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Review

The RNase a superfamily: Generation of diversity and innate host defense

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Summary

The Ribonuclease A superfamily includes an extensive network of distinct and divergent gene lineages. Although all ribonucleases of this superfamily share invariant structural and catalytic elements and some degree of enzymatic activity, the primary sequences have diverged significantly, ostensibly to promote novel function. We will review the literature on the evolution and biology of the RNase A ribonuclease lineages that have been characterized specifically as involved in host defense including: (1) RNases 2 and RNases 3, also known as the eosinophil ribonucleases, which are rapidly-evolving cationic proteins released from eosinophilic leukocytes, (2) RNase 7, an anti-pathogen ribonuclease identified in human skin, and (3) RNase 5, also known as angiogenin, another rapidly-evolving ribonuclease known to promote blood vessel growth with recently-discovered antibacterial activity. Interestingly, some of the characterized anti-pathogen activities do not depend on ribonuclease activity per se. We discuss the ways in which the anti-pathogen activities characterized *in vitro* might translate into experimental confirmation *in vivo*. We will also consider the possibility that other ribonucleases, such as the dimeric bovine seminal ribonuclease and the frog oocyte ribonucleases, may have host defense functions and therapeutic value that remain to be explored. (190 words)

Abbreviations: RNase, ribonuclease; EDN, eosinophl-derived neurotoxin; ECP, eosinophil cationic protein; RSV, respiratory syncytial virus; Ang, angiogenin; BS-RNase, bovine seminal ribonuclease

Historical perspective

The characterization of RNase A, one of the first enzymes isolated and described in the literature, represents a significant chapter in the history of modern protein chemistry. During the 1960s and into the 1970s, RNase A was a favorite subject for study, as it is remarkably thermostable and is present at high concentration in a relatively accessible source tissue, bovine pancreas. As such, the efforts of many investigators resulted in the purification of RNase A, the elucidation of the complete amino acid sequence, solution of the crystal structure, clarification of protein folding pathways and identification of the elements underlying the catalytic mechanism [1–8].

During this same period of time, methods for detecting RNase activity in human serum were developed and elevations in serum RNase activity were initially perceived as useful biomarkers for the diagnosis and evaluation of pancreatic cancer [9–11]. Although these measurements were eventually discarded due to lack of specificity [12–15], the presence of *specific* RNase A ribonucleases as biomarkers of disease has met with renewed interest. Elevated levels of eosinophil-derived neurotoxin (EDN/RNase 3) are detected in sera of patients with Graves' disease [16] and in urine of patients with ovarian cancer [17]. Likewise, serum angiogenin/RNase 5 has been explored as a prognostic indicator in a wide variety of tumors [18–20], inflammatory bowel disease [21], and endometriosis [22].

Emergence of the RNase a superfamily

During the 1970s and 1980s, Beintema and colleagues isolated and characterized pancreatic ribonucleases from numerous mammalian species and described the evolution of different features within this lineage, which we now refer to as RNase 1 [reviewed in 23–26]. The existence of multiple, highly divergent RNase lineages emerged in the late 1980s. Several of these proteins had already been identified and characterized to some extent; only upon purification or cloning did it become clear that they had structural features in common with the RNases 1. These newly identified RNase A ribonucleases included (1) angiogenin/RNase 5, a factor that had been identified as promoting blood vessel growth in tissue culture, (2) the eosinophil granule proteins eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic

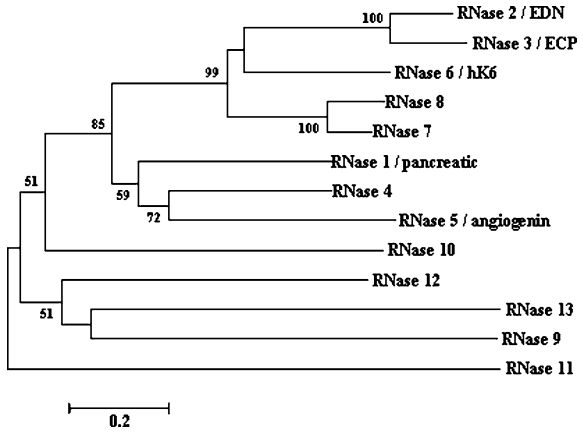

Figure 1. Phylogenetic tree documenting relationships among the human RNase A ribonucleases. As shown, the recently duplicated pairs, RNase 7 and RNase 8 are closely related to one another, as are EDN/RNase 2 and ECP/RNase 3 (78% and 67% amino acid sequence identity, respectively). Although all share cysteine structure and invariant catalytic histidines and lysine, the other human RNase A ribonucleases are highly divergent from one another. An unrooted neighbor-joining tree with complete amino acid sequences, distances determined with Poisson correction, as per algorithms of Mega 3.0 [145], bootstrap values greater than 50 are shown (5000 replications). Genbank accession numbers include: RNase 1, D26129; EDN/RNase 2, M24157; ECP/RNase 3, M28128; RNase 4, NM_194430; Ang/RNase 5, NM_001145; RNase 6, NM_005615; RNase 7, AJ131212; RNase 8, AF473854; RNase 9, NM_001001673; RNase 10, NM_001012975; RNase 11, AY665806; RNase 12, NM_001024822; RNase 13, NM_001012264.

protein (ECP/RNase 3), and (3) a group of frog oocyte proteins, including onconase, a cytotoxin isolated from *Rana pipiens*. Each of these ribonucleases will be considered in greater detail in the sections to follow. Subsequently, RNase 4 was isolated as "contaminant" ribonuclease in the same culture supernatants as angiogenin [27, 28], and RNase 7 was identified in a screen for anti-pathogen proteins from keratinocyte cultures [29] as well as from the analysis of the human genome sequence [30]. Other RNase A ribonucleases were identified directly as part of a search for mammalian superfamily orthologs: human RNase 6 was found as the result of a search for a human ortholog for bovine RNase k2 [31], and RNase 8 was likewise identified with the publication of the of the human genome [32]. Several groups [33–37] have identified several additional genes in the human genome that are related to the RNase A ribonucleases (RNases 9–13), although these latter sequences encode proteins with significant mutations rendering them unlikely to support RNase activity. The phylogenetic tree in Figure 1 documents the relationships among the human RNase A ribonucleases.

Characteristics of RNase a ribonucleases

The human RNase A ribonucleases are encoded by unique genes that are all located on human chromosome 14, in the configuration shown in Figure 2 [33]. All eight known functional genes encode relatively small polypeptides (~15 kDa). Among common elements, each gene encodes a ~20–28 amino acid signal sequence, as would be expected for secretory proteins. Seven of the eight genes encode eight cysteines that form four disulfide bonds in the secreted, appropriately folded forms of the proteins. The exception is the gene encoding angiogenin/RNase 5 which encodes only six cysteines and thus a protein with only three disulfide bonds. Each gene likewise encodes an invariant catalytic triad, which includes two histidines (one near the amino terminus, and one near the carboxy terminus) and one lysine within a conserved signature motif (CKXXNTF). All encoded proteins are catalytically active to varying degrees against standard polymeric RNA substrates. Basic information on structure and enzymatic activity of RNase A ribonucleases can be found in reference [26].

Other than the invariant cysteines, histdines and lysine within the signature motif, the RNase A ribonucleases display remarkable sequence diversity, which can play a role in promoting novel function. As the subject of this review is the generation of diversity to promote host defense, the focus here will be on the RNase A ribonucleases with known host defense functions, which currently include the eosinophil ribonucleases (RNases 2 and 3), RNase 7, and angiogenin/RNase 5. However, it is important to keep in mind

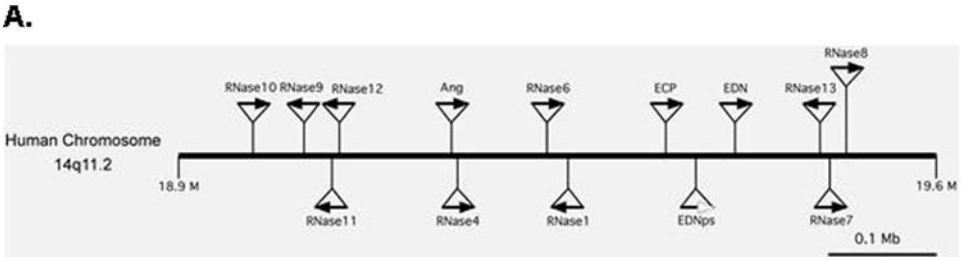

Figure 2. Localization and orientation of human RNase A ribonuclease genes on chromosome 14q11.2. RNase 1, EDN (RNase 2), ECP (RNase 3), RNase 4, Ang (RNase 5), RNase 6, RNase 7 and RNase 8 are ribonucleolytically active functional coding sequences. EDNps is a pseudogene with sequence similar to EDN and ECP. RNases 9–13 are potentially functional genes, but do not have all elements necessary to support ribonuclease activity. Drawn to scale as per documentation in human genome. Reprinted with permission from Cho S, Beintema JJ, Zhang J. 2005. The *Ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories.* Genomics 85: 208–220 [33].

that many, if not all of the other RNase A ribonucleases may play roles in host defense that have yet to be recognized and characterized. This is a particularly intriguing possibility to consider for the newly identified human RNases 9–13 [33–37]. We will also consider the data available on the cytotoxic ribonucleases, including species-limited paralog of RNase 1, bovine seminal RNase (BS-RNase) and ribonucleases from the bullfrog *Rana* species, which have not formally been evaluated in the context of host defense, but might have unexplored possibilities to be considered.

Evolution of the eosinophil ribonucleases (RNase 2 and RNase 3) leads to novel hypotheses vis à vis host defense

The eosinophil ribonucleases, eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic protein (ECP/RNase 3) are two of the major secretory effector proteins of human eosinophilic leukocytes, cells whose role in host defense remains somewhat enigmatic and controversial [38] . The discovery and unusually rapid divergence of the eosinophil ribonucleases has been reviewed previously [39, 40].

As noted earlier, both EDN and ECP had been identified and partially characterized prior to their identification as members of the RNase A superfamily. Olsson and colleagues [41, 42] isolated ECP as an arginine-rich protein of molecular mass ~21 kDa from the secretory granule fraction of human eosinophils. Given the association of peripheral blood and tissue eosinophilia with helminth infection, several groups explored the toxicity the granule proteins towards schistosomula, trypanosomes and other human parasites [43–49], and found significant toxicity in response to micromolar concentrations of ECP administered *in vitro*. However, given the current controversy on the role of eosinophils in host defense against parasitic infection and novel concepts on the function of degranulation in eosinophils in general [50, 51], the interpretation of these toxicity studies remains uncertain. ECP also has activity against gram-positive and gram-negative species in studies performed *in vitro* [52], but there is no documented role for eosinophils in antibacterial host defense *in vivo*.

Durack and colleagues [53] identified EDN as a distinct 18.4 kDa protein that induced the Gordon phenomenon, a cerebellar syndrome associated with Purkinje cell loss upon intrathecal injection in rabbits. In contrast to ECP, EDN is relatively ineffective against pathogens even at high micromolar concentrations, but recently has been shown to be chemotactic for dendritic cells [54, 55]. EDN has been included as a member of the novel class of proteins known as alarmins, which function as early chemotactic activators of innate and adaptive immune responses [56]. Unlike the eosinophil-specific ECP, EDN is also found in liver [57], spleen and placenta, and is a major contaminant of commercial urinary gonadotrophin preparations [58] although the tissue source of this EDN is not known.

Gleich and colleagues [59] were the first to report amino terminal sequences of purified EDN and ECP and to suggest that these proteins were similar to pancreatic RNase A. Molecular cloning elucidated the nucleotide and amino acid sequence similarities between EDN and ECP (89% and 67%, respectively) and confirmed membership in the emerging RNase A gene superfamily [60–63]. EDN/RNase 2 and ECP/RNase 3 are both catalytically active against single stranded RNA substrates [64, 65] and neither exhibit activity against double-stranded substrates under experimental conditions evaluated [66, 67].

Once identified as catalytically active ribonucleases, the role of the enzymatic activity in the characterized biologic activities becomes an interesting question. Molina and colleagues [46] demonstrated that the addition of placental ribonuclease inhibitor did not alter ECP's toxicity for trypomastigotes of the parasite *Trypanosoma cruzi*, although a complete interpretation of this result awaits a clearer understanding of the interaction of ECP with this inhibitor. Our group has demonstrated that a ribonucleolytically-inactivated form of recombinant ECP is just as effective at reducing colony forming units of gram-positive *Staphylococcus aureus* as the ribonucleolytically active wild type [68]. These results are consistent with those reported earlier by Young and colleagues [69] who suggested that cationic ECP functions by destabilizing lipid membranes. The ribonuclease-dependence of the chemotactic activity of EDN has not been addressed [54]. However, the existence of only ribonuclease-independent biologic activities for EDN and ECP is counterintuitive from an evolutionary perspective. If ribonuclease activity is unnecessary for function, what are the selective pressures permitting this catalytic activity and amino acid translations associated with these genes to remain in the genome? We hypothesized that there are ribonuclease-*dependent* functions of ECP (and EDN) that had not yet been identified.

The issue of undiscovered ribonuclease-dependent functions became all the more apparent once the evolutionary history of the eosinophil ribonucleases was elucidated [70–72]. In Figure 3, a Southern blot with genomic DNAs from primate and non-primate mammals probed with the coding sequence of human EDN is shown in which the EDN coding sequence probe detects itself, the ECP coding sequence, and the EDN pseudogene (see Figure 1A). As shown, hybridizing sequences can be detected in all primate genomes, but not in any of the non-primate genomes, despite the fact that eosinophils have been detected in nearly all vertebrate species [38]. Upon isolation of the primate coding sequences, we identified a single predecessor for EDN/ECP among the New World monkeys with relatively limited ribonuclease activity [73], and determined that EDN and ECP arose as a gene pair via a duplication event that occurred after divergence of the New World from the Old World monkeys (Figure 4). Since duplication, the genes encoding EDN and ECP have evolving rapidly under positive selection pressure [71], and have been accumulating non-silent mutations at rates exceeding those

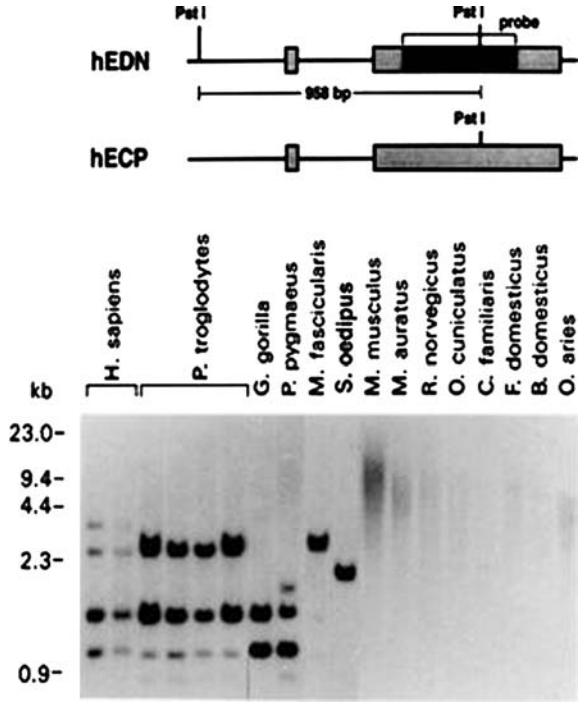

Figure 3. Genomic DNA from primate and non-primate mammals probed with the coding sequence of human EDN/RNase 2. Hybridizing sequences corresponding to EDN/RNase 2, ECP/RNase 3 and/or the EDNps are detected in Pst I restricted DNA from all primates, but not from non-primate mammals. These results suggested that these ribonucleases were highly divergent, a result later confirmed by molecular cloning. *H. sapiens*, human; *P. troglodytes*, chimpanzee; *G. gorilla*, gorilla; *P. pygmaeus*, orangutan; *M. fascicularis*, macaque; *S. oedipus*, tamarin; *M. musculus*, mouse; *M. auratus*, hamster; *R. norvegicus*, rat; *O. cuniculatus*, rabbit; *C. familiaris*, dog; *F. domesticus*, cat; *B. domesticus*, cow; *O. aries*, sheep. Reprinted with permission from Rosenberg HF, Dyer KD, Tiffany HL, Gonzalez M. 1995. *Rapid evolution of a unique family of primate ribonuclease genes.* Nature Genetics 10: 219–223 [70].

of all other functional coding sequences known among primates. Yet, despite the rapid rate of non-silent substitution, each primate gene retains the cysteines, histidines, and lysine that are essential for ribonuclease activity.

Lee and colleagues [74] ultimately identified two ribonucleases from mouse eosinophils, mouse eosinophil-associated ribonuclease (mEar) −1 and −2, and presented Southern analysis suggesting that others remained to be identified. Thirty-five mEars (including pseudogenes) have been identified in the mouse genome and 18 in the rat [33]. The mEars are not only highly divergent from their human orthologs (only ∼50% amino acid sequence homology) but are also evolving rapidly under positive selection pressure (Figure 5). The rodent Ears have diverged into unique non-overlapping clusters generated via a pattern of evolutionary development known as rapid birth death and gene-sorting [75]. This unusual evolutionary pattern has been observed previously in gene families with physiologic functions related to generation of diversity (e.g. . .T cell receptor, major histocompatability complex.). Little is known regarding the functions of individual mEars, although mEar 11, among the most divergent of the group, is expressed specifically in lung tissue response to Th2 stimuli [76] and in response to respiratory virus infection in the absence of interferon-mediated signaling [77]. Similarly, mEar 6 is expressed in liver tissue in response to Th2 stimului characteristic of *S. mansoni* infection [78].

Among the conclusions from the evolutionary studies, we believe that generation of sequence diversity and ribonuclease activity are both important elements that need to be considered in our understanding of the functions of both EDN and ECP and their rodent orthologs, the Ears. In consideration of these issues, we presented the possibility of a role for eosinophils and eosinophil secretory ribonucleases in host defense against respiratory virus pathogens, specifically the pneumovirus, respiratory syncytial virus (RSV); EDN has also been evaluated in HIV infected cultures [79, 80]. There are several studies implicating eosinophils in the pathogenesis of RSV *in vivo* [81–84]. While still in preliminary stages, we have shown that isolated eosinophils and recombinant EDN alone (Figure 6) can reduce the infectivity of RSV for target cells in culture, and that the antiviral effect is not observed with a ribonucleolytically-inactivated form of the recombinant protein (rhEDNΔK38) [85]. The mechanism via which EDN reduces the infectivity of RSV remains to be elucidated. Likewise, we have developed a natural mouse pneumovirus model which will permit us to evaluate the role of antiviral ribonucleases *in vivo* [86, 87], although development of relevant gene-deleted mice will not be a straightforward process due to the duplications and complexity of these genomic loci in mice.

As a final note on genetic diversity, there are two published studies on sequence variation of genes encoding EDN and ECP within human populations. Zhang and Rosenberg [88] identified nine haplotypes of EDN and eight haplotypes of ECP, both rather unremarkable levels of nucleotide diversity, particularly interesting given the extent of interspecies diversity among primates and the gene duplications observed among rodents. Kim and colleagues [89] identified two additional EDN haplotypes, but determined no specific association between haplotypes of EDN or ECP and the development of tropical pulmonary eosinophilia.

Ribonuclease 7 and anti-pathogen activity in healthy human skin

Harder and Schroder [29] identified RNase 7 as a result of an extensive effort to characterize active antimicrobial agents in healthy human skin. Heparin-affinity purification followed by high performance liquid chromatography yielded a protein peak with molecular mass of ∼14.5 kDa and an amino terminal sequence similar to RNases 6 and 8. Purified natural protein exhibited high levels of catalytic activity and broad spectrum antimicrobial activity against gram-positive and gram-negative bacteria as well as against *Candida albicans*

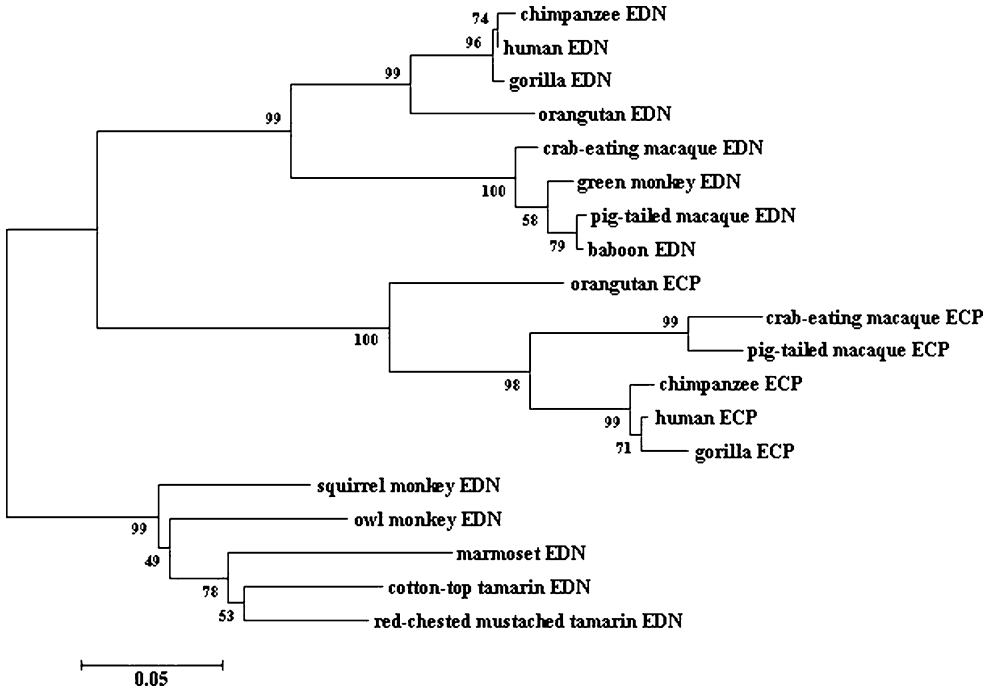

Figure 4. Phylogenetic tree documenting relationships among the primate EDN/RNase 2 and ECP/RNase 3 ribonucleases. As shown, the relationships follow the accepted phylogeny for these primate species. Distinct EDN and ECP genes could not be identified in the New World monkey genomes ($n = 5$); the single copy gene was called EDN because of its low isoelectric point, similar to the higher primate EDNs, however, as shown, it is no more closely related to the higher primate EDNs than it is to the ECPs. Parameters used for creating the unrooted neighbor-joining tree are as described in the legend to Figure 1; Genbank accession numbers include: human EDN, X55988; chimpanzee EDN, U24102; gorilla EDN, U24100; orangutan EDN, U24104; crab-eating macaque EDN, U24096; green monkey EDN, AF479630; pig-tailed macaque EDN, AF479631; baboon EDN, AF479629; human ECP, M28128; chimpanzee ECP, U24103; gorilla ECP, U24097; orangutan ECP, U24101; crab-eating macaque ECP, U24098; pig-tailed macaque ECP, AF479627; squirrel monkey EDN, AF479632; owl monkey EDN, U88827; common marmoset EDN, AF479634; cottontop tamarin EDN, U24099; red chested mustached tamarin EDN, AF479633; the five sequences in the lowermost cluster are the single-copy EDN/ECP genes identified in New World monkeys.

isolates when evaluated in culture systems *in vitro*. Furthermore, transcripts encoding RNase 7 were expressed in a variety of tissues, and induced in keratinocyte culture by addition of TNF-alpha, interferon-gamma, and interleukin-1beta (Figure 7A), as well as in the presence of bacteria. In an independent study, Zhang and colleagues [30] identified the sequence of RNase 7 from the human genome; the intron/exon boundaries of RNase 7 were defined, as well as the unusual lysine-rich nature of the cationic coding sequence.

Only two coding sequences of RNase 7 have been characterized thus far (Figure 7B), so we cannot comment specifically on whether RNase 7 is undergoing rapid evolution in a manner similar to that observed for EDN/RNase 2 and ECP/RNase 3. Neither the mouse nor the rat genome contains sequences orthologous to either RNase 7 or its closest relative, RNase 8 [33], which will unfortunately limit future studies of the role of this protein in host defense *in vivo*. Phylogenetic analysis suggests that the divergence of RNase 7 and RNase 8 occurred relatively recently, from a duplication event occurring during the evolution of primates [33]. Despite the sequence similarities between human RNase 7 and RNase 8, RNase 8 has very little catalytic activity, has none of the anti-pathogen activity that has been characterized for RNase 7, and is expressed only in the placenta when evaluated by Northern analysis in a panel of normal human tissues. Interestingly, the expression pattern (gender-specific, species-limited) and evolution (recent gene duplication and frequency of pseudogenization) of RNase 8 bears significant similarity to that of BS-RNase [90; discussed below].

Novel anti-pathogen activities for angiogenin/RNase 5

Angiogenin was first discovered by Fett and colleagues [91] who were searching for a tumor angiogenesis factor in conditioned medium from the human HT-29 colon cancer cell line. Once the amino acid sequence was determined [92, 93], it became clear that angiogenin had elements in common with RNase A, including the catalytic histidines and lysine, and six of the eight prototypic structural cysteines. Cho and colleagues [33] have recently presented evidence suggesting that angiogenin represents the most ancient of the RNase A lineages; the non-mammalian RNase A ribonucleases characterized to date have cysteine structures that are most closely related to the human angiogenins.

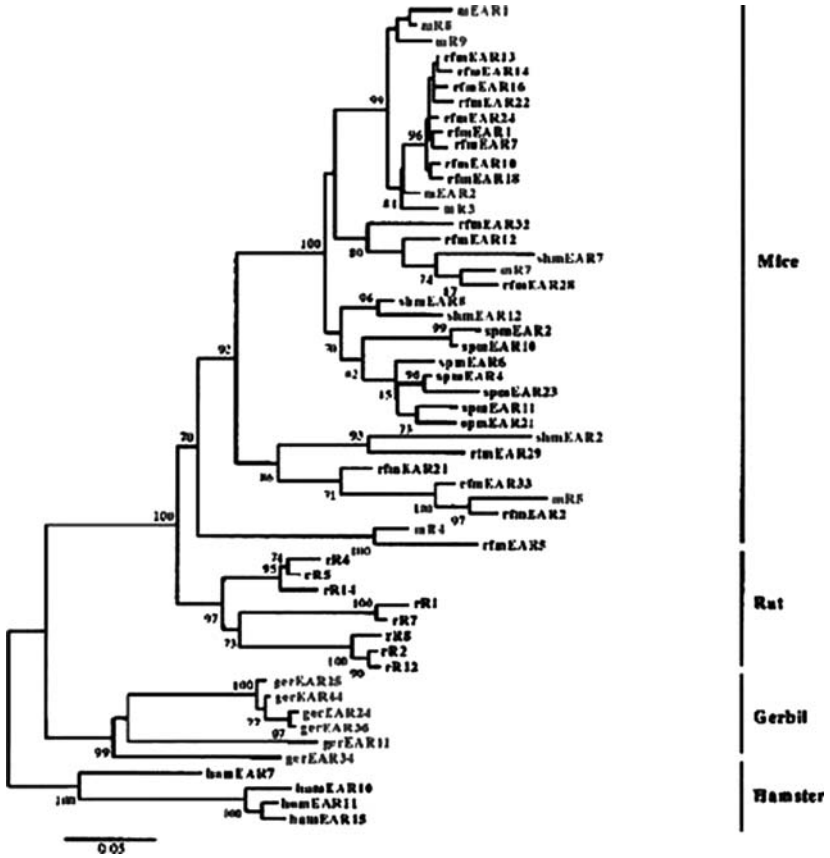

Figure 5. Phylogenetic tree documenting the expansion of the eosinophil ribonuclease lineages among rodent species. The rodent eosinophil-associated ribonucleases have duplicated into divergent gene clusters, in which each member is more closely related to another member of the cluster than it is to any ortholog in another, even closely related species. Note particularly the unique sequences isolated from distinct species of mice (m, house mouse, *M. musculus*; rfm, ricefield mouse, *M. caroli*; spm, spiny mouse, *M. saxicola*; shm, shrew mouse, *M. pahari*). Genbank accession numbers available in original reference. Additional trees demarcating placement of all currently identified mEars can be found in reference [33]. Reprinted with permission from Zhang J, Dyer KD, Rosenberg HF. 2000. *Evolution of the rodent eosinophil-associated RNase gene family by rapid gene sorting and positive selection*. Proc Natl Acad Sci USA 97: 4701–4706 [75].

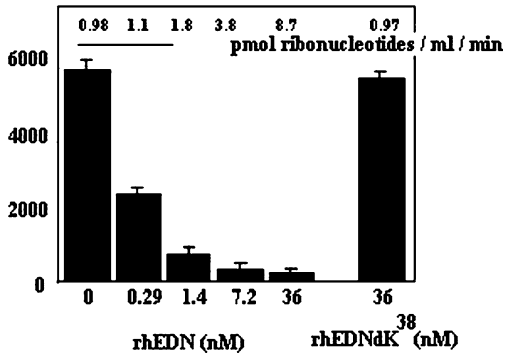

Figure 6. Recombinant human EDN reduce the infectivity of the respiratory pathogen, respiratory syncytial virus (RSV) for target epithelial cells *in vitro*. The antiviral activity of recombinant human EDN (rhEDN) depends directly on its ribonuclease activity, alteration of the active site lysine results in loss of both ribonuclease and antiviral activity. The mechanism via which recombinant EDN reduces virus infectivity remains unclear. Reprinted with permission from Domachowske JB, Dyer KD, Bonville CA, Rosenberg HF. 1998. *Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus.* J Infect Dis 177: 1458–1464 [85].

Human angiogenin is present in normal blood plasma, is expressed in a wide variety of cell types, and has recently been a focus of study for its role in tumorigenesis [94–96]. Although angiogenin has relatively weak ribonuclease activity against the standard RNA substrates [97], it has activity equivalent to that of the RNase 1 lineage when evaluated against ribosomal RNA [98]. That said, the true substrate of angiogenin (or any of the RNase A ribonucleases for that matter) remains unclear. This issue remains particularly acute for angiogenin, as its mechanism of angiogenesis – in which ribonuclease activity has been implicated [99] – appears to have little if anything to do with its enzymatic activity per se. In brief, several interweaving mechanisms of action have been proposed in which angiogenin interacts with a 42 kDa cell surface form of actin [100], and can also initiate proliferation of endothelial cells upon binding to a 170 kDa protein expressed on the cell surface of sub-confluent endothelial cells [101]. Angiogenin can be endocytosed, and, once inside the cell, it initiates signal transduction via Erk 1/2 kinases [102] and is translocated to the

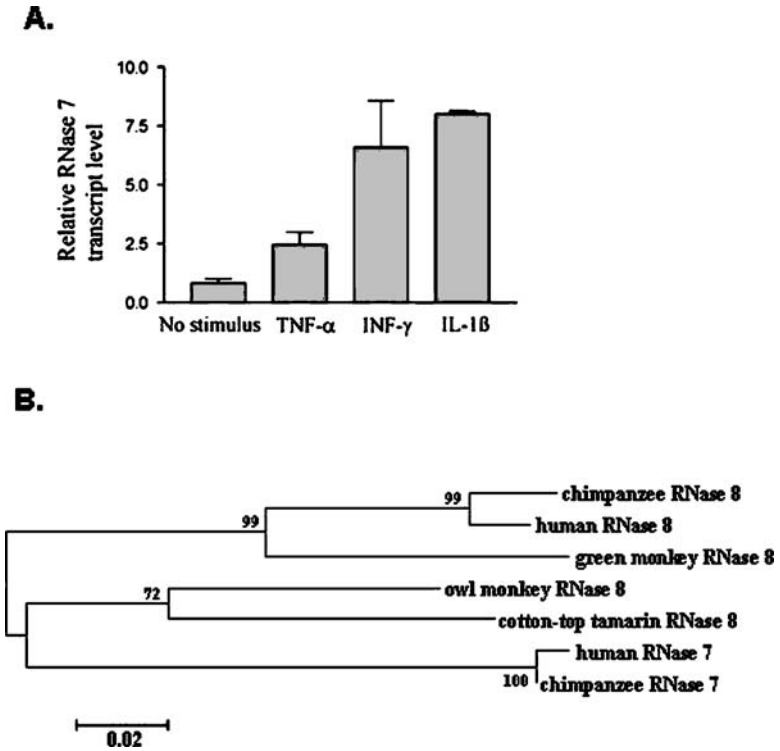

Figure 7. (A) Keratinocyte cultures respond to proinflammatory cytokines with increased expression of transcripts encoding RNase 7. Cationic RNase 7 has anti-pathogen activity, and its expression is induced by cytokines present in tissues infected with bacterial pathogens. Reprinted with permission from Harder J, Schroder JM. 2002. *RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin.* J Biol Chem 277: 46779–46784 [29]. (B) Phylogenetic tree documenting relationships among primate RNase 7s and RNase 8s. RNase 7 and RNase 8 are found only in primate species. There are only two RNase 7 sequences currently identified. RNase 8 is a close relative of RNase 7, and product of a recent duplication event, but has widely different expression pattern and reduced enzymatic activity. Parameters for unrooted neighbor-joining tree construction are as in the legend to Figure 1; Genbank accession numbers include: Human RNase 8, AF473854; chimpanzee RNase 8, AF473855; green monkey RNase 8, AF473858; owl monkey RNase 8, AF473860; cottontop tamarin RNase 8, AF473861; human RNase 7, AY170392; chimpanzee RNAse 7, XM528698.

nucleus where it is required for cell proliferation [103–105] and transcribes ribosomal RNA [106, 107] by binding to CT repeats that are abundant in the non-transcribed region of the ribosomal RNA gene [108]. While many points in this scheme require clarification and unification, this is the most complete mechanism of action described for any of the ribonucleases.

Numerous primate and other mammalian orthologs of human angiogenin have been identified and angiogenin, similar to EDN and ECP, appears to be responding to unusual functional constraints, resulting in a rapid rate of non-silent substitution [109]. All primates studied have a single functional angiogenin gene, save for the Asian colobine monkey, Douc langur (*Pygathrix nemaeus*), in which the single angiogenin gene appears to be pseudogenized [110]. There is also no gene sequence clearly encoding angiogenin in the recently released version of the dog genome [111], although three gene sequences with similarity to human angiogenin have been identified in chicken [33].

Similar to what has been observed for the eosinophil ribonucleases, the angiogenin lineage has expanded in rodents. Six mouse angiogenin genes (mAng1–6) and three mouse angiogenin pseudogenes (mAng-ps1– ps3) have been identified, as well as two rat angiogenin genes (rAng1 and 2) but no rat pseudogenes [33]. In the evolutionary tree of angiogenin genes (Figure 8) non-interleaved groups of mouse and rat genes are found indicating that multiple recent gene duplications have occurred in the angiogenin family since the mouse–rat separation (12–24 million years ago). In the mouse, most of the pair-wise comparisons show higher dN than dS, suggesting positive selection pressure. Cho et al. [33] suggested that positive selection pressure might result in divergent function among these paralogous mouse angiogenins.

With a similar perspective on divergent structure and function, Hooper and colleagues [112] demonstrated that the previously uncharacterized mouse angiogenin 4 (mAng 4) as well as mouse angiogenin 1 (mAng 1) and human angiogenin (hAng) have prominent and previously unsuspected anti-pathogen activities *in vitro*. Transcripts encoding mAng 4 are expressed in intestinal Paneth cells in germ-free mice in response to initial to colonization with bacterial flora. Mouse ang 4 displays selective toxicity toward two bacterial strains, *E. faecalis* and *L. monocytogenes*, in *in vitro* assays (Figure 9). In contrast, human ang and mouse ang1 displayed greater toxicity toward *C. albicans* and *S. pneumoniae*. The

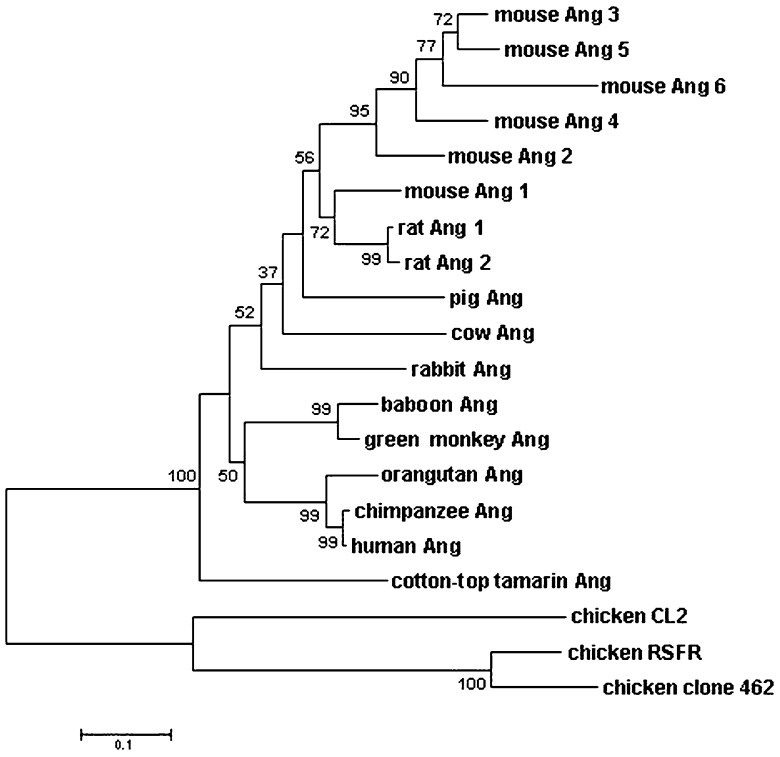

Figure 8. Phylogenetic tree documenting relationships among mammalian angiogenins/RNases 5. Included are angiogenins from primates, non-primate mammalian species; the chicken sequences have been included among the angiogenins, as they encode six rather than eight cysteines, but they are distant orthologs, and may not be directly related to this lineage [147, 148]. Parameters for unrooted neighbor-joining tree construction are as in the legend to Figure 2; Genbank accession numbers include: Human ang, NM_001145; chimpanzee ang, AF4416661; orangutan ang, AF441663; baboon ang, AF441666; green monkey ang, AF441664; cotttontop tamarin ang, AF441668; pig ang, AU059926; rabbit ang, P31347 (swissprot); mouse ang 1, NM_007447; mouse ang 2, NM_007449; mouse ang 3, U72672; mouse ang 5, AY665820; rat ang 1, AY665826; rat ang 2, AY665827; cow ang AF135124, chicken CL2, AY665837; chicken RSFR, X64743; clone 462, P27043.

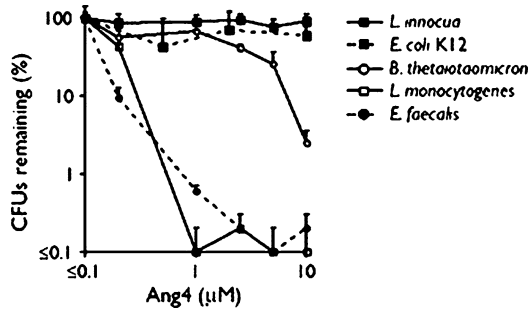

Figure 9. Selective antibacterial activity of mouse angiogenin 4. Mouse angiogenin 4 displays selective toxicity toward pathogens *L. monocytogenes* and *E. faecalis* at concentrations between 1 and 5 micromolar protein. The basis for this selective activity remains unclear. Reprinted with permission from Hooper LV, Stappenbock TS, Hong CV, Gordon JI. 2003. *Angiogenins: a new class of microbicidal proteins involved in innate immunity.* Nature Immunol. 4: 269–273 [112].

molecular basis for this selectivity remains unclear; it is likewise not known if the microbicidal activities are dependent at all on ribonuclease activity. Further work will be necessary to elucidate the nature and relative anti-pathogen activities of the angiogenins *in vivo.*

Bovine seminal ribonuclease: Is there a role in host defense?

Bovine seminal ribonuclease (BS-RNase) is a distinctive member of the RNase A gene superfamily, as it is the product of a species-limited gene-duplication of RNase 1, and the only RNase A superfamily member found naturally in oligomeric form [113, 114], although there has been significant recent interest in the properties of synthetic oligomers [115, 116]. The monomeric RNases 1 are not recognized as having major roles as cytotoxins or in anti-pathogen host defense, although it may still be possible that such functions exist; an RNase 1 gene-deleted mouse has not yet been described. However, the unusual properties of BS-RNase suggest that further exploration of its host defense properties might be warranted.

The BS-RNase gene is also unique to ruminant species, and is the only RNase A family ribonuclease that forms a natural covalent homodimer. This tertiary structure is promoted by interchain bonds between two unique cysteines present at positions 31 and 32 and covalent interchange of amino terminal domains [117–119]. The BS-RNase homodimer is catalytically active against double-stranded as well as single-stranded RNA [120, 121], is internalized by actively dividing cells, and is resistant to intracellular ribonuclease

inhbitor [122, 123], features which have been examined to explain BS-RNases apparent toxicity toward neoplastic cells [124–127] and proliferating lymphocytes [124, 128–130].

While interesting and important from the perspective of developing biotherapeutics, we still cannot say what if any role BS-RNase has to play in promoting host defense or even reproductive fitness in its host organism, the bull, *Bos taurus*. BS-RNase has an extremely limited pattern of expression, as it has been detected only in the ampulary gland and seminal vesicles and the protein is present at relatively high levels in seminal fluid [132]. Matousek and Klaudy [132] demonstrated that levels of BS-RNase protein in seminal fluid had no bearing on the fertility of a given bull. Slavik and colleagues [133] showed that BS-RNase can inhibit the meiotic maturation of bovine oocytes in studies performed *in vitro*. Although BS-RNase-mediated suppression of lymphocyte proliferation as noted above could certainly alter pathways relating to host defense, further research would be necessary to determine how this scenario might develop *in vivo*.

The evolution of BS-RNase is likewise unusual. The gene encoding BS-RNase is limited to ruminant species, yet in nearly all species but *Bos taurus*, the gene sequence has elements indicating pseudogenization [90]. The one exception is that of the swamp type water buffalo (*Bubalus bubalis*), yet even then, there is no evidence of protein expression [134]. Benner and colleagues [135] suggest that BS-RNase in *Bos taurus* is an example of a newly resurrected pseudogene that may be in the process of being tailored to fill a novel niche. While it is not clear that this niche relates at all to a host defense function, this remains a possibility.

*RNase a ribonucleases from the bullfrog **Rana** species*

Only a few of the non-mammalian RNase A ribonucleases have been characterized to any significant extent, and of those that have, such as those of the bullfrog, or *Rana* species, the focus has been biotherapeutic as opposed to biologic function. The primary sequence of *Rana catesbeiana* RNase is identical to that of a sialic-acid-binding lectin isolated from the oocytes of *R. catesbeiana* [136] and is highly homologous to that of an RNase isolated from the liver of *R. catesbeiana* [137]. Onconase isolated from *R. pipiens* [138] is homologous to the *Rana catesbeiana* RNases was the first of the *Rana* proteins to be identified as an RNase A family variant with anti-tumor activity, and renamed as onconase. The therapeutic potential of onconase, related all or in part to its resistance to human ribonuclease inhibitor, has been reviewed extensively [139–142], and is not within the scope of this review. However, what has not been explored at all is the role of these lectin-ribonucleases *in vivo* and their potential for host defense in their natural setting. Liao and colleagues [143] have shown that estrogen can control expression of these genes. Furthermore, multiple *Rana* sequences have evolved under positive selection pressure into multigene clusters [144, 145], in a manner similar to that observed for the rodent eosinophil-associated ribonuclease genes [70]. However, any assumptions about convergence of function would be quite premature.

Conclusion

Three distinct lineages of the complex RNase A superfamily have characterized host defense function *in vitro*. The primate genes encoding two of these lineages – the eosinophil ribonucleases (EDN/RNase 2 and ECP/RNase 3) and angiogenins (RNase 5) are responding to positive selection pressure ($dN > dS$) and the rodent repertoires have undergone significant expansion. The third primate gene, RNase 7, is the product of a recent duplication event and has no rodent orthologs. Save for the preliminary studies documenting the antiviral properties of the eosinophil ribonucleases, the antipathogen activities are not clearly related to ribonuclease activity. An understanding of the role played by these RNase A ribonucleases in promoting host defense *in vivo* will be the next important step forward.

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