RNase A ribonucleases and host defense: an evolving story

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Abstract: RNase A (bovine pancreatic RNase) is the founding member an extensive family of divergent proteins that share specific elements of sequence homology, a unique disulfide-bonded tertiary structure, and the ability to hydrolyze polymeric RNA. Among the more intriguing and perhaps counterintuitive findings, at the current state of the art, the connection between RNase activity and characterized host defense functions is quite weak; whether this is a scientific reality or more a reflection of what has been chosen for study remains to be determined. Several of the RNase A family RNases are highly cationic and have cytotoxic and bactericidal properties that are clearly (eosinophil cationic protein, leukocyte RNase A-2) or are probably (RNase 7) unrelated to their enzymatic activity. Interestingly, peptides derived from the leukocyte RNase A-2 sequence are nearly as bactericidal as the entire protein, suggesting that among other functions, the RNase A superfamily may be serving as a source of gene scaffolds for the generation of novel cytotoxic peptides. Other RNase A ribonucleases are somewhat less cationic (mouse angiogenin 4, zebrafish RNases) and have moderate bactericidal activities that have not yet been explored mechanistically. Additional host defense functions characterized specifically for the RNase eosinophil-derived neurotoxin include reducing infectivity of RNA viruses for target cells in culture, which does require RNase activity, chemoattraction of immature human dendritic cells via a G-protein-coupled receptor-dependent mechanism, and activation of TLR2. The properties of individual RNase A ribonucleases, recent experimental findings, and important questions for the near and distant future will be reviewed. *J. Leukoc. Biol.* 83: 1079–1087; 2008.

Key Words: *leukocyte · cationic · eosinophil · evolution · bactericidal · virus*

INTRODUCTION

RNase A (also known as bovine pancreatic RNase) is perhaps the best-characterized of all known mammalian proteins, and much of what has been learned about its chemistry and catalytic mechanism has had a direct impact on our understanding of the nature and evolution of the gene superfamily that bears its name [1]. However, it is not at all evident mechanistically how RNase A ribonucleases, whose members share specific elements of sequence homology, a unique three-dimensional disulfide-bonded structure, and the ability to hydrolyze polymeric RNA, might function to promote host defense. Given the timing of experimental discoveries, many of the better-known connections to host defense, including bactericidal, helminthotoxic, and cytotoxic functions, preceded discovery of the gene family, and (perhaps as a result) the vast majority of host defense functions currently characterized for RNase A ribonucleases are not related at all to their enzymatic activity. This observation may relate more closely to the experimental directions taken by individual researchers rather than the nature of the biology itself, as there may be important functions that do rely on active enzymatic activity that simply have not yet been considered and explored. A summary of our current knowledge vis à vis RNase A ribonucleases and host defense is included in **Table 1**.

THE RNase A SUPERFAMILY

As noted earlier, the protein RNase A, initially isolated from bovine pancreatic tissue, was a favorite subject for protein structure and functional studies during the 1950s and 1960s, as a result of its thermostability and the relative abundance of its source tissue [26–28]. However, during the late 1980s, several seemingly unrelated proteins from other source tissues were isolated and their genes cloned and sequenced, and these proteins were ultimately found to have significant sequence homology to pancreatic RNase. The founding members of what was to become the RNase A superfamily include angiogenin (RNase 5) [29], the eosinophil cationic protein (ECP; RNase 3), eosinophil-derived neurotoxin (EDN; RNase 2) [30–33], and several of the bullfrog oocyte cytotoxins [34, 35]. The biochemical nature of RNase A ribonucleases and the emergence of the RNase A gene superfamily have been reviewed extensively [36, 37]; a phylogenetic tree documenting the relationships among representative RNase A ribonucleases is shown in **Figure 1**. Upon completion of the human genome, eight fully,

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TABLE 1. Summary of Host Defense Functions Examined for RNase A Family Ribonucleases

RNase A ribonuclease	Species	pI	Host defense activities
Eosinophil-derived neurotoxin (RNase 2)	Human	9.0	Chemoattractant for immature human dendritic cells (DC) [2] Enhances maturation of DC [3][a] Reduces infectivity of RNA viruses (HRSV, HIV) in tissue-culture assays [4–6][b] Produced in macrophages in response to LPS and TNF-α [3] Activates TLR2 and augments Th2-biased immune responses [7]
Eosinophil cationic protein (RNase 3)	Human	10.8	Cytotoxin for helminthes [8–13], strong bactericidal activity [14] Reduces infectivity of HRSV in tissue-culture assay [15]
Eosinophil-associated RNase 2	Mouse	9.70	Chemoattractant for immature human DC [2]
Eosinophil-associated RNase 11	Mouse	9.10	Produced by alveolar macrophages in response to Th2 cytokine stimulation [16] Produced in response to respiratory virus infection, up-regulated in the absence of type I IFN signaling [17]
Eosinophil-associated RNases 1 and 2	Rat	8.6	Moderate bactericidal activity against *Escherichia coli* and *Staphylococcus aureus* [18, 19]
Angiogenin (RNase 5)	Human	10.1	Moderate bactericidal activity against *Streptococcus pneumoniae* [20]; conflicting report [21]
Angiogenin-4	Mouse	9.0	Moderate bactericidal activity against *Listeria monocytogenes* and *Enterococcus faecalis* [20] Produced in response to bacterial LPS [20]
RNase 7	Human	10.3	Broad-spectrum, strong bactericidal activity, very strong against *Enterococcus faecium* [22]
RNase 8	Human	8.4	Broad-spectrum, moderate bactericidal activity [23]
Leukocyte RNase A-2	Chicken	10.9	Bactericidal against *E. coli, S. aureus* [24], and *Salmonella sps.* (unpublished data)
Dr-RNases 1, 2, and 3	Zebrafish	9.0, 9.2, 8.8	Broad-spectrum, moderate bactericidal activity against *E. coli* [25]

Values for isoelectric point (pI) are calculated based on primary amino acid sequences. Moderate bactericidal activity, low micromolar concentrations in standard overnight incubation assay reduce colony count ten- to 100-fold; strong bactericidal activity, low micromolar concentrations reduce colony count 10^4- to 10^7-fold. [a] Activity also shared with human pancreatic ribonuclease or RNase 1. [b] Activity dependent on enzymatic (ribonuclease) activity. HRSV, Human respiratory syncytial virus; Dr, *Danio verio*.

catalytically active RNase A ribonucleases (numbered 1–8) were identified together with five divergent sequences that encode proteins that are structurally incapable of degrading polymeric RNA [39–41]. Each RNase A RNase displays some degree of nucleotide preference within RNA substrates, but they do not have rigidly selective recognition or cleavage sites. Among the signature features of this gene family, all active RNase A ribonuclease coding sequences are initiated with a signal sequence, all mature proteins include six to eight appropriately spaced cysteines that form distinct disulfide bonds, and all include two catalytic histidines and a single lysine, the latter within an invariant sequence motif (CKXXNTF). As a rule, RNase A ribonuclease coding sequences are found on a single exon and in the human genome, are found on chromosome 14 [41].

Host defense functions have been attributed to three specific lineages of the mammalian branch of the RNase A superfamily, including the aforementioned eosinophil secretory RNases, which are rapidly diverging proteins with prominent bactericidal, helminthotoxic, and antiviral activities; RNase 7, a bactericidal cationic protein initially isolated from human skin and the closely related RNase 8; and the angiogenins, proteins originally characterized as promoting blood vessel growth and just recently shown to have distinct, species-specific, antipathogen functions. Many fewer nonmammalian RNase A lineages have been studied; however, host defense functions have recently been identified for the highly cationic chicken leukocyte RNase A-2 and for three RNase A RNases from the zebrafish *D. rerio*.

THE EOSINOPHIL RNases

The concept that RNase A ribonucleases had a role to play in promoting host defense initially emerged from studies about the eosinophil granule proteins, ECP (RNase 3) and EDN (RNase 2). Although the results of in vitro experiments suggest that these mediators interact with and destroy target pathogens (parasites, bacteria), it is important to understand that the role of their source cells, the eosinophils themselves, in promoting host defense remains somewhat enigmatic and highly controversial. Although eosinophils are recruited in large numbers in response to parasitic infection and allergic provocation, there is no consensus as to their overall roles, functions, or contributions to these disease states [42–44]. Most recently, even the issue of whether eosinophils function via degranulation has come under significant scrutiny [45].

To review this history of these proteins, ECP was first identified and isolated as an arginine-rich cationic protein secreted from eosinophils [8, 46] that was toxic to human parasites when evaluated in vitro [9–11]. Likewise, ECP has documented bactericidal activity in vitro [14], which has been exploited as a useful and general model of cytotoxicity, although there is in fact no evidence that eosinophils themselves have any role to play in combating bacterial infection in vivo. Similarly, EDN was identified as a component of eosinophils that induced a cerebellar syndrome (the Gordon phenomenon) when injected intrathecally into rabbits [47], although there is likewise no evidence to suggest that this represents a physiologic phenomenon. Interestingly, although EDN and ECP have

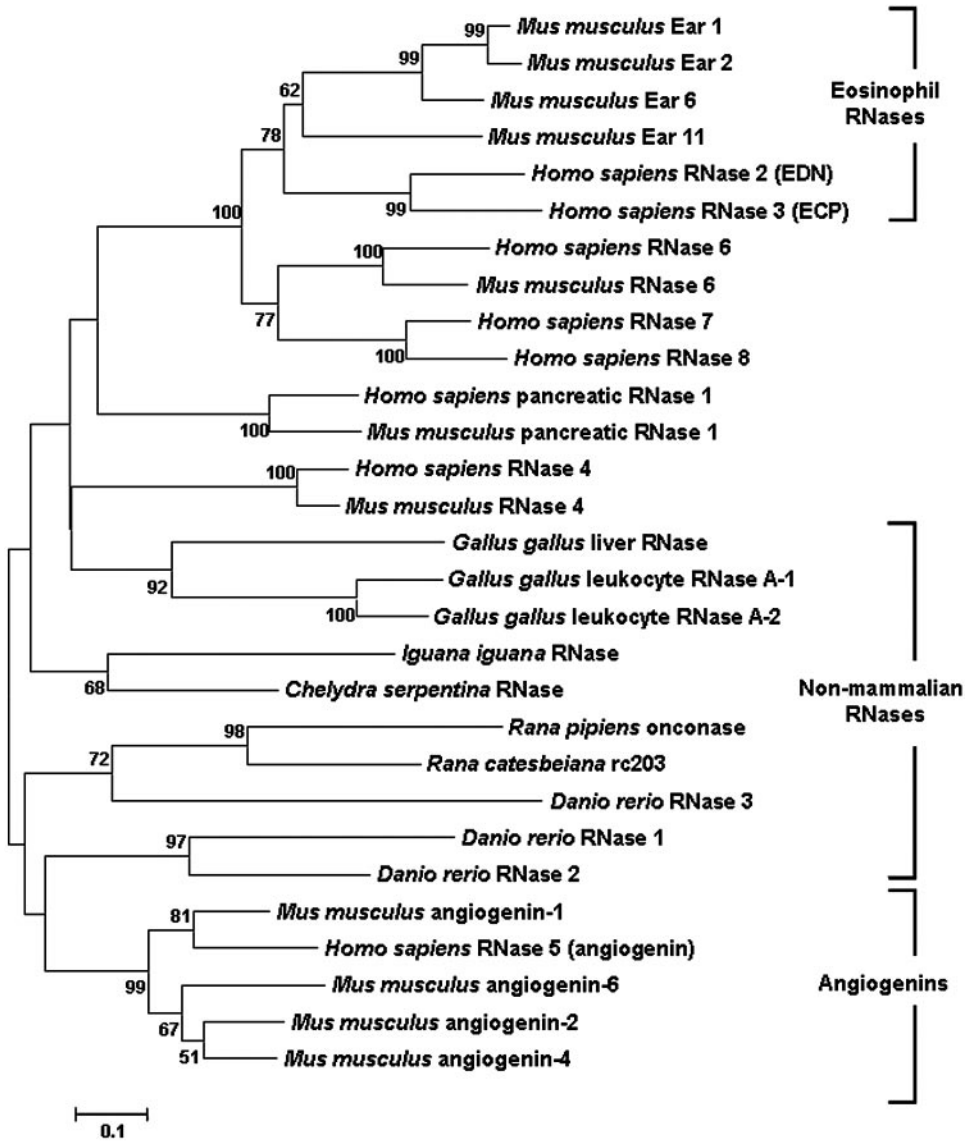

Fig. 1. A neighbor-joining phylogenetic tree featuring the relationships among the major mammalian and nonmammalian RNase A lineages. Amino acid sequences were aligned using ClustalW (http://clustalw.genome.jp/), and an unrooted tree was created using Mega 4.0 Evolutionary Genetics software [38] (http://www.megasoftware.net/). Bootstrap values over 50 (1000 replicates) are shown. Sequences are from GenBank and National Center for Biotechnology Information Protein databases (http://www.ncbi.nlm.nih.gov/sites/entrez); accession numbers are available from the author on request.

similar biochemical properties, EDN is relatively neutral and significantly less effective when evaluated in vitro against helminthes and bacterial pathogens [9–14].

Gleich and colleagues [48, 49] were the first to report the amino terminal sequences of EDN and ECP, to observe that the sequences were similar to one another as well as to that of pancreatic RNase, and to demonstrate that each had RNase activity. Molecular cloning confirmed membership of both of these proteins into what was then the emerging RNase A gene superfamily [30–33].

Among the more interesting features of these proteins, primate EDN and ECP and the expanded group of rodent orthologs, the eosinophil-associated RNases, or Ears, are known for their unusual degree of interspecies divergence, and the evolutionary constraints to which they appear to be responding have been hypothesized as directly related to their roles in promoting host defense [15]. Specifically, we have shown that EDN and ECP arose as a gene pair as a result of a duplication event that occurred shortly after the divergence of the Old World from the New World monkeys, an event dated at ~50 million years ago, and since that point in time, they have been accumulating nonsilent mutations at a very rapid rate [50]. Also interesting, despite the rapid rate of change, all evolutionary variants maintain the features necessary for enzymatic activity [51, 52]. Lee and colleagues [53] identified the first mouse Ears 1 and 2, which are highly divergent orthologs of EDN and ECP (only ~50% aa sequence homology). Zhang and colleagues [54] subsequently provided evidence demonstrating that the rodent Ears underwent divergence via rapid birth-death and gene sorting, a pattern observed only among gene families characterized by functional diversity (e.g., TCR, MHC), although not proof, again, suggestive of a role for these proteins in promoting host defense.

Ericksson and colleagues [55] have explored the role of genetic diversity within human species, focusing on ECP polymorphisms [56] and their correlations with the course of infection with the helminth, *Schistosoma mansoni*. Interestingly, the authors demonstrated a lower prevalence of schistosomiasis among individuals in a Ugandan population who were homozygous for the sequence polymorphism encoding a more cytotoxic variant of ECP (434GG, including an arginine at position 97, as opposed to a threonine). Of note, although this polymorphism

has an impact on ECP vis à vis its cytotoxicity, it does not alter its RNase activity to any significant extent [57]. This is consistent with studies performed with ribonucleolytically inactivated ECP, which demonstrated that the cytotoxic properties of ECP were not at all dependent on RNase activity [58].

This last finding is worthy of further comment. Despite the persistence of RNase activity in the face of rapid sequence change, ECP and ribonucleolytically inactivated ECP can function equally effectively in cytotoxicity assays. Certainly, a RNase activity-independent mechanism is consistent with the earlier findings of Young and colleagues [59], who reported that ECP functioned by destabilizing lipid membranes. This work was extended by Nogués and colleagues [60], who demonstrated that specific cationic and aromatic amino acids that map to the surface of ECP, as opposed to catalytic components, were crucial for bactericidal activity (**Fig. 2**), and by Boix and colleagues [61], who explored the distinct roles of individual tryptophan molecules and found the membrane-destabilization properties of ECP to be consistent with a "carpet-like" mechanism. Another intriguing and yet-to-be-explored avenue of research is the possibility that ECP (and other cationic RNases) interact with and activate bacterial autolysins [62]. The molecular mechanism of the cytotoxicity of ECP has been reviewed extensively [63]. However, it would seem unusual if not counterintuitive for a lineage to retain an enzymatic activity in the face of such otherwise rapid divergence for which it maintained no use whatsoever.

Toward this end, we hypothesized that EDN and ECP might also have activity against pathogens that were likewise subjected to evolutionary stress while remaining ribonucleolytically vulnerable; the literature linking eosinophils to the pathogenesis of disease caused by the identified target, single-stranded RNA respiratory virus pathogens, has been reviewed previously [15]. As such, we have shown that eosinophils and recombinant EDN can reduce the infectivity of the respiratory virus pathogen, HRSV for target epithelial cells in culture [4, 15] (**Fig. 3**). Addition of RNase inhibitor eliminated the eosinophil-mediated antiviral effect; likewise, antiviral activity is not observed with the rhEDNΔK38. We have recently expanded on this work by demonstrating a role for eosinophils in HRSV clearance in vivo [64] and will proceed to explore this question with natural respiratory pathogens [65]. However, other than the existence of ribonucleolytic involvement at some level, the precise mechanism via which EDN reduces virus infectivity remains unclear. Of note, EDN has been shown to have activity against other viruses in similar in vitro settings, including HIV [5, 6].

Another intriguing and original feature of EDN and its role in promoting host defense is its ability to promote maturation and chemotaxis of DC [2, 3]. EDN can promote chemotaxis of immature human DC nearly as effectively as the CXC chemokine, stromal-derived growth factor (SDF)-1α and does so via a direct or indirect interaction with an otherwise unidentified pertussis toxin (PTX)-sensitive G protein-coupled receptor (**Fig. 4**). Most recently, EDN was shown to activate TLR2 and to enhance antigen-specific, Th2-biased immune responses in vivo [7]. Yang and colleagues [66] have likewise not determined whether any of these features are directly related to RNase activity per se but have classified EDN as an alarmin, which is a potent immunostimulant, including defensins, cathelicidin, and high-mobility group box protein 1, serving as early warning signals to activate innate and adaptive immune systems.

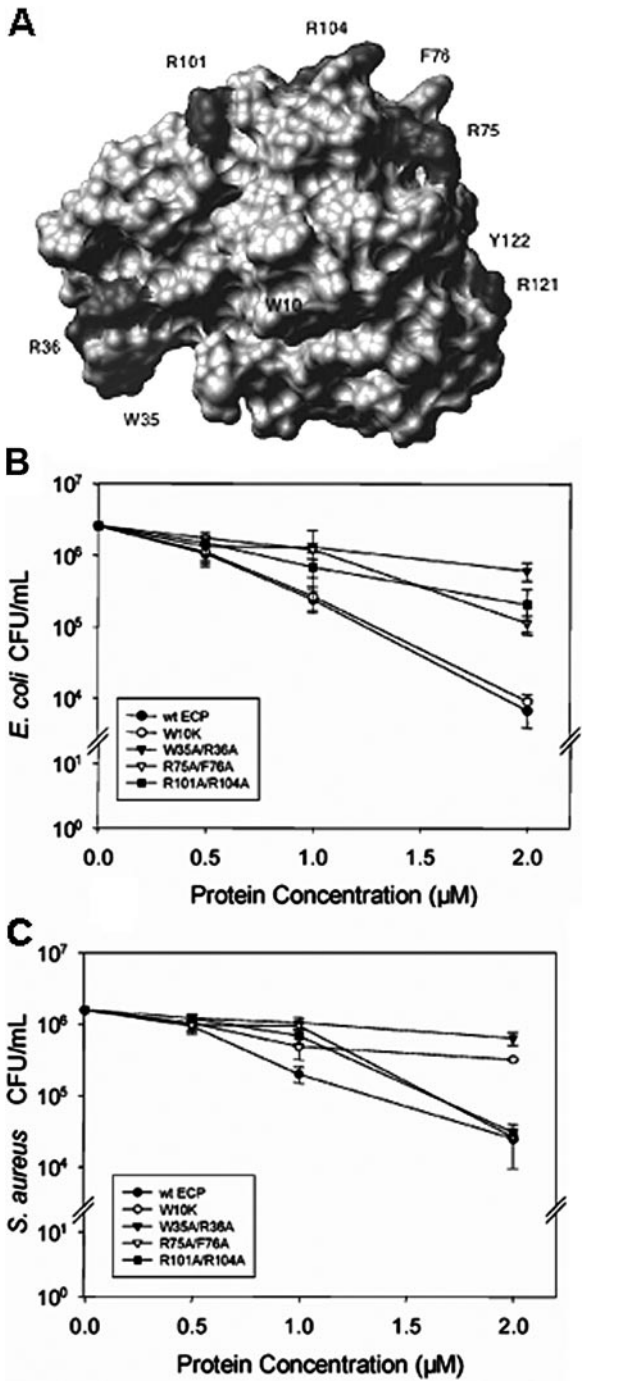

Fig. 2. Structure–function analysis of the bactericidal activity of ECP. (A) Space-filling diagram documenting the location of various amino acids evaluated in a mutational analysis by Carreras et al. [60], reprinted with permission, followed by a demonstration of the importance of specific cationic arginines (R) and aromatic tryptophans (W) to the overall activity of wild-type ECP (wt ECP) against (B) *E. coli* and (C) *S. aureus*. Overall, data suggest distinct mechanisms of action against gram-negative versus gram-positive bacterial species. wt ECP, Wild-type ECP.

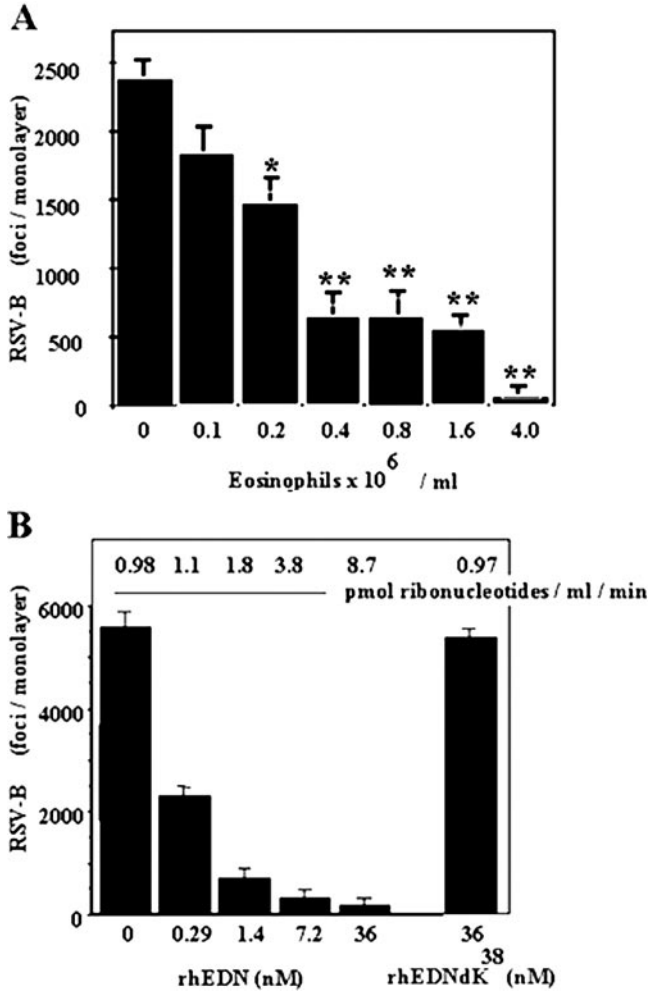

Fig. 3. (A) Isolated human eosinophils and (B) recombinant human, ribonucleolytically active EDN (rhEDN) reduce the infectivity of the pathogen HRSV for target epithelial cells in vitro (*, $P < 0.3$; **, $P < 0.01$ vs. control). As shown, ribonucleolytically inactivated recombinant human EDN [rhEDNΔK (rhEDNdK); catalytic lysine K38 converted to arginine] has no antiviral activity, although the mechanism via which EDN reduces virus infectivity remains otherwise unclear. Reprinted with permission from Domachowske et al. [4].

THE ANGIOGENINS

Similar to the eosinophil RNases, angiogenin had been identified and substantially characterized prior to its identification as a member of the RNase A gene family [67]. Once its amino acid sequence was determined, it was clear that angiogenin (also known as RNase 5) shared the two-histidine, one-lysine catalytic triad, signature CKXXNTF motif, and paired cysteines typical of members of the enlarging RNase A gene superfamily. The angiogenin lineage has also been the subject of rapid diversification [68], and recently, based on evidence from lower vertebrate sequences, Cho and colleagues [41] have suggested that angiogenin, with three as opposed to four paired cysteines, represents the more ancient of the RNase A RNase lineages.

There is one functional angiogenin gene in the human genome and six in the mouse. Of the six mouse angiogenin genes, Hooper and colleagues [20] found that mouse angiogenin 4 was expressed in Paneth cells and had the unexpected property of having bactericidal activity against specific intestinal microbes (**Fig. 5**). Upon further evaluation, the authors found that mouse angiogenin 1 and human angiogenin also displayed antimicrobial activity, with 100-fold reductions of colony counts of *S. pneumoniae* and *Candida albicans* observed in response to low micromolar concentrations of recombinant protein. Interestingly, Avdeeva and colleagues [21] recently reported that a commercial preparation of recombinant human angiogenin was no more effective than an albumin control at inhibiting the growth of *S. pneumoniae* or *C. albicans*. The reason for this remarkable discrepancy is not immediately apparent and will require further experimental clarification, although it might be noted that the pathogen in question, *S. pneumoniae*, is routinely identified by its sensitivity to detergent-mediated lysis (bile solubility test or 2% sodium deoxycholate), and its growth in culture can be inhibited by remarkably small amounts of detergent contaminants.

RNases 7 AND 8

Human RNase 7 was identified by Harder and Schröder [22] as part of a broad screening protocol aimed at identifying antimi-

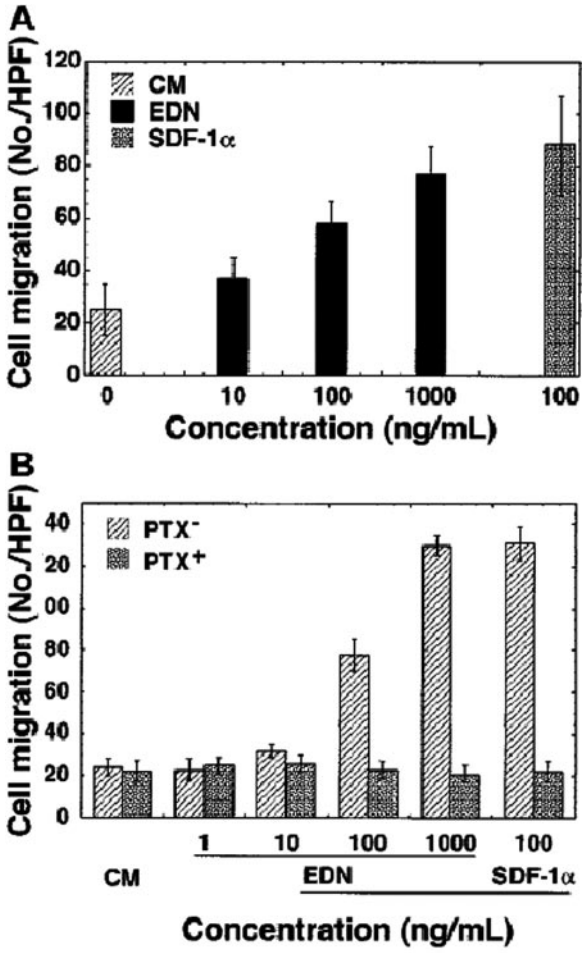

Fig. 4. EDN as a chemoattractant. (A) Migration of immature human DC to EDN was similar in magnitude to that seen to the chemokine SDF-1α and (B) was sensitive to inhibition by pertussis toxin (PTX). Reprinted with permission from Yang et al. [2]. HPF, High-power field; CM, .

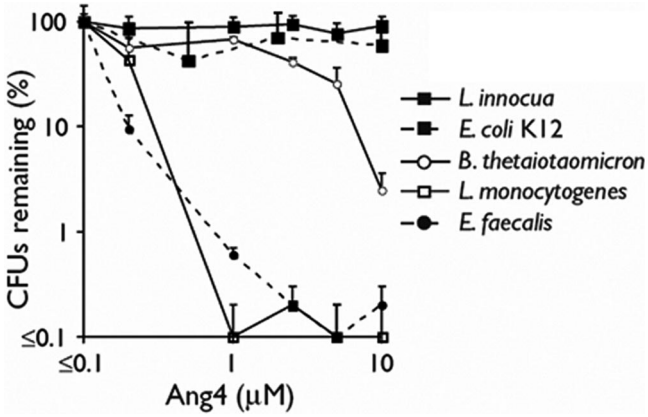

Fig. 5. Selective antipathogen activity of mouse angiogenin 4 (Ang4). Although the basis for this activity remains unclear, mouse angiogenin 4 displays activity against *E. faecalis* and *L. monocytogenes* but not against the other pathogens shown. Reprinted with permission from Hooper et al. [20].

crobial agents in human skin. Keratinocytes are a major source of RNase 7, which has strong bactericidal activity (colony reductions of $>10^4$-fold in response to low-to-mid micromolar concentrations) against a number of pathogens, with most profound activity against a clinical isolate of vancomycin-resistant *Enterococcus* ($LD_{90}<0.03$ μM). RNase 7 is expressed in response to several characterized inflammatory stimuli, specifically, IFN-γ, TNF-α, and IL-1β, as well as in direct response to bacteria, which suggests the possibility of a TLR2- or TLR4-mediated mechanism. A parallel study by Zhang and colleagues [69] documented prominent liver expression and commented on the unusual nature of the sequence, dominated by cationic lysines as opposed to arginines. Indeed, Huang and colleagues [70] have recently demonstrated that four specific clustered lysines (K1, K3, K111, and K112) are crucial for the membrane lytic activity of RNase 7. Although RNase dependence has not been addressed directly, the correlation of lytic activity with bactericidal function suggests that similar to ECP, RNase activity per se is not a crucial feature of RNase 7-mediated cytotoxicity.

Initially identified by Zhang and colleagues [71], human RNase 8 is closely related to RNase 7 but is expressed only in the placenta. Although only a limited number of samples from each species have been evaluated, RNase 8 appears to have been pseudogenized in about half of the known primate genomes. Rudolph and colleagues [23] have recently shown that human RNase 8 has antimicrobial activity against a variety of pathogens. Unfortunately (from an experimentalist's perspective), the mouse genome does not contain orthologs of RNase 7 or RNase 8; thus, the ways in which we can further our understanding of the roles of these proteins in promoting host defense in vivo are somewhat limited.

NONMAMMALIAN RNase A RIBONUCLEASES

As might be expected, the vast majority of work has been focused on the characterization of RNase A RNases in mammals, primarily in humans and human cell lines. Recently, the properties of several nonmammalian RNase A ribonuclease lineages have been revealed, and the potential role of these proteins in promoting endogenous host defense will be reviewed here.

THE ZEBRAFISH RNases

Two groups have recently reported their findings resulting from searches of the zebrafish (*D. rerio*) sequence databases [25, 72, 73]. The zebrafish RNase A sequences are typical of the ancestral RNases, with appropriate catalytic histidines and lysine within the conserved RNase A superfamily motif and six (as opposed to eight) conserved cysteines. The open reading frames encode relatively small proteins (molecular weight 13–15 kDa) with relatively low pIs for this gene superfamily (pI=8.8–9.2). Interestingly, despite their relatively low cationicity (comparable with human EDN, which has no detectable bactericidal activity in a similar assay [52]), recombinant proteins had some bactericidal activity, particularly against gram-negative *E. coli*, although not of the same magnitude as observed for the highly cationic RNases (e.g., RNase 7 or leukocyte RNase A-2, below). Given their limited cationicity, it would be most interesting to know whether the bactericidal activity observed for these proteins occurred via a distinct, perhaps RNase-dependent mechanism. However, mucosal localization (**Fig. 6**) and bactericidal activity among these distant RNase A ribonucleases stand in strong support of the authors' conclusions that host defense represents a primordial function of this gene lineage [25].

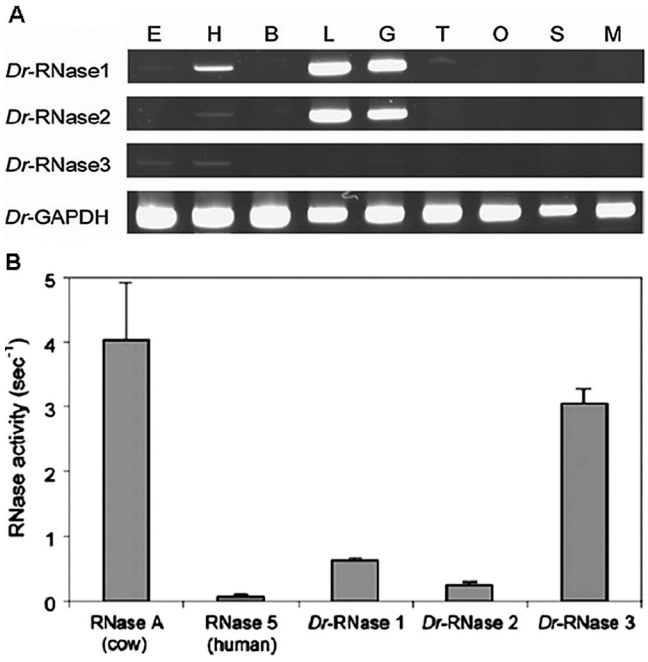

Fig. 6. Zebrafish RNase A ribonculeases. (A) Tissue distribution determined by RT-PCR including (E) eye, (H) heart, (B) brain, (L) liver, (G) gut, (T) testis, (O) ovary, (S) skin, and (M) skeletal muscle. (B) Comparative RNase activity against yeast tRNA substrate. Reprinted with permission from Cho and Zhang [25].

THE BULLFROG RNases

The RNase A ribonuclease isolated from oocytes from the bullfrog, *R. pipiens*, was one of the original members of the emerging RNase A gene superfamily [35], although for many years, this sequence and the related sequences from *R. catesbeiana* remained alone among the characterized, nonmammalian members of this group. The focus of these frog RNases has been their biotherapeutic, as opposed to biologic potential; the *R. pipiens* sequence, also known as onconase (trade name, *Ranpirnase*), is currently undergoing clinical trials for unresectable mesothelioma and non-small cell lung cancer [74]. The biotherapeutic potential of this RNase lineage is based all or in part on their structural resistance to a mammalian cellular RNase inhibitor and thus, once taken up by the cell, its apparently unrestricted capacity to degrade cellular RNA [75]. Associated with this property, Ardelt and colleagues [76] have recently shown that onconase can also suppress intracellular oxidative stress. Remaining unexplored, however, is the role of these proteins in their natural setting. Of interest, the *Rana* RNases have diverged into multigene clusters under positive selection [77], as have the rodent eosinophil RNases, although the overall significance of this, vis à vis host defense function, remains speculative.

CHICKEN LEUKOCYTE RNases A-1 AND A-2

Nitto and colleagues [24] characterized two RNase A ribonucleases originally isolated from chicken leukocyte-derived source material [78]. Renamed leukocyte RNases A-1 and A-2, both related proteins (diverging under positive selection pressure) were found in chicken peripheral blood heterophils, as would be appropriate for prominent host defense proteins (**Fig. 7**). Although RNase A-1 is the more enzymatically active RNase, RNase A-2 is more cationic (pI=11.0), angiogenic, and profoundly bactericidal against gram-positive and gram-negative species, with micromolar quantities reducing the colony count of *E. coli* from 10^7 CFU/ml to near zero. Most intriguing, not only was enzymatic activity irrelevant to bactericidal activity (similar to earlier findings with ECP [58]), but independent linear domains within the RNase A-2 sequence were nearly as effective at reducing the colony count as the full RNase A-2, suggesting that not only is RNase activity unimportant but also that the tertiary structure limited by the cysteines may be dispensible. Taken one step further, these results suggest that at least among duplicate genes, the RNase gene structure may be serving merely as a scaffold to support the evolution of novel, nonribonucleolytic host defense (and other?) peptides.

CONCLUSIONS

Our enlarging understanding of the RNase A gene superfamily has provided us with a greater appreciation of the role of these proteins in promoting host defense. Although the most prominent activity explored is RNase-independent bactericidal ac-

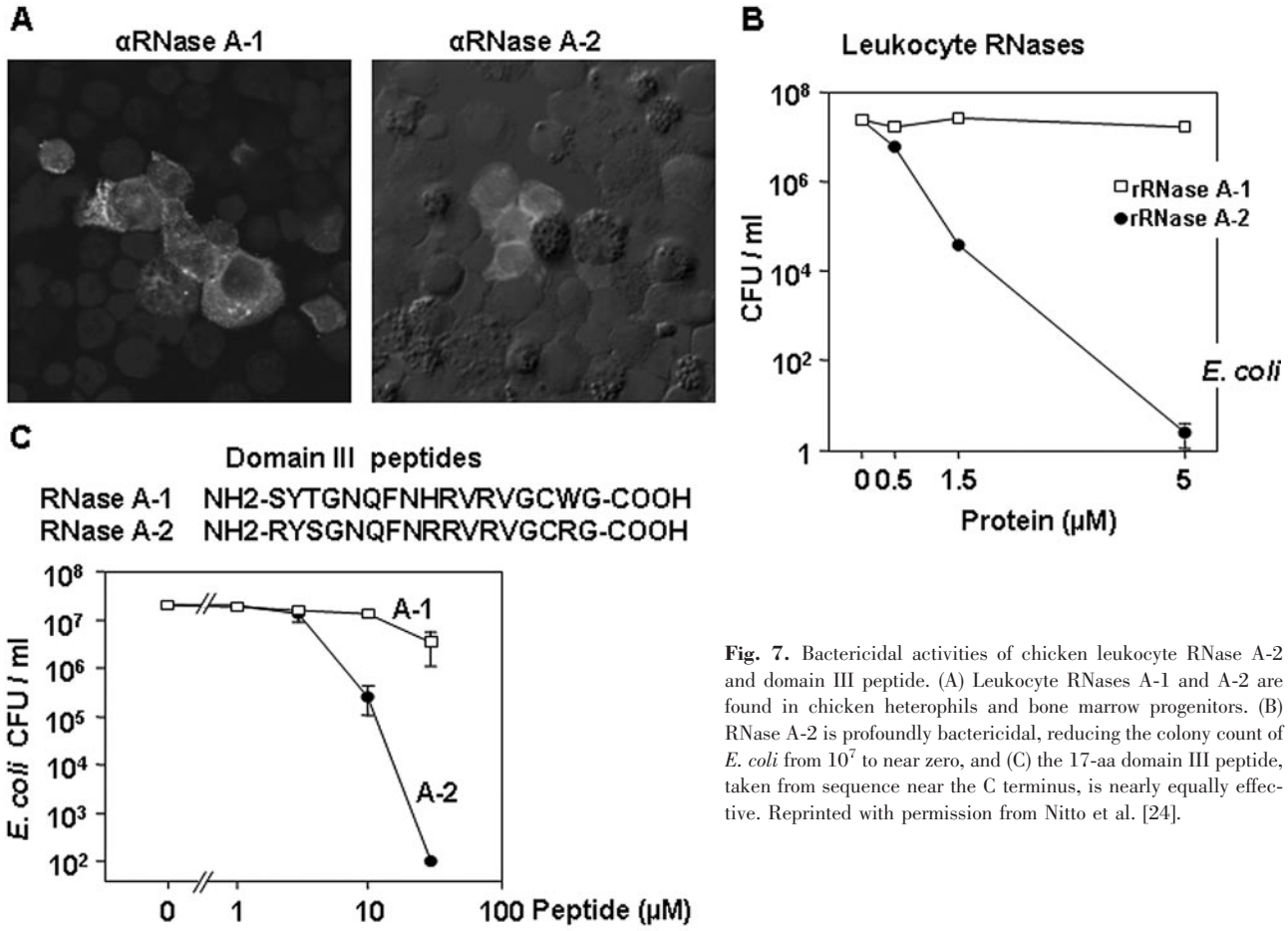

Fig. 7. Bactericidal activities of chicken leukocyte RNase A-2 and domain III peptide. (A) Leukocyte RNases A-1 and A-2 are found in chicken heterophils and bone marrow progenitors. (B) RNase A-2 is profoundly bactericidal, reducing the colony count of *E. coli* from 10^7 to near zero, and (C) the 17-aa domain III peptide, taken from sequence near the C terminus, is nearly equally effective. Reprinted with permission from Nitto et al. [24].

tivity, more recent explorations have focused on the role of these mediators, particularly EDN, in antiviral host defense as chemoattractant agents and as endogenous ligands for TLR. Future work will permit us to focus on specific, unique, and perhaps more subtle roles played by individual RNase A ribonuclease lineages.

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