Evolution and Function of Leukocyte RNase A Ribonucleases of the Avian Species, Gallus gallus*

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In this study, we explore the evolution and function of two closely related RNase A ribonucleases from the chicken, Gallus gallus. Separated by ~10 kb on chromosome 6, the coding sequences of RNases A-1 and A-2 are diverging under positive selection pressure (dN > dS) but remain similar to one another (81% amino acid identity) and to the mammalian angiogenins. Immunoreactive RNases A-1 and A-2 (both ~16 kDa) were detected in peripheral blood granulocytes and bone marrow. Recombinant proteins are ribonucleolytically active (kcat = 2.6 and 0.056 s−1, respectively), and surprisingly, both interact with human placental ribonuclease inhibitor. RNase A-2, the more cationic (pI 11.0), is both angiogenic and bactericidal; RNase A-1 (pI 10.2) has neither activity. We demonstrated via point mutation of the catalytic His110 that ablation of ribonuclease activity has no impact on the bactericidal activity of RNase A-2. We determined that the divergent domains II (amino acids 71–76) and III (amino acids 89–104) of RNase A-2 are both important for bactericidal activity. Furthermore, we demonstrated that these cationic domains can function as independent bactericidal peptides without the tertiary structure imposed by the RNase A backbone. These results suggest that ribonucleolytic activity may not be a crucial constraint limiting the ongoing evolution of this gene family and that the ribonuclease backbone may be merely serving as a scaffold to support the evolution of novel, nonribonucleolytic proteins.

The RNase A ribonuclease gene family has been a tremendous source of information on unusual evolutionary constraints and their effects on protein structure and function at the molecular level. Although RNase A ribonucleases maintain invariant disulfide bonds and catalytic components that are necessary for RNA degradation, other regions have diverged dramatically. RNase A ribonucleases have been implicated in a wide variety of physiologic functions and have been observed to promote angiogenesis, cellular apoptosis, and anti-tumor and anti-pathogen host defense via a complex array of seemingly unrelated molecular mechanisms (reviewed in Refs. 1–8).

The specific patterns of diversification are best understood among the RNase A ribonucleases of mammalian species. Four major RNase A lineages have been described in mammals (6) as follows: the pancreatic RNases, or RNases 1, which include the prototype, bovine pancreatic RNase A; a second group, including the eosinophil ribonucleases EDN (RNases 2), ECP (RNases 3), and RNases 6, 7, and 8; a third group that includes the RNases 4; and a final group that includes the angiogenins (RNases 5). There are also several genes, such as RNases 9–13 in the human genome, that are distantly related to the RNase A family based on amino acid sequence homology but that are missing one or more elements necessary for enzymatic activity (9–13). Interestingly, not all RNase A lineages are found in every mammalian species, and there are some recently described mammalian RNase A ribonucleases that cannot be clearly assigned to a specific lineage or group (14).

In contrast, the nonmammalian RNase A ribonucleases have not been as clearly defined. Several RNase A ribonucleases of the frog genus Rana have been isolated (15–18), including the cancer biotherapeutic agent, onconase (19). Also characterized are several RNase A ribonucleases from birds (14, 20–22) and reptiles (23–25). With so few sequences characterized, we do not have an adequate sense of the specific interrelationships or unique lineages. Likewise, the physiologic activities of many of these nonmammalian ribonucleases remain unexplored.

Given our ongoing interest in the role of RNase A ribonucleases in promoting host defense (5), in this work we explore the biologic activity and structure-function relationships of two closely related leukocyte-associated ribonucleases from the chicken, G. gallus. Chicken RNase A/angiogenin, named for structural similarity, as opposed to functional analysis, was identified by Nakano and Graf (20) from RNA from v-Myb transformed myeloblasts; ribonuclease superfamily-related (RSFR)3 was cloned by Lobanenkov and co-workers (21) from chicken bone marrow as part of an unrelated subtractive hybridization study. Cho et al. (11) performed an exhaustive search of the recently published chicken genome and have concluded that there are only three RNase A ribonucleases, the two

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) DQ395275 and DQ395276.

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3 The abbreviations used are: RSFR, ribonuclease superfamily-related; hPRL, human placental RNase Inhibitor; EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; RACE, rapid amplification of cDNA ends.
leukocyte-associated RNases on chromosome 6 and a more distantly related sequence on chromosome 4. In this study, we isolated two cDNAs from chicken bone marrow and generated specific antibodies that enabled us to localize these leukocyte-associated RNases in peripheral blood granulocytes. We also characterized novel biologic function for these proteins, performed structure-function analysis, and identified unique domains that contributed independently to bactericidal and ribonucleolytic activities. Further studies on the surprising independent function of these peptide domains have led us to reconsider some long held assumptions regarding evolutionary diversity and RNase A ribonuclease genes.

**EXPERIMENTAL PROCEDURES**

**Isolation of Leukocytes from Bone Marrow**—Bone marrow cells were collected from femurs and tibiae of White Leghorn chickens (*Gallus gallus*) by flushing sterile phosphate-buffered saline (PBS, pH 7.4) through opened bones. Cells were washed twice with PBS, and red blood cells were lysed by incubation with ACK lysing buffer (Cambrex Bio Science, Walkersville, MD) for 5 min. The cells were subsequently washed again with PBS and counted with a hemocytometer. Viability was assessed by trypan blue staining.

**Isolation of Leukocytes from Peripheral Blood**—Leukocytes were separated from peripheral blood according to the methods described previously (26, 27) with minor modifications. Heparinized peripheral blood was collected by cardiac puncture and then mixed with a 3% dextran solution. Red blood cells (density, 1.077–1.080 g/ml at 20 °C; Mediatech, Herndon, VA) at 400 g were harvested by centrifugation at 400 g for 10 min. Medium (density, 1.077–1.080 g/ml at 20 °C, harvested by centrifugation at 400 g for 10 min) was collected by centrifugation at 400 g for 10 min and separated into phases via Lymphocyte Separation Coat. 

Peripheral blood leukocytes (2–5 × 10⁶ cells/100 μl) were mixed with PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (Sigma), and left on ice for 30 min. After centrifugation at 13,600 g for 10 min, the supernatant was collected and stored at −80 °C prior to use.

**Preparation of RNA from Bone Marrow Cells**—One ml of RNAzol B (Tel-Test, Friendswood, TX) was added to each aliquot of 2 × 10⁶ cells (15–25 × 10⁶ cells total). Chloroform was added (1:10 (v/v)), and the samples were mixed thoroughly and incubated on ice for 15 min. After centrifugation at 13,600 × g for 20 min at 4 °C, the aqueous layer was transferred to fresh tubes. Equal volumes of ice-cold isopropl alcohol were added, and RNA was precipitated at −20 °C, harvested by centrifugation, washed twice in 80% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. RNA was quantitated spectrophotometrically.

**Rapid Amplification of cDNA Ends (5′- and 3′-RACE)**—cDNA was synthesized from 1 μg of RNA from *G. gallus* bone marrow RNA with Moloney murine leukemia virus reverse transcriptase as per the manufacturer’s instructions (SMART RACE cDNA amplification kit, Clontech) and then amplified using specific oligonucleotides for chicken leukocyte RNase A-1 (previously identified as angiogenin/RNase 20) and chicken leukocyte RNase A-2 (previously identified as RSFR (21)). The sequences used are as follows: 5′-RACE for leukocyte RNase A-1, 5′-TGGAGAGGTGTCATCCAGATGCAAG- GAAGCCTCCGGC-3′; 5′-RACE for leukocyte RNase A-2, 5′-TGAATGCAACCCTCTACAGAGGTAGATTG-3′; 3′-RACE, 5′-GGTCCACCTACAGATTGTGG-3′.

The PCR conditions were as follows: 5 cycles of 94 °C for 30 s followed by 72 °C for 3 min, then 5 cycles of 94 °C for 30 s followed by 70 °C for 30 s and followed by 72 °C for 3 min, and finally 25 cycles of 94 °C for 30 s, followed by 68 °C for 30 s, and followed by 72 °C for 3 min. The amplified PCR fragments were gel-purified (BI01, Inc., Vista, CA) and subcloned into the pCR2.1 (Invitrogen); multiple colonies were sequenced in both directions. The sequences were assembled using Sequencher 4.1 (GeneCodes, Ann Arbor, MI) to obtain the full-length cDNA sequence. To amplify the two chicken leukocyte RNases (minus signal sequences), the following primers were used: 5′-aagcttCTGTTCACACCTACAGAGGTAGATTTTGC-3′ and 5′-gaattcTGAAAGGTGCCATCCAGATGCAAG-3′ for leukocyte RNase A-1 and 5′-aagcttCTGTTCACACCTACAGAGGTAGATTTTGC-3′ and 5′-gaattcTGAAAGGTGCCATCCAGATGCAAG-3′ for leukocyte RNase A-2 (lowercase letters indicate restriction sites added to facilitate subcloning). PCR with Pfx DNA polymerase was performed under the following conditions: an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. PCR product was subcloned into pCR-Blunt vectors (Invitrogen) as described above.

**Sequence Analysis**—Automated DNA sequencing was performed using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA) and a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences). Sequence analyses were performed with the assistance of Sequencher 4.1 (Ann Arbor, MI).

**Production of Recombinant Chicken Leukocyte RNases A-1 and A-2**—Recombinant chicken RNase proteins were prepared in *Escherichia coli* BL21 strain using the pFLAG-CTS expression vector (Sigma), which utilizes an isopropyl β-D-galactopyranoside-inducible promoter, an amino-terminal bacterial OmpA secretion piece and a carboxyl-terminal FLAG tag. This expression system has been used extensively for production of recombinant RNase A ribonucleases (28–32). Quantity of protein was determined by densitometric analysis as described previously (29).

**Site-directed Mutagenesis**—Chicken ribonucleases with exchanged domains were prepared by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Primers used to effect site-specific mutations are shown in Table 1. Mutations were verified by DNA sequencing. Recombinant proteins with specific mutations were prepared as described above.

**Preparation of Peptides and Anti-peptide Antisera**—Peptides and anti-peptide antisera were prepared at Zymed Laboratories Inc. Purity of peptides was confirmed as >95% by mass spec-
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TABLE 1
Primers used for site-directed mutagenesis

| Description | A1 → A2 | Sequences (′→′) |
|-------------|---------|-----------------|
| Domain I    | A1 → A2 | ACCAAAGTTTGTCTAAAGGATATGGTCCCGAAGAC |
|             | A2 → A1 | GTCCTTGGAAGCTCCTACGTTGAACAATCTTGTGCT |
| Domain II   | A1 → A2 | GCCCTTCGTAACCCGCGGCACTTTCGTTAATCAAGCCT |
|             | A2 → A1 | CTTACAGGTGTCCGACTGGTCTGTTACTGAAAGGCC |
| Domain III  | A1 → A2 | AGGGCCATACCCACCCCGGACCCCGGCTGTTAGTATG |
|             | A2 → A1 | CAAACTTCAGCGCGGCGGCTGTTAGTATG |
|             |         | GGAGGGCTTCCCGTGGCTCTGGATGGCACC |
|             |         | GCCCTTCGTAACCCGCGGCACTTTCGTTAATCAAGCCT |
|             |         | CTTACAGGTGTCCGACTGGTCTGTTACTGAAAGGCC |
|             |         | AGGGCCATACCCACCCCGGACCCCGGCTGTTAGTATG |
|             |         | CAAACTTCAGCGCGGCGGCTGTTAGTATG |
|             |         | GGAGGGCTTCCCGTGGCTCTGGATGGCACC |
|             |         | GCCCTTCGTAACCCGCGGCACTTTCGTTAATCAAGCCT |
|             |         | CTTACAGGTGTCCGACTGGTCTGTTACTGAAAGGCC |
|             |         | AGGGCCATACCCACCCCGGACCCCGGCTGTTAGTATG |
|             |         | CAAACTTCAGCGCGGCGGCTGTTAGTATG |
|             |         | GGAGGGCTTCCCGTGGCTCTGGATGGCACC |
|             |         | GCCCTTCGTAACCCGCGGCACTTTCGTTAATCAAGCCT |
|             |         | CTTACAGGTGTCCGACTGGTCTGTTACTGAAAGGCC |
|             |         | AGGGCCATACCCACCCCGGACCCCGGCTGTTAGTATG |
|             |         | CAAACTTCAGCGCGGCGGCTGTTAGTATG |
|             |         | GGAGGGCTTCCCGTGGCTCTGGATGGCACC |
|             |         | GCCCTTCGTAACCCGCGGCACTTTCGTTAATCAAGCCT |
|             |         | CTTACAGGTGTCCGACTGGTCTGTTACTGAAAGGCC |
|             |         | AGGGCCATACCCACCCCGGACCCCGGCTGTTAGTATG |
|             |         | CAAACTTCAGCGCGGCGGCTGTTAGTATG |
|             |         | GGAGGGCTTCCCGTGGCTCTGGATGGCACC |

Antibodies to G. gallus leukocyte RNases A-1 and A-2 were prepared by immunization of rabbits with 10 mg of keyhole limpet hemocyanin-conjugated sequence-specific peptides: A-1, H₂N-QPNRALRTQQQQLP-COOH and A-2, H₂N-DQALRTTRRHFRIT-COOH (Zymed Laboratories Inc.).

**Immunoblotting**—Bone marrow lysates and peripheral granulocyte lysates from G. gallus and recombinant FLAG-tagged chicken RNases were mixed with a reducing Tris-glycine 2× SDS sample buffer (Invitrogen) and subjected to SDS-PAGE (14% acrylamide, 1× Tris-glycine system). After proteins in-gel were transferred onto a nitrocellulose membrane (Invitrogen), nonspecific binding to the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.5, prior to probing the membrane with a 1:200 dilution of rabbit anti-leukocyte RNase A-1, 1:200 rabbit anti-leukocyte RNase A-2, or 1:200 mouse anti-FLAG M2 antibody (Sigma), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Bio-Rad). The membranes were developed with AP substrate kit (Bio-Rad) according to the manufacturer's instructions.

**Immunostaining of G. gallus Leukocytes and Observation under Confocal Microscopy**—One million cells were isolated as described and washed with 3 ml of 1% BSA in PBS (BSA/PBS) and then fixed and permeabilized by FIX & PERM (Caltag Laboratories, Burlingame, CA) as per the manufacturer's protocol. Primary antibody was used at 1:100 and consisted of anti-leukocyte RNase A-1 or anti-leukocyte RNase A-2. Preimmune serum was used as a control for background staining. After an overnight incubation in primary antibody at 4 °C, the cells were washed in BSA/PBS, and a FITC-conjugated secondary antibody, goat anti-rabbit IgG-FITC (BD Biosciences), was applied at a 1:100 dilution. After a 1-h incubation in the dark at room temperature, the cells were washed, and the nuclear stain, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR), was added at 1 μg/ml and incubated for 15 min in the dark. The cells were washed twice in BSA/PBS and then fixed onto glass slide using cytofunnels (Thermo Electron Corp., Waltham, MA). Coverslips were attached after the addition of the ProLong antifade reagent (Invitrogen). Slides were stored in dark at 4 °C until imaging. Images were collected on a TCS-SP2 AOBS confocal microscope (Leica Microsystems, Mannheim, Germany) using a ×63 oil immersion objective NA 1.32 at different zoom factors. The confocal pinhole was set to 0.9 Airy units to ensure maximum resolution. FITC was excited using an argon laser at 488 nm, and DAPI, nuclear stain, was excited using an argon laser (Enterprise model 651, Coherent, Inc., Santa Clara, CA) at 364 nm. Differential interference contrast images were collected simultaneously with the fluorescence images using the transmitted light detector. The settings were adjusted on a preimmune slide for each cell type and were not changed for images collected with relevant primary antibody.

**Ribonuclease Assays**—The ribonuclease (RNase) assay with yeast tRNA substrate has been described in detail elsewhere (29). In this study, 10 μl of a 20 mg/ml solution of yeast tRNA, polyuridylic acid (poly(U)), polyadenylic acid (poly(A)), poly-cytidylic acid (poly(C)), polyguanylic acid (poly(G)), or poly-1nosinic acid (poly(I)) (Sigma) was added to 800 μl of 40 mM sodium phosphate buffer, pH 7.0, containing recombinant FLAG-tagged chicken RNase protein. In experiments in which the pH optimum was to be determined, 40 mM sodium phosphate buffers of pH 5.0, 5.5, 6.0, 6.5, 7.4, or 8.0 were used instead of the buffer of pH 7.0. In some experiments, 200 units of human placental RNase inhibitor (Roche Applied Science) were added prior to the addition of the tRNA substrate. Conversion of A₂₆₀ per unit time to pmol/s was performed as described previously (29), based on the following conversion factors: the average molecular weight of a molecule of tRNA is 28,100 (75–90 ribonucleotides/tRNA molecule per ribonucleotide) with an A₂₆₀ of 40 μg of RNA.

**Angiogenesis Assay**—The chick aortic ring sprouting assay was performed as described previously (33) with minor modifications. Aortic arches were dissected from 13-day-old chicken embryos, cleaned free of unwanted tissue, and cut into 0.8-mm slices. Each ring was transferred into the center of a 96-well plate. 10 μl of ice-cold Matrigel (BD Biosciences) was added immediately to embed the ring. After the Matrigel was solidified, 100 μl of human endothelial SFM basal...
growth medium (Invitrogen) was added to each well. After addition of recombinant leukocyte RNase A-1 or RNase A-2 or control preparation, the rings were incubated at 37 °C for 24 h. Vessels sprouted from rings were observed under microscopy and assessed by blinded observers. Each group composed of 6–8 rings.

Bactericidal Assays—Bactericidal assays were performed as described previously with modifications (28). Briefly, 100 μl from a single bacterial colony of overnight culture grown in Luria-Bertani (LB) broth was diluted 1:100 in 100 mM sodium phosphate, pH 7.4, collected by centrifugation, and resuspended in 1 ml of sodium phosphate, pH 7.4. Ten microliters of bacteria in buffer are incubated for 4 h at 37 °C with 10 μl of recombinant protein or peptide at the concentrations indicated or diluent control and were then diluted 10-, 100-, or 1000-fold prior to plating on LB agar for overnight growth for colony counts. All points were determined in triplicate.

Phylogenetic Analysis—Sequence data were analyzed using algorithms included in the MEGA program (34), which is available on line. Sequences were aligned using ClustalW for import into MEGA. All sequence data are available on line at the GenBank™ or NCBI data bases. Accession numbers for sequences used in statistical analyses include the following: G. gallus leukocyte RNase A-1 (RNase/angiogenin), DQ395275; G. gallus leukocyte RNase A-2 (RSFR), DQ395276; G. gallus liver RNase (RNase CL2), DQ395277, P81476; Iguana iguana RNase, AY780490; Chelydra serpentina RNase, P00461; Rana piperi onconase, AA54383; Rana japonica RNase, P18839; Rana catesbeiana RC203, AAK30253; Homo sapiens pancreatic RNase 1, NP_002924; H. sapiens EDN/RNase 2, NP_002925; H. sapiens ECP/RNase 3, NP_002926; H. sapiens RNase 4, P34096; H. sapiens angiogenin/RNase 5, NP_001136; NP_002924; H. sapiens RNase k6, AAH20848; H. sapiens RNase 7, AY170392; H. sapiens RNase 8, AF473854; Mus musculus pancreatic RNase 1, NP_035401; M. musculus Ear 1, P97426; M. musculus Ear 2, P97425; M. musculus Ear 6, AAP82022; M. musculus Ear 11, AAH20070; M. musculus RNase 4, NP_067447; M. musculus angiogenin-1/RNase 5, NP_031473; M. musculus angiogenin-2, NP_031475; M. musculus angiogenin-3, NP_031474; M. musculus angiogenin-4, AY219870; M. musculus angiogenin-6, AY665821; M. musculus RNase 6, NP_084374.

Statistical Analysis—Data are shown as the mean ± S.E. for the numbers of samples. The Student’s t test was performed for statistical analysis of the differences between the groups.

RESULTS

RNase A Ribonucleases in the Genome of the Domestic Chicken, G. gallus—Three RNase A ribonucleases have been identified in the genome of the chicken, G. gallus (11). An unrooted neighbor-joining tree (Fig. 1) comparing these three chicken sequences to other known nonmammalian and mammalian RNase A ribonucleases indicate the following: 1) these three sequences are most closely related to one another; 2) they are more closely related to the other known nonmammalian RNase A ribonucleases than to any of the mammalian superfamily members; and 3) together, the nonmammalian RNase superfamily members are most closely related to the mamma-
FIGURE 2. Characterization of anti-peptide antibodies and immunodetection of RNases A-1 and A-2 in bone marrow and peripheral blood leukocytes. A, amino acid sequences of RNase A-1 and RNase A-2 (minus amino-terminal signal sequence) are as shown, which document 81% sequence identity, six structural cysteines (in red lettering), catalytic histidines 11 and 110 (in boxes), and catalytic lysine 42 (in box) within an invariant CKXXNTF (in larger box) RNase A family signature motif. Amino acid sequence differences are indicated with blue lettering. Rabbit anti-peptide antisera were prepared against keyhole limpet hemocyanin-conjugated peptides A1 (overlined: NH$_2$-QQQLPV-COOH) and A2 (underlined: NH$_2$-RRHFRI-COOH) as shown. B, characterization of the specificity of the anti-peptide antibodies against recombinant carboxyl-terminal FLAG-tagged RNase (rRNase) A-1 and A-2 by Western blot. C, detection of RNase A-1 and RNase A-2 in bone marrow and peripheral blood granulocytes by Western blot. D, immunolocalization of RNases A-1 and A-2 in bone marrow (panels i and ii) and peripheral blood granulocytes (panels iii and iv). Cytospin preparations were probed with primary antibody followed by FITC-conjugated goat anti-rabbit IgG (green), or DAPI (blue); merged images are shown, and original magnification is ×63.
malian angiogenin lineage, which, as noted by Cho et al. (11), likely represents the ancestral structural subtype of this superfamily. We have re-isolated full-length cDNAs of the two chicken leukocyte RNases by reverse transcription-PCR from RNA prepared from the bone marrow of White Leghorn chickens (G. gallus) and have deposited sequences into GenBank™ as chicken leukocyte RNase A-1 (DQ395275) and chicken leukocyte RNase A-2 (DQ395276). These sequences share 93% nucleotide sequence identity to one another and are identical to RNase A/angiogenin (19) and RSFR (20), respectively; the new names are more consistent with current RNase A family nomenclature and make no specific assumptions vis-à-vis gene function. The chicken leukocyte RNase A-1 and A-2 map to chromosome 6 (GenBank™ NW_060392) is separated by only 10 kb. The signal sequences are 100% identical; the ratio of rates of nonsynonymous (dN = n/N = 0.095) to synonymous (dS = s/S = 0.055) substitution calculated for the mature coding sequence is >1.0 (dN/dS = 1.73), suggesting divergence by positive (Darwinian) selection.

Detection and Characterization of Leukocyte RNases A-1 and A-2 in Bone Marrow and Peripheral Blood Granulocytes—The amino acid sequences of leukocyte RNases A-1 and A-2 are shown in Fig. 2A. The sequences are shown without the encoded 23-amino acid signal sequences. The amino termini were determined by direct analogy with the amino terminus of the paralogous chicken RNase CL2 (GenBank™ accession number AAB28438), which was determined by Irie and co-workers (22) directly from the protein sequence. The amino acid sequences (without signal sequences) of RNase A-1 and A-2 are 81% identical, although the isoelectric point of leukocyte RNase A-1 is 10.2 and that of RNase A-2 is 11.0; the divergence is because of the extra arginines (10 versus 14) in the RNase A-2 sequence. Each sequence includes canonical features of the RNase A superfamily, including six cysteines (characteristic of the angiogenin-type RNase A ribonucleases), the catalytic histidines (positions 11 and 110 shown here), and the catalytic lysine (position 42) within the CKXXNTF signature motif.

Rabbit antibodies prepared against divergent peptides shown in Fig. 2A (anti-A-1, NH2-QPNRALRTTQQQLP-COOH; anti-A-2, NH2-DQALRTRRRHRTRIT-COOH) were evaluated for activity and specificity by immunoblotting against recombinant carboxyl-terminal FLAG-tagged recombinant leukocyte RNase A-1 and A-2 (Fig. 2B). Preimmune sera did not detect either recombinant RNase. A 1:200 dilution of anti-A-1 antibody detected RNase A-1 only, and a 1:200 anti-A-2 antibody detected RNase A-2 only. Both proteins were readily detected with the anti-FLAG antibody control.

By using these antibodies, ~16-kDa immunoreactive bands corresponding to leukocyte RNase A-1 and RNase A-2 were detected in chicken bone marrow lysates (Fig. 2C). For reasons not yet clear, only RNase A-2 was detected in the lysates from peripheral blood granulocytes. Both immunoreactive RNase A-1 and RNase A-2 were detected in the cytoplasm of cells in the bone marrow, and both were detected in ~80% of peripheral blood granulocytes (Fig. 2D); no positive cells were detected with preimmune sera (data not showed).

FIGURE 3. Angiogenic activity of recombinant leukocyte RNases. Chicken aortic rings were incubated with 2 μM recombinant chicken leukocyte RNase A-1 or A-2. A sham protein isolate prepared with the same procedure with vector transfectant only was used as control. Photographs were taken at 24 h of incubation. See Table 2 for quantitative analysis.

Angiogenic Activity of Recombinant Leukocyte RNases—Both chicken leukocyte RNases are structurally related to the mammalian angiogenin lineage (Fig. 1). However, none of the nonmammalian ribonucleases evaluated thus far have been reported to have angiogenic activity. The potential angiogenic activity of recombinant leukocyte RNases A-1 and A-2 was evaluated with the chick aortic ring assay (33). Aortic rings prepared from 13-day-old chick embryos were incubated with 2 μM recombinant leukocyte RNases (rRNases). Prominent blood vessel formation was observed in the rings incubated with leukocyte rRNase A-2 (Fig. 3 and Table 2). The blood vessel formation in aortic rings exposed to 2 μM leukocyte rRNase A-1 was comparable with that in control (vector only transfectant) protein preparations. We conclude that leukocyte rRNase A-2 but not rRNase A-1 has angiogenic activity ex vivo.

Enzymatic Activity of Recombinant Leukocyte RNases—We next characterized the enzymatic activity of the two recombinant leukocyte RNases. Optimum pH was determined as 7.0 with 50 pmol of rRNase A-1 or A-2, with 200 μg of yeast tRNA substrate in 40 mM sodium phosphate buffers ranging from pH 5.5 to 8.0 (Fig. 4A). Both rRNase A-1 and rRNase A-2 demon-
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strated optimal activity at pH 7.0; all further activity measurements were performed at pH 7.0. Enzymatic constants $K_m$ and $k_{cat}$ were determined using the yeast tRNA substrate (Table 3) based on linear initial rates, shown for rRNase A-1 and rRNase A-2 (50 pmol, 40 mM sodium phosphate buffer, pH 7.0, yeast tRNA substrate) in Fig. 4B. Although the value for $k_{cat}$ $\text{ (s}^{-1})$ for RNase A-2 was 50 times lower than that for RNase A-1, the value determined for $K_m$ $\text{ (M)}$ for chicken leukocyte RNase A-2 was 34 times lower than that for RNase A-1, which resulted in comparable catalytic efficiencies ($k_{cat}/K_m$ $\text{ (M}^{-1} \text{ s}^{-1})$).

Recombinant leukocyte RNases A-1 and A-2 hydrolyzed poly(U) RNA at initial rates that were roughly equivalent to one another (Fig. 4C). Polyribonucleotides other than poly(U) were poor substrates for leukocyte rRNase A-1. Recombinant RNase A-2 cleaved poly(C) and poly(A), although at initial rates that were lower than those observed for poly(U). Neither of the leu-

### TABLE 2

Angiogenesis scores

| Vessels sprouted from aortic rings treated with 100 μl of human endothelial SFM basal growth medium and 2 μM recombinant leukocyte RNase were observed under microscopy and assessed by blinded observers (33). Each group included 6–8 rings; % change $\left(\frac{(\text{rRNase} - \text{control}) \times 100}{\text{control}}\right)$, $\ast$, $p < 0.05$ (with Student’s t test). | Arbitrary units (mean ± S.E.) |
|---|---|
| Control | rRNase A-1 | % change | rRNase A-2 | % change |
| Exp. 1 | 0.37 ± 0.07 | 0.38 ± 0.13 | +2.7 | 0.68 ± 0.26$^*| +84 |
| Exp. 2 | 0.33 ± 0.06 | 0.41 ± 0.06 | +24 | 0.60 ± 0.14$^*| +82 |

![FIGURE 4. Ribonucleolytic activity of recombinant leukocyte RNases.](image)
kocyte RNases cleaved poly(G), poly(I), or double-stranded RNA (data not shown).

Surprisingly, given the large evolutionary distance between species, preincubation with 200 units of human placental RNase inhibitor (hPRI) completely inhibited RNase activity of both *G. gallus* leukocyte RNases (Fig. 4D). This will be considered further under “Discussion.”

**Bactericidal Activity of Recombinant G. gallus Leukocyte RNases**—The human ECP/RNase 3, human RNase 7, and mouse angiogenin-4 all demonstrate antibacterial activity (28, 35–37). We explored the bactericidal activity of chicken leukocyte RNases cleaved poly(G), poly(I), or double-stranded RNA (data not shown).

Colony formation of *E. coli* and *Staphylococcus aureus* was reduced in a dose-dependent fashion in response to incubation with leukocyte rRNase A-2 prior to plating on RNase-free LB medium (Fig. 5). *E. coli* was more susceptible to the bactericidal activity of leukocyte rRNase A-2 than was *S. aureus*, as 5 μM rRNase A-2 reduced the colony count of *E. coli* 107-fold while reducing the colony count of *S. aureus* only 100-fold. Recombinant rRNase A-1 had no effect on colony number of *E. coli* or *S. aureus* concentrations up to and including 5 μM.

**Preparation of Domain-exchange Mutants of rRNases A-1 and A-2**—We have shown that the initial rates catalyzed by rRNase A-1 are significantly more rapid than those catalyzed by rRNase A-2 (Fig. 4B). We have also shown that rRNase A-2 has bactericidal activity that rRNase A-1 does not (Fig. 5A). To identify the amino acid sequences that have a direct impact on these biological activities, we prepared rRNases in which three specific divergent domains (I, II, and III) were exchanged (Fig. 6A). Fig. 6B documents the amino acid substitutions that contributed to the specific domain exchanges between leukocyte rRNase A-1 and A-2. In addition to these exchanges, we introduced a mutation at His110 to Ala, which disrupts the catalytic triad (Fig. 2A), in order to provide an independent assessment of RNase activity and its role in the bactericidal activity observed.

**Enzymatic and Bactericidal Activity of Domain-exchange Mutants of rRNases A-1 and A-2**—We have shown that the initial rates catalyzed by rRNase A-1 are significantly more rapid than those catalyzed by rRNase A-2 (Fig. 4B). We have also shown that rRNase A-2 has bactericidal activity that rRNase A-1 does not (Fig. 5A). To identify the amino acid sequences that have a direct impact on these biological activities, we prepared rRNases in which three specific divergent domains (I, II, and III) were exchanged (Fig. 6A). Fig. 6B documents the amino acid substitutions that contributed to the specific domain exchanges between leukocyte rRNase A-1 and A-2. In addition to these exchanges, we introduced a mutation at His110 to Ala, which disrupts the catalytic triad (Fig. 2A), in order to provide an independent assessment of RNase activity and its role in the bactericidal activity observed.

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**TABLE 3**

| Enzymatic constants determined for recombinant G. gallus RNases |
|---------------------------------------------------------------|
| Enzymatic activity was measured using 10 pmol of RNase A-1 or RNase in 40 mM sodium phosphate buffer, pH 7.0. Conversion from $A_{260}$ to pmol of ribonucleotide product was as described in Ref. 29 and under “Experimental Procedures.” |
| Enzyme | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|--------|--------|---------|-------------|
| rRNase A-1 | 17.2 | 2.61 | $1.5 \times 10^5$ |
| rRNase A-2 | 0.5 | 0.056 | $1.1 \times 10^5$ |

**FIGURE 5.** Bactericidal activity of rRNases A-1 and A-2. *E. coli* DH5α (A) and *S. aureus* 502A (B) were resuspended in 100 mM sodium phosphate buffer, pH 7.4, and incubated with the indicated concentrations of chicken leukocyte RNase A-1 (open squares) and A-2 (closed circles) for 4 h at 37 °C, plated on RNase-free LB agar, and grown overnight for colony counts. CFU, colony-forming units.

**FIGURE 6.** Identification of the divergent domains within the *G. gallus* leukocyte RNase A-1 and A-2 sequences and the exchange mutation strategy. A, the mature amino acid sequences of chicken leukocyte RNases A-1 and A-2. The divergent amino acids are enclosed in boxes and domains I, II and III as indicated. Likewise boxed is catalytic histidine 110. B, the strategies for creating domain-exchange mutants of RNases A-1 and A-2. Independent ribonuclease-minus forms of rRNase A-1 and rRNase A-2 are created by point mutation of His110 to alanine (H110A).
domain II or III or domains II and III of rRNase A-1 reduced the activity of RNase A-1 to a level indistinguishable from that of rRNase A-2. As anticipated, substitution of His110 to Ala completely eliminated RNase activity.

In Fig. 7B, we explore bactericidal activity of the domain-exchanged mutants of rRNase A-1 against *E. coli*. Each protein was tested at 5 μM, which is sufficient for RNase A-2 to exert a 10^7-fold reduction in colony count (see Fig. 5). As anticipated from earlier results, the presence of wild type rRNase A-1 had no impact on colony count, whereas rRNase A-2 reduced the colony count to fewer than 100 colony-forming units/ml. Substitution of the RNase A-1 backbone with either domain I, and more so, II, or III of RNase A-2 conferred bactericidal activity on RNase A-1. Substitution with domains II and III together resulted in a protein capable of reducing the *E. coli* colony count by 100-fold (p < 0.01).

Enzymatic and Bactericidal Activity of Domain-exchange Mutants of Chicken Leukocyte RNase A-2—The RNase activities of domain-exchange mutants of rRNase A-2 were measured under the same conditions as those of rRNase A-1. Substitution of domain II or III or domains II and III of rRNase A-1 all augmented the RNase activity of rRNase A-2, although the activities of these domain-exchanged mutants are still less than that of wild type rRNase A-1 (Fig. 8A). As shown above for rRNase A-1, substitution of His110 to Ala completely abolished RNase activity.

Bactericidal activities of rRNase A-2 domain-exchanged mutants are shown in Fig. 8B. As above, rRNase A-2 reduced the colony count to fewer than 100 colony-forming units/ml; interestingly, the H110A ribonuclease-minus mutant form of rRNase A-2 was also bactericidal and equally active as the wild type form, indicating that ribonuclease activity can be decoupled from bactericidal activity (see “Discussion”). Substitution of domain I of rRNase A-1 onto the rRNase A-2 backbone significantly attenuated bactericidal activity. Taken further, substitution of either domain II or III of rRNase A-1 alone (or domains II and III together) eliminated the bactericidal activity of rRNase A-2.
Characterization of Ribonucleases in Avian Leukocytes

Bactericidal Activity of Isolated Domain II and III Peptides—Substitution of domains II and III of rRNase A-2 conferred bactericidal activity on RNase A-1, and removal of these domains from rRNase A-2 likewise eliminated bactericidal activity. Similarly, we demonstrated that ribonuclease activity per se is not at all essential for bactericidal activity. Given these findings, we sought to determine whether the primary structure of these domains was sufficient for bactericidal activity using synthetic peptides minimally encoding domains II and III. We also tested a scrambled peptide including the amino acids of RNase A-2 domain II in random order in order to assess whether the amino acid sequence or composition defined the activity. Fig. 9 documents the bactericidal activity of synthetic peptides. The RNase A-2 domain II peptide (pI 12.8) reduced the colony count of E. coli in a dose-dependent manner, whereas A-1 domain II peptide (pI 6.1) was inactive up to 1000 μM. LD₉₀ of A-2 domain II peptide was calculated as 200 μM. The scrambled domain II peptide reduced the colony count of E. coli to the same degree as the wild type A-2 domain II peptide, suggesting that amino acid composition, rather than sequence, defined the bactericidal activity. The A-2 domain III peptide (pI 12.3) was also active and reduced the colony count of E. coli at an even lower concentration (LD₉₀ for E. coli was 10 μM) than the A-2 domain II peptide. The A-1 domain III (pI 9.9) peptide showed no significant activity.

Although both A-2 domain sequences were active bactericidal agents when delivered as free peptides, the domain II peptide was more effective when delivered within the three-dimensional structure of the RNase A backbone. The A-2 domain III peptide was equally bactericidal whether delivered as a free peptide or inserted into the sequence of RNase A-1 (Fig. 9B and Fig. 8B).

DISCUSSION

In this work, we explore the evolution, physiology, and function of two nonmammalian ribonucleases from the chicken, G. gallus. Evolutionary analysis suggests that RNases A-1 and A-2 have diverged under positive selection (dₛ/dₚ = 1.73). Both ribonucleases are found in peripheral blood granulocytes; both are ribonucleolytically active (A-1 > A-2), and both can be inhibited by human placental ribonuclease. However, RNase A-2 is angiogenic and bactericidal, whereas RNase A-1 has neither biologic activity. We have identified two highly cationic domains of RNase A-2 that are both crucial for bactericidal activity. Interestingly, these domains function as independent bactericidal cationic peptides. The bactericidal activity of the A-2 domain II peptide is ~10-fold lower in the absence of the ribonuclease scaffold; the activity of the A-2 domain III peptide remains the same whether in free form or within the tertiary structure of the RNase A molecule.

Bactericidal activity is very likely to be a prominent physiologic function of RNase A-2, as we have documented its localization in peripheral blood granulocytes. There are three morphologically distinct granulocyte lineages in the peripheral blood of bird species, including heterophils, also called pseudoeosinophils (13–49% of the total leukocyte count), true eosinophils (2–14%), and basophils (1–7%) (38). As in mammalian host defense, recruitment and activation of granulocytes are crucial features of resistance to infection, particularly with Gram-negative bacteria (39–41). Chicken granulocytes respond to exogenous stimuli with a characteristic oxidative burst (42), and they express defensin-family peptides (43) and proinflammatory chemokines (44, 45). Interestingly, Zijlstra et al. (46) have recently shown that chicken granulocytes also promote angiogenesis in response to growth factors and tumor cells. Leukocyte RNase A-2 may be a prominent component of both the bactericidal and angiogenic activities of chicken peripheral blood granulocytes.

Given the evolutionary distance between human and bird species, we were surprised to find that the enzymatic activities of both RNase A-1 and A-2 were inhibited with hPRI. Ribonuclease inhibitor is a cytoplasmic protein with multiple leucine-rich repeats and multiple, distinct contact points with individual RNase A ribonucleases with dissociation constants typically in the femtolar range (47–49). Although hPRI does cross species and inhibits the activity of non-human mammalian ribonucleases, such as bovine RNase 1 (see Fig. 4D), earlier studies demonstrated that hPRI does not inhibit the activity of the more distant nonmammalian RNases such as the R. pipiens ribonuclease, onconase (50), or iguana pancreatic ribonuclease (24). The x-ray crystallographic analysis revealed 24 contact points between hPRI and human angiogenin (48); the genes for hPRI-sensitive chicken leukocyte RNases A-1 and A-2, which are both structurally similar to human angiogenin (see Fig. 1), encode only four of these contact points, and two of these
shared contact points (Lys\textsuperscript{42} and His\textsuperscript{110}) are also shared by hPRI-resistant iguana pancreatic ribonuclease and \textit{R. pipiens} onconase. An x-ray crystallographic analysis of both chicken leukocyte ribonucleases and their interactions with hPRI (and \textit{G. gallus} RI, which can be obtained from the livers of estrogen-treated roosters (51)) will likely clarify the basis of this unexpected interaction. Interestingly, the recently characterized ribonuclease inhibitor from \textit{G. gallus} shares 50% amino acid sequence identity with its human counterpart (52); human and chicken RNases share no more than 40% amino acid sequence identity.

As of this writing, chicken leukocyte RNase A-2 is the only nonmammalian RNase A ribonuclease with angiogenic activity. We used the chick aortic arch assay to evaluate angiogenic activity, which is of course uniquely suited from an evolutionary perspective to evaluate angiogenic activity of chicken RNases. Although the factor or factors involved in promoting angiogen-

**FIGURE 10. Three-dimensional localization of the domain II and the domain III sequences.** A, amino acid sequence alignments, including human angiogenin, mouse angiogenin, and two chicken leukocyte RNases, with corresponding amino acid sequence for domain II (red) and domain III (orange) as shown. B, localization of domains II and III based on amino acid sequence alignment shown in A. Sequences corresponding to each domain II and domain III are shown as yellow lines.

The chicken genome includes only three RNase A ribonucleases, which is relatively few in comparison to other genomes that have been evaluated (11, 14). RNases A-1 and A-2 are found on chromosome 6 and separated by only \sim 10 kb of sequence and an intervening B-keratin gene (11), and our data indicate that the mature coding sequences of RNases A-1 and A-2 have diverged by positive selection \((d_N > d_S)\), with a potential role for gene conversion. This scenario is reminiscent of the gene duplication followed by positive selection leading to the emergence of the primate eosinophil ribonucleases, EDN and ECP (29, 53, 54). The single sequence predecessor maintained in the New World monkeys underwent duplication to generate the gene pair observed in higher primates (29, 53). In response to specific evolutionary constraints, EDN remains relatively neutral (pI 8.3–9.2 among higher primates) and enzymatically active, whereas ECP lost much enzymatic activity but acquired positive charge (pI 10.7–11.4) and bactericidal function (29). Similarly, the rat and mouse eosinophil ribonucleases have been duplicated extensively and have generated several highly cationic gene sequences, although function and enzymatic activity have not been explored (55, 56).

We have shown previously in our structure-function study of the eosinophil ribonucleases that RNase activity is not an essential feature of bactericidal activity (28); analogous results have emerged from this study of the chicken leukocyte RNases. We have taken the structure-function analysis substantially further here and have identified two domains within the sequence of leukocyte RNase A-2 that are necessary for bactericidal activity, as their removal eliminates the bactericidal activity RNase A-2 and their introduction confers bactericidal activity on the oth-
 Otherwise inactive backbone of leukocyte RNase A-1. Both RNase A-2 domains II and III can function as independent bactericidal agents. Domain III alone is nearly equipotent on a molar basis to RNase A-1 with the RNase A-2 domain III insertion.

By inference with the x-ray crystallographic structures of human angiogenin and mouse angiogenin-1, the domain II and domain III peptides map to surface regions of the proteins as shown in Fig. 10, at sites that would be available for interaction with a bacterial pathogen. However, most intriguing, the overall message of these data is that the RNase scaffold is actually not essential to the bactericidal mechanism. As shown in Fig. 9, the RNase A-2-derived cationic peptides can function independently to reduce the colony count of E. coli. Our results with the sequence-scrambled A-2 domain II peptide indicate that bactericidal activity depends on the amino acid content (i.e. cati

tonicity) rather than sequence, suggesting a membrane lytic as opposed to receptor-mediated mechanism.

This finding that the RNase backbone or scaffold is not absolutely essential to the bactericidal mechanism is intriguing and somewhat unexpected from an evolutionary perspective. Although the RNase A backbone provides the secretory mechanism and the means for simultaneous delivery of two peptides, our results clearly document that the evolutionary process is generating peptides that can function more or less effectively on their own. We have always considered the gene duplication and divergence to be dedicated toward the selection of novel ribonucleases with enzymatic activity or at least with ribonuclease tertiary structure as crucial function elements. Here, we demonstrate that the ribonuclease backbone is ultimately not crucial at all, except as a base or “construction site” for the evolution of novel, nonribonucleolytic proteins. From a larger perspective, these findings provide an interesting viewpoint on the roles and potential functions of the newly identified nontraditional or “quasi-ribonucleases,” which are the full coding sequences found in various vertebrate genomes that are clearly related to the RNase A ribonucleases but that are missing one or more of the elements necessary for enzymatic activity. Among this group are human RNases 9–13, the rat eosi

nophil ribonucleases with shifted catalytic lysines (55), and several of Md-ribonucleases identified in the genome of the opossum (14). These may be proteins with definitive novel functions but no significant enzymatic connections with ances
tral roots in the RNase A gene family.

In summary, we present here a structure-function analysis of two leukocyte RNA A ribonucleases encoded in the genome of the domestic chicken, G. gallus. These ribonucleases developed via gene duplication followed by positive selection, with RNase A-2 emerging as an angiogenic and bactericidal protein with diminished ribonuclease activity. We have identified two cationic domains from RNase A-2 that confer bactericidal activity on the otherwise inactive RNA A-1 backbone. Interestingly, these cationic domains function as bactericidal peptides without the tertiary structure imposed by the RNase A backbone. These results provide a larger perspective on evolutionary divergence within the RNase A gene family and, in particular, the role of recently discovered quasi-ribonucleases that may have already diverged to accommodate functions that exist totally independent of ribonuclease activity.

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