Ribonucleases and Angiogenins from Fish*

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For the first time fish RNases have been isolated and characterized. Their functional and structural properties indicate that they belong to the RNase A superfamily (or tetrapod RNase superfamily), now more appropriately described as the vertebrate RNase superfamily. Our findings suggest why previously repeated efforts to isolate RNases from fish tissues have met with no success; fish RNases have a very low ribonucleolytic activity, and their genes have a low sequence identity with those of mammalian RNases. The investigated RNases are from the bony fish Danio rerio (or zebrafish). Their cDNAs have been cloned and expressed, and the three recombinant proteins have been purified to homogeneity. Their characterization has revealed that they have indeed a very low RNA-degrading activity, when compared with that of RNase A, the superfamily prototype, but comparable with that of mammalian angiogenins; that two of them have angiogenic activity that is inhibited by the cytosolic RNase inhibitor. These data and a phylogenetic analysis indicate that angiogenic fish RNases are the earliest diverging members of the vertebrate superfamily, suggesting that ribonucleases with angiogenic activity were the ancestors of all ribonucleases in the superfamily. They later evolved into both mammalian angiogenins and, through a successful phylogenesis, RNases endowed with digestive features or with diverse bioactivities.

One of the largest and most studied superfamilies of proteins is that of extracellular, pyrimidine-specific, animal RNases. It has been labeled with different names on different bases: the “RNase A superfamily,” a mostly historical name, recognizes bovine pancreatic RNase A, one of the most successfully investigated proteins, as the superfamily prototype; the “pancreatic-type RNase superfamily,” which includes not only the large family of RNases isolated from the pancreas of many animals but also all other RNases phylogenetically related to them; or “the tetrapod RNase superfamily,” as to date the investigated members of this superfamily comprise RNases from mammals, birds, reptiles, and amphibians but not from fish (1).

Some of the tetrapod RNases have diverse bioactivities, distinct from the ribonucleolytic activity, but strictly dependent on it, including immunosuppressive, cytotoxic, microbial, and angiogenic activity (2). The RNases with angiogenic activity, the angiogenins, form a distinct family within the superfamily and are identified by their ability to stimulate the growth of blood vessels (3, 4). Angiogenins with confirmed angiogenic activity, investigated so far only in mammals, are RNases characterized by a very low catalytic activity, albeit essential to their angiogenic activity, and the presence in their structure of only three disulfides, compared with the four disulfides bridges of most mammalian RNases (3–5).

For an investigation of fish RNases we selected zebrafish (Danio rerio), a tropical bony fish, and one of the most favored model organism for the study of vertebrate development including angiogenesis (14). Zebrafish has been also proposed as an animal system for assaying angiogenesis (15, 16). Furthermore, two sequences apparently related to RNase superfamily members are present in a zebrafish DNA database (Washington University Zebrafish EST Project 1998).

As fish RNases have not been investigated to date, we cloned and expressed in Escherichia coli the cDNAs encoding three RNase sequences from the genome of zebrafish. All three recombinant proteins, purified to homogeneity, were active as ribonucleases. Two of them also were found to possess angiogenic activity. These findings, and an analysis of the phylogenetic relationships of the zebrafish RNases/angiogenins with the other RNases of the superfamily, have led to the proposal that fish angiogenins were the ancestral members of the vertebrate RNase superfamily.”

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‡ The abbreviations used are: hANG, human angiogenin; ZF-RNase, a ribonuclease from zebrafish; cRI, cytosolic RNase inhibitor; EST, expressed sequence tag; MES, 4-morpholineethanesulfonic acid.
EXPERIMENTAL PROCEDURES

Isolation and Cloning of Zebrafish RNase cDNAs—Identification of three genes encoding zebrafish RNases, and cloning of their cDNAs were carried out as follows. The sequence subsequently labeled zf-rnase-1 was first identified in the Washington University Zebrafish EST Project 1998 data base, with accession number wz5727.1. It contained the EST sequence fb58b02.y1 clearly encoding a member of the pancreatic-type superfamily, termed in the data base an “angiogenin-related protein precursor.” A large fragment, obtained from the Resource Center and Primary Database (RZPD GmbH, Berlin, Germany), cloned in pSPORT1 vector, was found to contain the sequence. The N terminus of the encoded protein was identified using the SignalP 3.0 Server program. However, an inspection of the sequence in the Zebrafish genome data base, under completion in the Sanger Institute (Cambridge, UK) revealed that the sequence was incomplete, as it lacked the 15-nucleotide stretch upstream of the cDNA stop codon that encodes the last five amino acid residues at the C terminus of the expected protein. Thus, two PCR steps were performed.

By a first PCR, the fragment starting with the 5’-end of the cDNA encoding the expected RNase, devoid of the signal peptide, was amplified. The oligonucleotides (synthesized by MWG Biotech, Firenze, Italy) employed were: 5’-GGATTCATATGCATGTAAAGGAGCGT-3’ (forward) and 5’-CCCAAGCCTATCGAGAATGCCAATAGGACAAGAGCTC-3’ (reverse). The reaction was performed with the Taq polymerase High Fidelity Kit (Roche Applied Science) following the manufacturer’s instructions. The PCR procedure included 5 min at 95 °C and 35 cycles consisting of 45 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C, and 7 min at 72 °C. The amplified fragment was isolated by low melting, 1% agarose gel electrophoresis and purified using the Product Purification Kit (Roche Applied Science).

The second PCR was carried out for the addition of the missing sequence encoding the five C-terminal amino acid residues. The forward oligonucleotide sequence was the same as for the previous PCR, and the reverse was: 5’-CGTACTTATCGAGAATGCCAATAGGAAGAGCTC-3’. The PCR procedure and the isolation and purification of the amplified product were as described above for the first PCR. The amplified cDNA fragment, previously treated with restriction enzymes NcoI and EcoRI (Novagen, Madison, WI), was cloned into the pET22b(+) expression vector.

The gene sequence labeled zf-rnase-2 was also found in the Washington University Zebrafish EST Project 1998 data base, with accession number fd55b09.y1, and was recognized in the data base as encoding a “mouse-angiogenin-related protein.” A DNA fragment from the RZPD data base was found to contain the sequence and was acquired from RZPD as cloned in plasmid pSPORT1. The N- and C-terminal ends of the encoded protein were identified as described above for zf-rnase-1. The cDNA of interest (devoid of the signal peptide) was obtained by PCR using the following oligonucleotides: forward, 5’-GTATTAGCCTGATATCGAGAATGCCAATAGGACAAGAGCTC-3’; reverse, 5’-GGAGAAGAATCTGCTGAGCGCTTC-3’. The PCR procedure and the isolation and purification of the cDNA were as described above for zf-rnase-1. The obtained cDNA was then treated with NdeI and HindIII restriction enzymes and inserted into the pET22b(+) expression vector.

The third RNase-type sequence (zf-rnase-3) from zebrafish genome was identified on the basis of its relatedness to RNase-encoding genes in the zebrafish genome data base (accession number ENSDARG36171) under completion in the Sanger Institute (Cambridge, UK). Reverse transcription-PCR was performed with mRNA from adult (6-month) zebrafish specimens. The oligonucleotides were: forward, 5’-TGCGCCCTGTCATATTCAATATGACAAGAGCTC-3’; reverse, 5’-TTAGGGGGCGGTTTTACCTTC-3’. The PCR was carried out under the following conditions: one cycle at 94 °C for 4 min followed by 35 cycles at 94 °C for 1 min, 50.7 °C for 2 min, 72 °C for 3 min, and finally one cycle at 72 °C for 15 min. The amplified fragment was isolated by electrophoresis on a low melting, 1% agarose gel and purified as described above. The excised band of interest was cloned in a pGEM T-easy plasmid. The sequencing reaction encoding the signal peptide, identified as described above for the other cDNAs, was excised from the plasmid through a PCR with the following oligonucleotides: 5’-GGATTTCCATATGGGAAATGACCGGCGCTTGC-3’ (forward); 5’-CCCAAGCTTATATGACCGGCGCTTGC-3’ (reverse). The resulting cDNA, treated with NdeI and HindIII, was cloned into the pET22b(+) expression vector.

All cloned, purified DNAs were certified through sequencing (MWG Biotech) before processing. It should be noted that the sequence of zf-rnase-3 in the zebrafish genome data base contained at position 91 a guanine and at position 214 a thymine, whereas in the sequence isolated from adult zebrafish and used for expression of the recombinant protein, we found a cytosine at both positions. This did not produce any change in the amino acid encoded by the triplet 214–216 (leucine), but it altered the amino acid encoded by the triplet 91–93 (from glycine to arginine). Likely, the observed changes are due to single nucleotide polymorphism, nonsynonymous in the latter case.

Expression and Purification of Zebrafish RNases—The three expression plasmids, each containing a cDNA encoding a presumed zebrafish RNase, were used to transform competent E. coli strain BL21(DE3) (provided by Invitrogen). Cells were grown at 37 °C to an A_{600} = 1 and then induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and grown overnight. Pelleted cells were sonicated at 20 kHz in an Ultrasonic sonicator (Heat System Ultrasonic, Farmingdale, NY) with 30-s impulses, each followed by a 30-s rest, for a 15-min total time.

The sonicated cells were centrifuged for 1 h at 12,000 rpm to separate inclusion bodies. As the three proteins were found to be expressed exclusively in inclusion bodies, these were solubilized with 7 M guanidine-HCl in 100 mM Tris acetate, pH 8.4, containing 1 mM glutathione. After flushing nitrogen for 10 min, each preparation was left for 2 h at room temperature. Renaturation was obtained through an initial dilution 1:20, drop by drop, in 100 mM Tris acetate, pH 8.4, containing 0.5 M L-arginine and 1 mM oxidized glutathione.

After 24 h at room temperature, the three preparations were dialyzed against 50 mM Tris-HCl, pH 7.4, and loaded on SP-Sepharose columns (Amersham Biosciences) equilibrated in...
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the same buffer and run on an Akta purifier (Amersham Biosciences). Elution was carried out with a gradient from 0 to 1 M NaCl in the same buffer. In the range 0.3 or 0.4 M NaCl a protein peak was eluted, which for each preparation was found by SDS-PAGE (17) to contain essentially a single protein with the approximate molecular size of an RNase; a low level of RNase activity was revealed through zymograms (see Fig. 1). These proteins were thus labeled ZF-RNase-1, -2, and -3. The fractions containing ZF-RNase-1 and -3 from the chromatographic runs on SP-Sepharose were loaded on a reverse-phase C-4 column (Amersham Biosciences) equilibrated in 100% solution A (composed of 5% acetonitrile (v/v) and 0.1% (v/v) trifluoroacetic acid). The column was eluted with a gradient in which the concentration of solution B (composed of 90% acetonitrile containing 0.1% trifluoroacetic acid) was raised to 100% in 1 h. For each preparation a single, major protein component was eluted, which by SDS-PAGE was found to contain a single protein (see Fig. 1). As for the ZF-RNase-2 protein eluted from the SP-Sepharose column, the corresponding fractions were loaded on a Resource-S column (Amersham Biosciences) equilibrated in 50 mM Tris-HCl, pH 7.4, eluted first with a gradient from 0 to 0.3 M NaCl in the same buffer, and then with an isotonic run at 0.3 M NaCl. The major component eluted with 0.3 M NaCl was finally purified on a reverse-phase C-4 column, run as described above for the final purification of ZF-RNase-1 and -3.

Assays of RNase Activity—Zymogram assays of RNase activity were carried out as described previously (18) on 15% SDS-polyacrylamide gels (17). Quantitative assays of RNase activity were carried out with the fluorogenic substrate 6-carboxy-fluorescein-dArUdAdA-6-carboxy-tetramethylrhodamine (Integrated DNA Technologies, Coralville, IA) (19). The assay mixture contained 0.1 M MES, pH 6.0, 0.1 M NaCl, 20–60 nM substrate depending on the enzyme activity, and suitable enzyme aliquots. Inhibition by cRI (Fermentas International, Burlington, Ontario, Canada) of the RNase activity of the zebrafish enzymes was measured by repeating the assays in the presence of stoichiometric amounts of cRI.

Assays of Angiogenic Activity—The assay was based on previously described assays (20, 21) with the following modifications. A 48-well plate was coated with Matrigel (150 µl/well, purchased from BD Biosciences) for 30 min at 37 °C. Human umbilical endothelial vein cells (3.5 × 10^4 cells/well, obtained from Cambrex, Milan, Italy) were seeded in 250 µl of EBME-2 medium (Cambrex) in the presence of hANG (a kind gift of Dr. Guo-fu Hu, Harvard Medical School) or one of the three purified proteins had ribonucleolytic activity when related to the medium a 5-fold molar excess of inhibitor. All assays were performed in duplicate, and repeated three times.

Bioinformatic Tools—DNA and protein sequences were analyzed using the programs BLAST and FUGUE (22). FUGUE utilizes environment-specific substitution tables and structure-dependent gap penalties, so that scores for amino acid matching and insertions/deletions are evaluated depending on the local environment of each amino acid residue in a known structure. It thus produces the best possible alignment to sequences of proteins with known three-dimensional structures, which can be used as input for modeling. The template chosen was the structure of human angiogenin (Protein Data Bank code 1a4y), and the program was MODELLER (23).

Cladistic analyses were performed by ClustalW alignments and with the previously described program, MEGA 3.0 (Molecular Evolutionary Genetics Analysis (24)). For this procedure the neighbor-joining option was used with Poisson-corrected distances to generate an unrooted phylogenetic tree. Phylogeny was tested by 2000 bootstrap replications.

RESULTS

Isolation, Cloning, and Expression of Zebrafish RNases—Two putative zebrafish RNase-type sequences were available; one of them was incomplete in the published data bases, in which they were identified as proteins phylogenetically related to RNases or angiogenins. The cDNAs encoding these putative RNases, isolated from purchased DNA fragments, were trimmed, reconstructed, and cloned in expression vectors. A third RNase-type sequence was identified in the zebrafish genome, under completion in the Sanger Institute (Cambridge, UK). The latter was isolated through reverse transcription-PCR with mRNA from adult zebrafish specimens. Details on the cloning are under “Experimental Procedures.” An inspection of the genes encoding the zebrafish RNases indicated (data not shown) that, as for all RNase genes investigated thus far, they are comprised in a single exon and have signal peptides for extracellular expression.

Each cDNA was cloned in the expression vector pET22b(+) and used to transform competent E. coli strain BL21(DE3). The proteins, expressed exclusively in inclusion bodies, were subjected to denaturation/renaturation steps and purified to homogeneity by cation exchange chromatography followed by reverse-phase high pressure liquid chromatography as described under “Experimental Procedures.”

Fig. 1 shows an SDS-PAGE analysis and a zymogram of the purified proteins carried out with yeast RNA as a substrate. The three purified proteins had ribonucleolytic activity when related to the high protein aliquots (10 µg) were used. Their molecular sizes, determined by mass spectrometry, were those expected from their amino acid sequences (reported in parentheses following). They were: 14,323.91 ± 0.48 Da (14,330.44); 14,455.49 ± 0.27 Da (14,461.42); and 14,464.78 ± 0.49 Da (14,471.41) for ZF-RNase-1, -2 and -3, respectively. These findings also indicated that the six Cys residues in each sequence form three disulfide bridges, as there is a difference of 6 Da between the observed and the theoretical mass values. Furthermore, amino acid sequencing carried out for the N-terminal 25 residues indicated that the three proteins had the expected N-terminal sequences.
An examination of these sequences revealed that the catalytically essential residues of RNase A, namely His-12, His-119, and Lys-41, were present at correspondingly close sequence positions in all three proteins (see Fig. 2). Furthermore, all three proteins had the standard "RNase signature," i.e. CKXXNTF, whereas the other RNase signature, namely PVHXD/E, was present in ZF-RNase-2 but was only partially conserved in ZF-RNase-1 and -3, as it occurs in several sequences of non-mammalian RNases.

The overall sequence identity values of the three proteins with respect to RNase A were: 31.3, 33.3, and 30.8%, for ZF-RNase-1, -2, and -3, respectively. Higher identity values could be calculated for the three proteins with respect to human angiogenin, an RNase with angiogenic activity. The latter values were: 35.4, 36.2, and 36.2 for ZF-RNase-1, -2, and -3, respectively. In particular, all three proteins shared with all mammalian angiogenins thus far analyzed a consensus sequence within the first 12 N-terminal residues characterized as “YXX-FLXQH.” All three ZF-RNases possess three disulfides (see above) as has been found for non-mammalian RNases and mammalian angiogenins as well.

Characterization of Zebrafish RNases—The purified proteins were tested for RNase activity with a sensitive assay (19). This led to the evaluation of the low activity of these enzymes and provided a direct determination of $k_{\text{cat}}/K_m$ values. As tabulated in Table 1, the three zebrafish proteins have a very low RNase activity with respect to that of RNase A, but it is comparable with that of human angiogenin.

When we tested the three RNases in the presence of cRI, the human cytosolic RNase inhibitor, we found that under the conditions of the assay stoichiometric amounts of cRI fully inhibited (100%) the RNase activity of ZF-RNase-1 and -2, whereas ZF-RNase-3 was inhibited only by 23%.

The finding of a very low RNase activity of the zebrafish enzymes, typical of angiogenins and the sequence similarities reported above between the zebrafish RNases and mammalian angiogenins motivated us to determine whether the zebrafish enzymes possess angiogenic activity. The assays were based on the formation of capillary-like microtubules when primary endothelial cells were grown on Matrigel in the presence of the effectors (20, 21). Fig. 3 shows that ZF-RNase-1 and -2 were found to possess a marked angiogenic activity, comparable with that of human angiogenin. ZF-RNase-3, instead, was found to have no angiogenic activity. Interestingly, the angiogenic activity of ZF-RNase-1 and -2 was completely inhibited by the cytosolic RNase inhibitor (see Fig. 3).

These findings led us to analyze the structure of the zebrafish RNases through modeling based on the structure of human angiogenin (Protein Data Bank code 1a4y) as a template. The
models were validated using ANOLEA (25), and the occurrence of dihedral \( \phi \) and \( \psi \) angles outside allowed areas of the Ramachandran plot was assessed with SEGNO (26).

As expected from the sequence-structure alignments of the sequences as produced by FUGUE (see Fig. 2), the models constructed for Zf-RNase1, -2, and -3 (shown in Fig. 4) closely resemble the structure of angiogenin, with two significant differences: the loop between the first and second \( \alpha \)-helix is shorter in zebrafish RNases; and the loop that follows the second \( \alpha \)-helix in the sequence was found to be longer in the zebrafish proteins (see Figs. 2 and 4).

ZF-RNases in the RNase Vertebrate Superfamily—A preliminary phylogenetic analysis of zebrafish RNases was carried out by comparing their amino acid sequences with those available in the vertebrate RNase superfamily. The rootless tree illustrated in Fig. 5, with radiant branches, includes representative members from the main classes of vertebrate RNases. The bootstrap values are satisfactory, allowing inspection of the relationships among clades with low or no ambiguity. Only for the divergences, bird/mammal and fish/amphibian bootstrap values lower than 50% were found (42 and 46%, respectively).

The monophyletic group of fish RNases, including the three zebrafish RNases and a sequence identified in salmon (NCBI accession number BG936674), appears to be closely related to the family that comprises non-mammalian RNases from amphibians, reptiles, and birds. Mammalian pancreatic RNases (RNases 1) are quite distant from fish and other non-mammalian RNases. The independent clade of mammalian angiogenins (RNases 5) is instead more closely related to the early diverged non-mammalian RNases than to mammalian pancreatic RNases 1. It should be noted that ZF-RNase-3, which has been found devoid of angiogenic activity, appears to be more closely related to salmon RNase than to the other two ZF-RNases endowed with angiogenic activity.

**DISCUSSION**

We report here the first investigation on RNases from fish. As RNases thus far have been found and studied in all tetrapods, the characterization of three fish proteins as RNA-degrading enzymes leads to a more appropriate definition of the animal, extracellular, pyrimidine-specific RNase superfamily as the vertebrate RNase superfamily.

Based on the inspection of RNase genes, it has been proposed (27) that the earliest members of the RNase superfamily are related to the families of RNases 5 (angiogenins) and/or non-mammalian RNases. The findings reported here on the characterization of three fish RNases provide experimental support to this hypothesis. Moreover, based on the evidence that indeed two fish RNases, namely ZF-RNase-1 and -2 from zebrafish, are

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**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{\text{cat}}/K_{\text{m}} ) M s (^{-1} )</th>
<th>( \times 10^2 )</th>
</tr>
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<tbody>
<tr>
<td>RNase A</td>
<td>1.6 ( \pm 0.12 \times 10^7 )</td>
<td></td>
</tr>
<tr>
<td>Zf-RNase-1</td>
<td>2.3 ( \pm 0.25 \times 10^3 )</td>
<td></td>
</tr>
<tr>
<td>Zf-RNase-2</td>
<td>6.3 ( \pm 0.50 \times 10^2 )</td>
<td></td>
</tr>
<tr>
<td>Zf-RNase-3</td>
<td>6.0 ( \pm 0.74 \times 10^3 )</td>
<td></td>
</tr>
<tr>
<td>hANG</td>
<td>7.4 ( \pm 0.72 \times 10^2 )</td>
<td></td>
</tr>
</tbody>
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**FIGURE 3.** Angiogenic activity of ZF-RNase-1 and -2. Assays were carried out with primary human umbilical endothelial vascular cells. Positive (A) and negative (B) controls were obtained by using complete EGM-2 medium and non-supplemented EBM-2 medium, respectively. C, assay for human angiogenin. D and E, positive results obtained with ZF-RNase-1 and -2. F, negative result with ZF-RNase-3. G and H, respectively, show the effects of the cytosolic RNase inhibitor on the angiogenic activity of ZF-RNase-1 and -2.

**FIGURE 4.** Structural models of zebrafish RNases. ZF-RNase-1, ZF-RNase-2, and ZF-RNase-3 are shown in cyan, gray, and blue, respectively. The structure of human angiogenin (Protein Data Bank code 1a4y) is shown in red. The N- and C-terminal ends are marked. The extended loop in region 36–44 (ZF-RNases numbering) is shown.
endowed with angiogenic activity, we propose that all vertebrate RNases evolved from early diverged angiogenic RNases from fish. Upon evolution, they diversified and experienced selection, thus engendering two distinct evolutionary major clades: (i) that of pancreatic RNases possessing digestive abilities, or diverse noncatalytic bioactions, that were still based on their ability to degrade RNA (2); (ii) that of mammalian angiogenins, with angiogenic activity and a low but essential RNase activity (4).

In fact, as one of the three investigated zebrafish RNases, ZF-RNase-3, does not have angiogenic activity, we can suggest that RNases with or without angiogenic activity diverged at a very early stage in the evolution of fish, before the divergence of the closely related amphibian RNases. However, early diverged fish RNases have all the features of superfamily members: they are encoded in a single exon, possess a signal peptide, are stabilized by disulfides, and are endowed with ribonucleolytic activity determined by the presence of two critically positioned His residues and a Lys residue. Work is in progress on the identification of RNases from hagfish, lampreys, and elasmobranchs (dogfish) to verify whether the RNase/angiogenin scaffold predated the divergence of bony fishes. It should be noted that RNase superfamily members have not been found in invertebrates (28).

A comparison of the two zebrafish RNases with angiogenic activity, ZF-RNase-1 and -2, with the best characterized mammalian angiogenins shows that similarities, but also differences, are detectable. As found for mammalian angiogenins (7), the zebrafish angiogenic ZF-RNase-2 contains in its sequence a putative nuclear translocation signal at positions 30–36 (KRRKITAK). A string of basic amino acids is also present in ZF-RNase-3 (RRRIR at positions 30–34), but ZF-RNase-3 does not display angiogenic activity. The fish angiogenins do not present in their primary structures the two Asn residues (at sequence positions 61 and 109 of human angiogenin) in which deamidation leads to a loss of angiogenic activity (4). It has been presumed that mouse Ang-4 is devoid of angiogenic activity because it presents a replacement of Asn with Lys at position 61 (3); however, ZF-RNase-1 and -2 do not present this critical residue, yet they have angiogenic activity. It should be noted that the angiogenic activity of human and other mammalian angiogenins were assayed with a chicken experimental system (29), whereas fish angiogenins were assayed with a human system (see “Experimental Procedures”). Tests carried out on mammalian and fish angiogenins with identical assay systems should help to verify the actual significance of sequence signals in the tested angiogenins.

The availability of the angiogenins described here can help to investigate angiogenesis in zebrafish and the role(s) of the newly isolated angiogenins in the angiogenic process, both in the adult animals and in developing zebrafish embryos. In fact angiogenesis has been successfully studied in zebrafish as a very convenient experimental system (14), and developing zebrafish embryos have been proposed as a model for angiogenic/antiangiogenic drug screening (15).

It is surprising that the human cytosolic RNase inhibitor can exert its inhibitory effects on fish RNases. The presence of cRI has been reported only in mammals (11, 12, 30). Furthermore, cRI has been found to inhibit all tested mammalian RNases, with the exception of seminal RNase, a finding explained by the dimeric structure of this RNase, which is the only RNase of the superfamily with such a structure (31). It does not inhibit an amphibian RNase such as onconase (32), whereas RNase inhibitors from birds and amphibians do not inhibit RNase A, the superfamily prototype (30). Thus, either fishes possess an RNase inhibitor homologous to mammalian cRI that has not yet been detected in evolutionarily related species, or we have a

FIGURE 5. Rootless phylogenetic tree of the vertebrate RNase superfamily. The tree shows the close relationship of fish RNases with mammalian angiogenins on one hand and non-mammalian RNases on the other. Mammalian pancreatic (and seminal) RNases 1 appear to be more distant. ZF-RNase-3, devoid of angiogenic activity, is more closely related to salmon RNase than to the other ZF-RNase-1 and -2. The neighbor-joining method was used with Poisson-corrected distances.
case of proteins that just happen to interact apart from any basic biological significance, as in the case of biotin and avidin.

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