \)-strands in blue, and other secondary structures are colored in gray. Right, the ribbon representation of the best NMR structure that possesses the lowest total energy is shown. The hRNase7 is composed of three \( \alpha \)-helices (red) and two triple-stranded antiparallel \( \beta \)-sheets (cyan). B, surface structure of hRNase7 is displayed as a 180° rotation with positively and negatively charged residues shown in blue and red, respectively. C, side chain conformations of all charged residues, Lys (K) in blue, Arg (R) in cyan, Asp (D) in red, and Glu (E) in pink, of 15 NMR structures of hRNase7 are displayed as a 180° rotation. The figure was generated based on the superposition of the backbone atoms in the full-length protein, and clarification of only one ribbon structure is shown. The charged residues that were mutated for antimicrobial activity studies are boxed with dotted lines.
critical for the bactericidal activity, whereas the other two clusters, K32, K35, and K96, R97, K100, are not (Figs. 6A and 8B). The result of Fig. 6C shows that the side chains of the Lys1, Lys3, Lys111, and Lys112 residues in the first cluster are more flexible than those of cationic residues in other clusters. In addition, we also found that no anionic residue resides in the first cluster of hRNase7 near the K1, K3, K111, K112 residues by NMR studies (Fig. 6) and no anionic residue resides at each N-terminal region of bactericidal RNases by the comparison of amino acid sequences among the RNaseA superfamily members (Fig. 9). Thus, it is concluded that clustering of cationic residues without an adjacent anionic residue is critical for the bactericidal activity of hRNase.

Several members in the RNase superfamily have antimicrobial activities, but the key residues/domains responsible for antimicrobial activity were different in human RNase3 and RNase7 (6–10). The content of cationic residues of hRNase3 (Lys1 and Arg19) differs from that of hRNase7 (Lys18 and Arg4). Furthermore, the cationic residues Arg101 and Arg104 and aromatic residues Trp10 and Trp35 of hRNase3, which are suggested to be responsible for membrane binding and disruption, reside on the dispersed secondary structure (α1, β4) (26), whereas the cationic residues K1, K3, K111, K112, of hRNase7 reside on the flexible coil and loop at the N-terminal cluster. With regard to chicken RNaseA2, the Arg residues in domains II (residues 71–76) and III (residues 89–104) are critical for the bactericidal activity, but the structures of the RNaseA2 remains unknown (10).

In addition to the flexibility of the side chains of cationic residues, we were also interested in the influence of similar tertiary structure folds for RNases on the antimicrobial activity. Our results indicate that the bactericidal activity of RNase was not correlated with its backbone tertiary structure; for example, the root mean square deviation values between hRNase7 and bactericidal hRNase3 and hRNase5 are 2.57 and 3.37 Å, respectively, whereas those between bactericidal hRNase7 and non-bactericidal hRNase2, hRNase4, and bovine RNaseA are 2.34, 3.09, and 2.33 Å, respectively. This indicates that the tertiary structure fold of the whole RNase backbone is not critical for the antimicrobial activity.

The hRNase7 was effective in bactericidal activity at 4 °C, whereas the indolicidin, a Trp-rich oligopeptide (ILPWKWWPWWPWR-NH₂) from bovine neutrophils, was not (27). The bactericidal RNases hRNase7 and RC-RNase6 from bullfrog...
oocytes were bound to susceptible bacteria, whereas the non-bactericidal RNases were not. These results suggest that some component(s) on the bacteria is/are responsible for the binding of hRNase7 and the action mechanism of hRNase7 is energy-independent (28, 29). The bacterial membrane became permeable to the DNA binding dye SYTOX Green just a few minutes after the addition of hRNase7 (Fig. 3). This indicates that the marked increase of membrane permeability is an important step for the bactericidal activity of hRNase7. Thus, we suggest that the hRNase7 may bind to the negatively charged components of the bacterial membrane through the flexible and cationic residues. The hRNase7 may change its own conformation, incorporates itself into the bacterial membrane through the hydrophobic scaffold, and triggers the disruption of membrane. Alternatively, we also propose that several pores, which are composed of hRNase7-binding proteins, may reside on the bacterial surface for the regulation of the transportation of ions and metabolites. The opening of pores may be triggered by hRNase7 binding through the flexible/clustered cationic residues and hydrophobic scaffold.

The efflux of ions and fatal depolarization of bacterial membrane may thus cause immediate cell death, even at low temperatures. The antibacterial mechanisms of these positively charged peptides/proteins by physical disruption of bacterial membrane is different from those for conventional antibiotics, which are inhibition of cell wall synthesis, DNA replication, RNA transcription, or protein synthesis. Due to the unique bactericidal activity of hRNase7, it has the potential to be a new therapeutic agent for bacterial infection, because it may not face the rapid emergence of drug resistance. The induction of endogenous hRNase7 gene expression or the administration of a synthetic oligopeptide designed from the hRNase7 structure would be possible in the clinical therapy for microbial infection.

Acknowledgments—We thank Dr. Chen-Pei David Tu for critical reading of the manuscript, Dr. Yuan-Chao Lou for editing the manuscript, and Chiu-Feng Wang for the construction of various hRNase7 mutants.

REFERENCES
1. D’Alessio, G. (1993) Trends Cell Biol. 3, 106–109
2. Durack, D. T., Ackerman, S. I., Loegering, D. A., and Gleichen, G. J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5165–5169
3. McLaren, D. J., McKean, J. R., Olsson, I., Venges, P., and Kay, A. B. (1981) Parasite Immunol. 3, 359–373
4. Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F., and Vallee, B. L. (1985) Biochemistry 24, 5480–5486
5. Liao, Y. D., Huang, H. C., Leu, Y. J., Wei, C. W., Tang, P. C., and Wang, S. C. (2000) Nucleic Acids Res. 28, 4097–4104
6. Zhang, J., Dyer, K. D., and Rosenberg, H. F. (2003) Nucleic Acids Res. 31, 602–607
7. Harder, J., and Schroder, J. M. (2002) J. Biol. Chem. 277, 46779–46784
8. Hooper, L. V., Stappenbeck, T. S., Hong, C. Y., and Gordon, J. I. (2003) Nat. Immunol. 4, 269–273
9. Holloway, D. E., Hares, M. C., Shapiro, R., Subramanian, V., and Acharya, K. R. (2001) Protein Expression Purif. 22, 307–317
10. Nitto, T., Dyer, K. D., Czapiga, M., and Rosenberg, H. F. (2006) J. Biol. Chem. 281, 25622–25634
11. Hsu, C. H., Liao, Y. D., Pan, Y. R., Chen, L. W., Wu, S. H., Leu, Y. J., and Chen, C. (2003) J. Mol. Biol. 326, 1189–1201
12. Huang, H. C., Wang, S. C., Leu, Y. J., Wu, S. H., and Liao, Y. D. (1998) J. Biol. Chem. 273, 6395–6401
13. Liao, Y. D., and Wang, J. J. (1994) Eur. J. Biochem. 225, 215–220
14. Rosenberg, H. F., and Dyer, K. D. (1995) J. Biol. Chem. 270, 21539–21544; Correction (1995) J. Biol. Chem. 270, 30234
15. Nkhotiaeva, N., Elmutt, A., Rajaratnam, G. K., Hallbrink, M., Langel, U., and Good, L. (2004) FASEB J. 18, 394–396
16. Clore, G. M., and Gronenborn, A. M. (1994) Methods Enzymol. 239, 349–363
17. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
18. Johnson, B. A. (2004) Methods Mol. Biol. 278, 313–352
19. Cornilsev, G., Delaglio, F., and Bax, A. (1999) J. Biomol. NMR 13, 289–302
20. Herrmann, T., Guntert, P., and Wuthrich, K. (2002) J. Mol. Biol. 319, 209–227
21. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) J. Biomol. NMR 8, 477–486
22. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 51–55
23. Epand, R. M., and Vogel, H. J. (1999) Biochim. Biophys. Acta 1462, 11–28
24. Hancock, R. E. (1997) Lancet 349, 418–422
25. Powers, J. P., and Hancock, R. E. (2003) Peptides 24, 1681–1691
26. Carreras, E., Boix, E., Rosenberg, H. F., Cuchillo, C. M., and Nogues, M. V. (2003) Biochemistry 42, 6636–6644
27. Hsu, C. H., Chen, C., Jou, M. L., Lee, A. Y., Lin, Y. C., Yu, Y. P., Huang, W. T., and Wu, S. H. (2005) Nucleic Acids Res. 33, 4053–4064
28. Falla, T. J., Karunaratne, D. N., and Hancock, R. E. (1996) J. Biol. Chem. 271, 19298–19303
29. Robinson, W. E., Jr., McDougall, B., Tran, D., and Selsted, M. E. (1998) J. Leukocyte Biol. 63, 94–100

FIGURE 9. Sequence alignment of 16 amino acid residues from the N terminus of hRNases and bovine RNaseA. The figure was generated using the programs ClustalW and ESPrint, and the sequence number and α-helix annotation at the top are based on the sequence of hRNase7. In the alignment, positive (Lys and Arg) and negative (Asp and Glu) residues are underlined and dotted, respectively (26). Conservative residues and identical residues are shown in boxes and with black background, respectively.