Chapter 2
Vertebrate Secretory (RNase A) Ribonucleases and Host Defense

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Contents

Abstract Bovine pancreatic ribonuclease, also known as RNase A, is the prototype of an extensive, multi-lineage family of vertebrate secretory proteins that share elements of structure and catalytic activity despite substantial functional divergence. In this review, we feature the RNase A family and its members that are implicated in promoting host defense – activities that include sustaining mucosal

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barriers, as well as participating in various aspects of innate and acquired immunity – and explore relationships linking gene evolution, enzymatic activity, and physiologic function.

Keywords Leukocytes • Eosinophil • Pathogen • Bacteria • Respiratory viruses

2.1 Introduction

In this chapter, we will review the actions of specific vertebrate secretory (RNase A) ribonucleases in promoting host defense, which is a general term that covers multiple aspects of mucosal, innate and acquired immunity. The reader is referred to several of earlier reviews on this subject (Dyer and Rosenberg 2006; Pizzo and D'Alessio 2007; Rosenberg 2008a; Sorrentino 2010), and also two reviews that focus on a related topic, the eosinophilic leukocyte and its complex interactions with respiratory virus pathogens (Jacoby 2004; Rosenberg et al. 2009).

2.2 A Brief History

The RNase A family of vertebrate secretory ribonucleases has long intrigued the research community and as such, it has an important place in the history of modern biology. As described in detail elsewhere (Beintema and Kleineidam 1998; Marshall et al. 2008) and here in this volume (Chap. 1,15), many of the principles of protein structure, protein folding, and enzyme catalysis emerged from studies of bovine pancreatic ribonuclease, also known as RNase A, the prototype, and founding member of this group. For the purposes of this review, we pick up the story in the mid-1970s, as medical science began to take an interest in serum ribonuclease activity as a marker for neoplastic disease (Reddi and Holland 1976; Maor and Mardiney 1978). Although serum ribonuclease levels alone proved to be insufficiently specific for diagnostic purposes (Peterson 1979), these explorations provided the first indication that there might be more than one human secretory ribonuclease. The medical and basic science literature suggested that there were at least two secreted ribonucleases: an acid type (from leukocytes) and an alkaline type (from pancreas) defined by their distinct catalytic properties (Biswas and Hindocha 1974; Akagi et al. 1978). At the same time, Jaap Beintema and colleagues (Welling et al. 1975; Beintema et al. 1988) initiated their studies on ribonuclease evolution, which provided insight into the structural diversity of the numerous orthologs of pancreatic-type ribonucleases in species throughout the animal kingdom.

Equally important were the ongoing political events of this era, as they ultimately served to direct the course of scientific inquiry. The U.S. National Cancer

Act of 1971 provided the scientific establishment with a tremendous economic boost, which is largely credited with funding the nascent field of Molecular Biology. In this environment, vertebrate ribonuclease research took another giant step forward. Specifically, upon characterization of the amino acid and cDNA sequences for angiogenin (ANG), a protein from HT-29 adenocarcinoma cells known to promote blood vessel growth (Fett et al. 1985; Strydom et al. 1985; Kurachi et al. 1985; Moenner et al. 1994), it became clear that this protein shared sequence features with vertebrate secretory (RNase A) ribonucleases. Likewise, the amino terminal sequences and cDNA clones of eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), otherwise unremarkable secretory proteins from human eosinophilic leukocytes, indicated that these two proteins might likewise be part of this emerging RNase A gene family (Gleich et al. 1986; Rosenberg et al. 1989a, b; Hamann et al. 1989; Barker et al. 1989).

With the completion of the human genome sequencing project in 2003, interrelationships among several of the distinct vertebrate RNase lineages were clarified. Interestingly, in the report describing the initial findings relating to the human genome sequence, Lander and colleagues (2001) remarked specifically on the unique nature of the RNase A ribonucleases as the only known family of vertebrate-specific enzymes, and likewise concurred with our interpretations regarding rapid evolution and the generation of antimicrobial properties (Rosenberg et al. 1995; Zhang et al. 2000; Cho and Zhang 2007). As shown in Fig. 2.1, human RNase 1 is the direct ortholog of bovine RNase A. Also shown are other canonical ribonucleases, which are secretory proteins with classical signal sequences, specific disulfide-bonded tertiary structure, a His-Lys-His triad in a catalytic crevice, and some degree of enzymatic activity against a single-stranded RNA target, including EDN/RNase 2, ECP/RNase 3, RNase 4, ANG/RNase 5, RNase 6 and RNase 7. Recent findings from our laboratory have suggested that we may need to reconsider some of our earlier assumptions regarding RNase 8 primary structure (Chan, Moser et al., manuscript in preparation). In contrast, the known noncanonical ribonucleases (RNases 9–13) are sequences that are clearly related to this family, but with divergent features (insertions, deletions, mutations in critical regions) that indicate that they are unlikely to function as active enzymes.

2.3 Host Defense: A Working Definition

In its simplest form, host defense is the general term that encompasses all the components of the immune system, working in concert to provide a barrier against incoming pathogens. Among its component parts, this includes physical surfaces, such as the skin, and the respiratory, gastrointestinal, and urogenital epithelia. In addition to a barrier function, these tissues express and release antimicrobial proteins and have specialized detection mechanisms (e.g., toll-like receptors) that serve to identify pathogens and to prevent invasion. Host defense also includes the actions of the innate immune system, those of complement proteins, and leukocytes

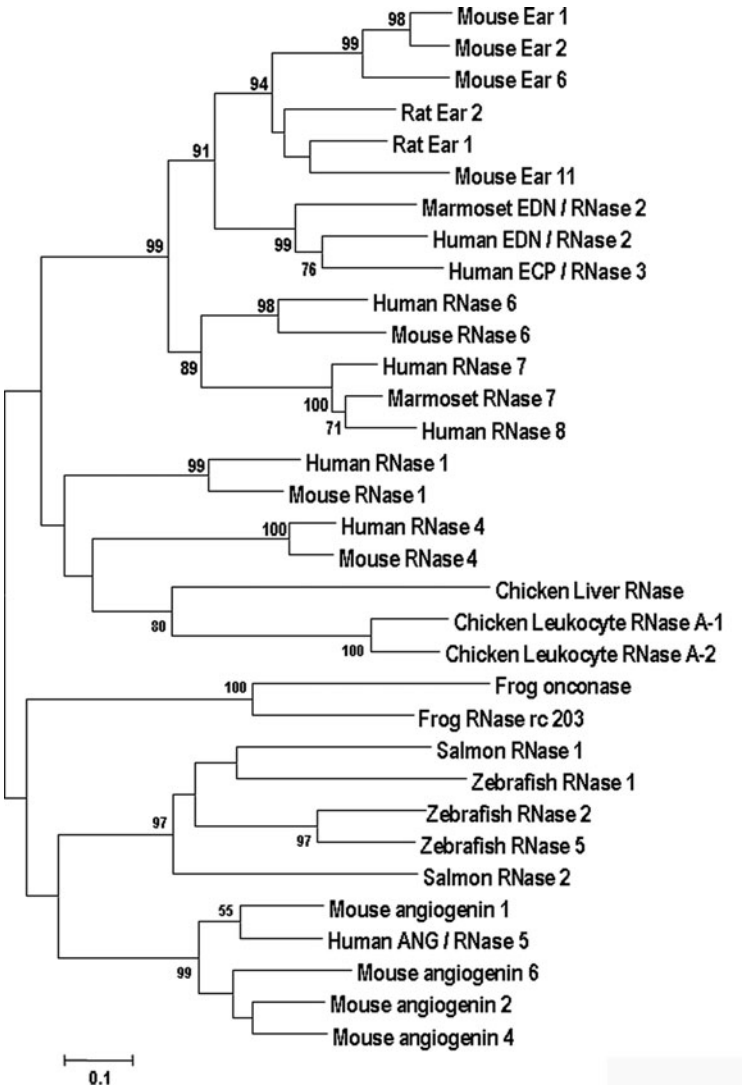

Fig. 2.1 Phylogenetic tree documenting relationships among various vertebrate secretory (RNase A) ribonucleases, including those described in greater detail in this review. Neighbor-joining tree constructed with encoded amino acid sequences (Poisson correction, 2000 bootstrap replicates) using algorithms within MEGA 4.0 (Tamura et al. 2007)

including mast cells, natural killer cells, granulocytes (neutrophils, eosinophils, basophils), and macrophages. Resident dendritic cells present pathogen-specific antigens and thus serve to link the innate immune system to the adaptive immune system, the latter including the actions of T and B lymphocytes that are thus influenced by antigen exposure.

2.4 Vertebrate Secretory (RNase A family) RNases and Host Defense: General Questions

To date, vertebrate secretory ribonucleases have been identified as participants in host defense at the skin and mucosal surfaces, and as secretory components from innate immune cells (eosinophils, heterophils, monocytes, dendritic cells, mast cells). Interestingly, there are no reports of vertebrate secretory RNases as functional components of T cells or B cells, but this may reflect the fact that no one has actually looked carefully for this possibility. The current state of the art is summarized in Table 2.1.

Although each situation presents its own unique issues, there are some general questions that have promoted inquiry and that continue to guide research in this field. Among these questions, those of us working in this field have asked, which vertebrate secretory ribonucleases promote host defense, and how specifically is this accomplished? What strategies are used and what targets are involved? Is enzymatic activity crucial or even necessary for promoting all or at least some host defense functions? Similarly, is the characteristic disulfide-bonded tertiary structure a crucial feature, or is it completely dispensable for this activity? Finally, can we determine which aspects are incidental findings and which are absolutely necessary for vertebrate survival in vivo?

2.5 The Eosinophil RNases

Eosinophils are tissue leukocytes that develop in the bone marrow and expand in number in response to Th2 cytokines (primarily interleukin-5), which are produced and accumulate during allergic states and parasitic infection in vivo (Fig. 2.2). Despite years of study, the precise role of eosinophils in host defense remains uncertain, as studies performed with cytokine- and eosinophil-deficient mice have cast significant doubt on the long-held belief that eosinophils promote host defense against helminthic parasites (Klion and Nutman 2004; Jacobsen et al. 2007; Fabre et al. 2009). Recently, Yousefi and colleagues (2008) have shown that hypereosinophilic mice are protected against bacterial infection, although the full relationship between bacteria and eosinophils remains to be explored. The complex evolutionary divergence between human and mouse eosinophils (reviewed in Rosenberg et al. 2007) precludes strong conclusions in many of these experimental trials.

The major eosinophil granule-secretory proteins were isolated and described by Gleich and colleagues (1984) and Venge and colleagues (1987). Eosinophil cationic protein (ECP) was described as a small ~16 kDa, highly charged protein with cytotoxicity to bacteria, parasites, and mammalian cells in vitro. Eosinophil-derived neurotoxin (EDN, also known as eosinophil-protein-X) had a similar amino acid content to ECP, but was less cationic and less toxic in general, although

Table 2.1 Summary of host defense functions identified by vertebrate secretory RNase A family ribonucleases

RNase A ribonuclease	Species	pI	Host defense activities
Eosinophil-derived neurotoxin (RNase 2)	Human	9.2	Chemoattractant for immature human dendritic cells (Yang et al. 2003) Enhances maturation of dendritic cells (Yang et al. 2004)[a] Reduces infectivity for RNA viruses (HRSV, HIV) in tissue culture assays (Domachowske et al. 1998; Rugeles et al. 2003)[b]
Eosinophil cationic protein (RNase 3)	Human	10.5	Cytotoxin for helminthes, strong bactericidal activity (reviewed in Boix et al. 2008) Produced by alveolar macrophages in response to Th2 cytokine stimulation (Cormier et al. 2002)
Eosinophil-associated RNase 11	Mouse	9.3	Produced in response to respiratory virus infection, upregulated in the absence of type I interferon signaling (Garvey et al. 2005)
Eosinophil-associated RNases 1 and 2	Rat	9.0, 9.9	Moderate bactericidal activity against *E. coli* and *S. aureus* (Ishihara et al. 2003)
Angiogenin (RNase 5)	Human	9.7	Moderate bactericidal activity against *S. pneumoniae* (Hooper et al. 2003); conflicting report (Avdeeva et al. 2006) Moderate bactericidal activity against *L. monocytogenes* and *E. faecalis* (Hooper et al. 2003)
Angiogenin-4	Mouse	9.2	Produced in response to lipopolysaccharide (Hooper et al. 2003)
RNase 7	Human	9.8	Broad-spectrum strong bactericidal activity, very strong against *E. faecium* (Harder and Schroeder 2002)
RNase 8	Human	8.6	Broad-spectrum moderate bactericidal activity (Rudolph et al. 2006)
Leukocyte RNase A-2	Chicken	10.4	Bactericidal against *E. coli*, *S. aureus* (Nitto et al. 2006) and *Salmonella* sps. (unpublished data)
ZF-RNases 1–5 SS-RNases 1 and 2	Zebrafish salmon	9.0–9.4 9.3, 8.5	Broad-spectrum moderate bactericidal activity against gram-negative bacteria (Pizzo et al. 2006, 2008; Cho and Zhang 2007)

Values for isoelectric points (pI) were calculated using the web-based ExPaSy tool (http://expasy.org/tools/pi_tool.html) using encoded amino acid sequence data. Moderate bactericidal activity, low micromolar concentrations reduce the colony count 10–100-fold in a standard overnight incubation assay; strong bactericidal activity, low micromolar concentrations reduce colony count 10^4–10^7-fold. Reprinted with modifications from Rosenberg (2008a)[a]Activity shared with human pancreatic ribonuclease, also known as RNase 1[b]Activity dependent on enzymatic (ribonuclease) activity

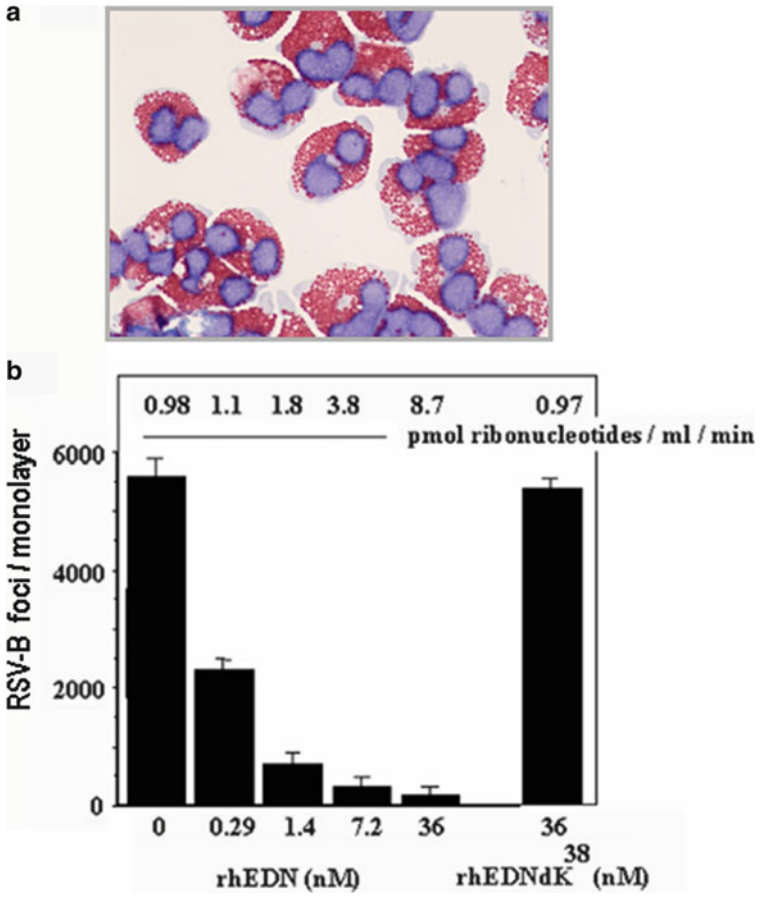

Fig. 2.2 (**a**) Isolated human eosinophils stained with modified Giemsa (Diff Quik). The cells feature characteristic bilobed nucleus and the prominent cytoplasmic granules that contain the secretory ribonucleases, EDN/RNase 2 and ECP/RNase 3. (**b**) Recombinant EDN reduces the infectivity of the human respiratory syncytial virus (RSV-B) for target epithelial cells in a dose-dependent fashion. Site-specific mutagenesis resulting in elimination of the catalytic lysine restores virus infectivity, consistent with ribonuclease-dependent antiviral activity. (Reprinted from Domachowske et al. 1998)

it promoted destruction of Purkinje cells when administered intrathecally to rodents and rabbits (Durack et al. 1981). Gleich and colleagues (Gleich et al. 1986; Slifman et al. 1986) were the first to recognize the homology between the amino terminal sequences of both EDN and ECP and human ribonuclease, and to document enzymatic activity; identification of cDNA clones encoding both proteins documented complete sequence homology to RNase A (Rosenberg et al. 1989a, b; Hamann et al. 1989; Barker et al. 1989).

2.5.1 Eosinophil Cationic Protein (ECP/RNase 3): Mechanism of Action

The earliest studies of ECP focused on its cationicity and its ability to disrupt membrane function (Young et al. 1986). However, once it became clear that ECP had dual potential, the role of ribonuclease activity and its relationship to the cytotoxic nature of ECP was addressed. Interestingly, in experiments performed with active site histidine and lysine mutations, it became quite clear that enzymatic activity played no significant role in the bactericidal activity promoted by ECP (Rosenberg 1995). This finding has been confirmed and extended many times over both directly and indirectly, as Torrent, Boix, Nogues and colleagues (Carreras et al. 2003; Torrent et al. 2007, 2008, 2009a, b, 2010a, b; Navarro et al. 2008; Boix et al. 2008; Garcia-Mayoral et al. 2010; Sanchez et al. 2010) examined the structure– function relationships of ECP with target pathogens and pathogen-mimetic membranes. ECP and ECP-derived minimal cationic peptides promote cytotoxicity via interactions with target bacterial membrane and wall components, including peptidoglycan and lipopolysaccharides. Interestingly, ECP-mediated interactions elicit cytotoxicity via membrane aggregation more prominently than via cellular lysis.

This finding – the emergence of ECP/RNase 3 as a cationic toxin with activity that is not dependent on ribonuclease activity – seems to be counterintuitive from an evolutionary perspective. However, this can be understood by applying the principles first introduced by the evolutionary biologist, Susumo Ohno (1970), specifically, that novel function can evolve by duplication of genetic material followed by relaxation of functional constraints. As shown in Fig. 2.1, ECP and EDN are paired genes, having undergone duplication from a single predecessor gene over 60 million years ago (described further in Sect. 2.5.2). As such, one might envision a case such that ECP might gain novel function (i.e., cationic cytotoxin) while EDN maintained the original enzymatic activity.

2.5.2 Evolution of Eosinophil Ribonucleases and the Rodent Eosinophil-Associated Ribonucleases (Ears)

As part of an attempt to obtain a better sense of the relationships connecting enzymatic activity, cationicity, and host defense, we proceeded to explore the evolutionary relationships among these proteins. In doing so, we uncovered the remarkable interspecies divergence within the EDN/ECP lineage, now known to be among the most rapidly evolving coding sequences among primate species (Rosenberg et al. 1995; Rosenberg and Dyer 1995; Zhang et al. 1998). All primate genomes encode a highly cationic ECP and a more neutral EDN, save for the new World monkey genomes, which maintain a single sequence, with properties similar to EDN, as predicted above (Sect. 2.5.1). Meanwhile, Larson and colleagues (1996)

identified the first mouse orthologs of this lineage, a highly divergent cluster of eosinophil-associated ribonucleases, or Ears. Zhang and colleagues (2000) documented that the rodent Ears, similar to the human eosinophil ribonucleases, are undergoing rapid evolution and are diverging under unique constraints, via a pattern known as rapid-birth–death and gene sorting.

2.5.3 EDN/RNase, Ears, and Host Defense

Despite the rapid evolution, and clearly unusual constraints to which this gene lineage is responding, the EDN/RNase 2 genes maintain features consistent with their role as active enzymes. Taking into account (a) the role of eosinophils in promoting respiratory pathology in asthma, (b) the fact that RNA viruses such as respiratory syncytial virus (RSV) commonly incite asthmatic sequelae in susceptible individuals, we closed the circle and (c) considered the possibility that single-stranded RNA virus pathogens might be enzymatically susceptible, evolutionarily mobile targets of a ribonuclease such as EDN/RNase 2. To date, we have shown that EDN can reduce infectivity of RSV for target epithelial cells in culture, via a mechanism that is directly dependent on active ribonuclease activity (Domachowske et al. 1998; Rosenberg 2008b). Interestingly, EDN can also reduce infectivity of human immunodeficiency virus (HIV), a single-stranded RNA virus of the family *Retroviridae*, in similar in vitro culture assays (Rugeles et al. 2003; Bedoya et al. 2006). The precise mechanism of antiviral activity awaits further exploration.

EDN has also been implicated in several other aspects of innate immunity. Yang and colleagues (Yang et al. 2003, 2004) reported that EDN elicited chemotaxis and enhanced maturation of human dendritic cells. In further studies, this group (Yang et al. 2008) examined the interaction of EDN with TLR2, and suggested that EDN might serve as an alarmin, one of a group of endogenous immunostimulant molecules that serve as signals of tissue damage (Oppenheim and Yang 2005).

Rather little is known regarding the rodent Ears, their unique expression patterns, and their individual and/or collective roles in host defense. There are ~14 mEar coding sequences in the mouse genome; mEar 1 and mEar 2 are expressed most prominently in mouse eosinophils and in lung tissue (Moreau et al. 2003). Expression of mEar 11 was induced in mouse alveolar macrophages in response to Th2 cytokine stimulation (Cormier et al. 2002) and in virus-infected lung tissue in the absence of the type I IFN receptor (Garvey et al. 2005); mEar 6 was detected in liver in response to *Schistosoma mansoni* infection (Nitto et al. 2004). Likewise, Ishihara and colleagues (2003) demonstrated antimicrobial activity for rat Ears in standard in vitro assays, and Gaudreault and Gosselin (2007, 2008) detected release of mEars in virus-infected mouse lung tissue in response to leukotriene-B4.

2.6 RNases 7 and 8

Ribonuclease 7 (RNase 7) is another intriguing vertebrate secretory ribonuclease with unique structure and antimicrobial properties. Although RNase 7 was discovered after the emergence of the RNase A family, similar to ECP, it was identified initially as a host defense protein, emerging from a study by Harder and Schroeder (2002) who were evaluating healthy keratinocyte cultures for novel anti-pathogen molecules. In a parallel study, Zhang and colleagues (2003) identified RNase 7 as a novel RNase A family member from the first draft of the human genome sequence. RNase 7 is a cationic ribonuclease with wide-spectrum antimicrobial activity, particularly powerful against gram-negative bacteria including *Pseudomonas* and *Enterococcus* species (Harder and Schroeder 2002; Köten et al. 2009). Recently, Zanger and colleagues (2009) and Simanski and colleagues (2010) demonstrated that RNase 7 protects healthy skin against infection and colonization, respectively with *Staphylococcus aureus*. RNase 7 is expressed widely, and is induced in keratinocyte culture by prominent proinflammatory stimuli, including TNF, IFNγ, IL-1β, and IL-1α (Harder and Schroeder 2002; Mohammed et al. 2010; Bando et al. 2007) as well as UV-irradiation (Gläser et al. 2009) and bacterial components (Harder and Schroeder 2002).

Ribonuclease 8 (RNase 8) is closely related to RNase 7. RNase 8 appears to have undergone "functional" pseudogenization in several primate species (i.e., several orthologs have mutations in elements that are crucial for enzymatic activity), although further evaluation of more samples is warranted prior to reaching a final conclusion on this subject. Although Zhang and colleagues (2002) reported no cytotoxicity in the bacterial cells synthesizing the recombinant protein, Rudolph and colleagues (2006) reported that recombinant RNase 8, modeled on the structure of RNase 7, displays moderate antimicrobial activity against several staphylococcal, enterococcal, and *E. coli* strains.

2.6.1 Structure and Evolution of RNase 7

RNase 7 is fairly typical for a member of the RNase A family. Its open reading frame encodes a 28-amino acid signal sequence, and the mature protein coding sequence includes eight cysteines, and appropriately localized histidines and lysine, the latter within the family signature motif. Human RNase 7 is relatively cationic, with a calculated isoelectric point (pI) of 9.8, and a predominance of lysines, rather than arginines, as was observed in the coding sequence of ECP/RNase 3. Compared to EDN/ECP, the divergence among primate RNase 7 genes is comparatively modest, with 23% sequence divergence observed in a comparison between human and common marmoset (New World monkey) *C. jacchus* RNase 7 sequences. Two RNase 7 genes have been identified in the genomes of the horse (*E. caballus*) and cow (*B. taurus*), at 30% and 35% sequence divergence from the human sequence, respectively; no orthologs of RNase 7 have been found in the mouse genome.

2.6.2 *RNase 7: Molecular Basis of Antimicrobial Action*

Although RNase 7 shares some features with ECP/RNase 3 (structure, cationicity, ribonuclease activity), the molecular nature of its antimicrobial activity is unique and distinct. Similar to what has been observed for ECP, ribonuclease activity is not essential to RNase 7's mechanism of antimicrobial action. Torrent and colleagues (2009b, 2010a) have compared the actions of ECP and RNase 7 using membrane phospholipid vesicles and bacterial cell walls. They have noted that ECP has a much larger tendency to induce aggregation of both targets, whereas RNase 7 results in vesicle leakage and release of bacterial cell contents without inducing substantial aggregation. Liao and colleagues (Huang et al. 2007; Lin et al. 2010) focused on the unique features of RNase 7, and concluded that the amino terminal lysines were crucial features promoting antimicrobial activity; an outer membrane protein of *P. aeruginosa* has been identified as a target for RNase 7/cell surface interactions preceding interaction with lipopolysaccharide and ultimately internalization.

2.7 Angiogenin/RNase 5

Angiogenin is best known for its role in promoting blood vessel growth (reviewed in Strydom 1998; Badet 1999); recently, polymorphisms in genes encoding angiogenin have been linked to susceptibility for developing amyotrophic lateral sclerosis (Bosco and Landers 2010). The human genome encodes only one functional angiogenin gene, while the mouse genome encodes six. Hooper and colleagues (2003) described an intriguing host defense function for angiogenin 4 (Ang 4), a member of the mouse cluster expressed in intestinal Paneth cells. Ang 4 expression is induced by normal microflora in conventionally raised mice, but interestingly, Ang4 is not expressed in germ-free mice, and can be induced in the latter by the introduction of the microflora component, *B. thetaiotaomicron*. Furthermore, human ANG, its mouse ortholog Ang1, and mouse Ang 4, all displayed antimicrobial activity at micromolar concentrations. While Avdeeva and colleagues (2006) presented a conflicting view, noting that human ANG was no more effective than control protein (bovine serum albumin) at inhibiting pathogen growth, it is crucial to recognize that one of the pathogens examined in this latter study, *S. pneumoniae*, is difficult to grow and quite sensitive to minimal levels of detergent contaminants. Since this first report, Lagishetty and colleagues (2010) presented their study of mice raised on vitamin-D deficient diets that were then induced to develop colitis. Interestingly, mouse Ang 4 expression was diminished more than twofold, a finding that correlated with a 50-fold elevation of bacteria in colonic tissue.

2.8 Avian Leukocyte RNase A-2

There are three RNase A family sequences in the genome of the chicken, *Gallus gallus*; two of these sequences are duplicated leukocyte-associated ribonuclease genes, which are renamed leukocyte RNase A-1 and A-2 (Nitto et al. 2006). Interestingly, both RNase A-1 and RNase A-2 are cationic (isoelectric points 9.8 and 10.4, respectively), both are angiogenic in an aortic ring assay (RNase A-2 > RNase A-1), and both immunoreactive proteins were found in both bone marrow progenitors and in circulating heterophils. In contrast, (and similar to the EDN/ECP pair) RNase A-1 was a more effective RNase against the standard tRNA substrate, while RNase A-2 was the powerful antimicrobial protein (Fig. 2.3).

Consistent with earlier studies focusing on ECP, site-specific mutagenesis that eliminated the ribonuclease activity of RNase A-2 had no impact on the antimicrobial activity. Most intriguing was the fact that the unique tertiary structure likewise seemed to have no specific impact on antimicrobial function, as cationic domains within the structure of RNase A-2 were able to function as independent antimicrobial peptides. Specifically, domain III, a 16-amino acid peptide including amino acids 89–104 of the native protein reduced the bacterial colony count 10^5-fold when introduced at micromolar concentrations, matching the effectiveness of the full, native protein. These results suggest that not only is enzymatic activity dispensable, but the unique disulfide-bonded RNase A backbone may not be a crucial limiting constraint driving the evolution of this gene family.

2.9 Zebrafish and Salmon RNases

RNase A family genes from the zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) have been identified and recombinant proteins generated and explored for antimicrobial activity. The salmon RNases 1 and 2 displayed moderate antibacterial activity at micromolar concentrations against both gram-negative and gram-positive bacteria (Pizzo et al. 2008); analogous to ECP and leukocyte RNase A-2, ribonuclease activity is not essential, nor is tertiary structure, as fully denatured protein is as effective as folded ribonuclease at promoting anti-pathogen activity in tissue culture.

Ribonucleases from zebrafish were isolated and characterized by D'Alessio and colleagues (Pizzo et al. 2006), Quarto et al. (2008), Kazakou and colleagues (2008), and Cho and Zhang (2007). Recombinant zebrafish ribonuclease proteins also display moderate antimicrobial activity against both Gram-negative and Gram-positive bacteria; the detection of antimicrobial activity in among these proteins led Cho and Zhang (2007) to postulate that host defense was not only present but likely a primordial function of the RNase A gene family. Zanfardino and colleagues (2010) recently examined a molecular mechanism for the antimicrobial action of ZF-RNase 3, a zebrafish ribonuclease expressed in lung and gut tissue. As is typical

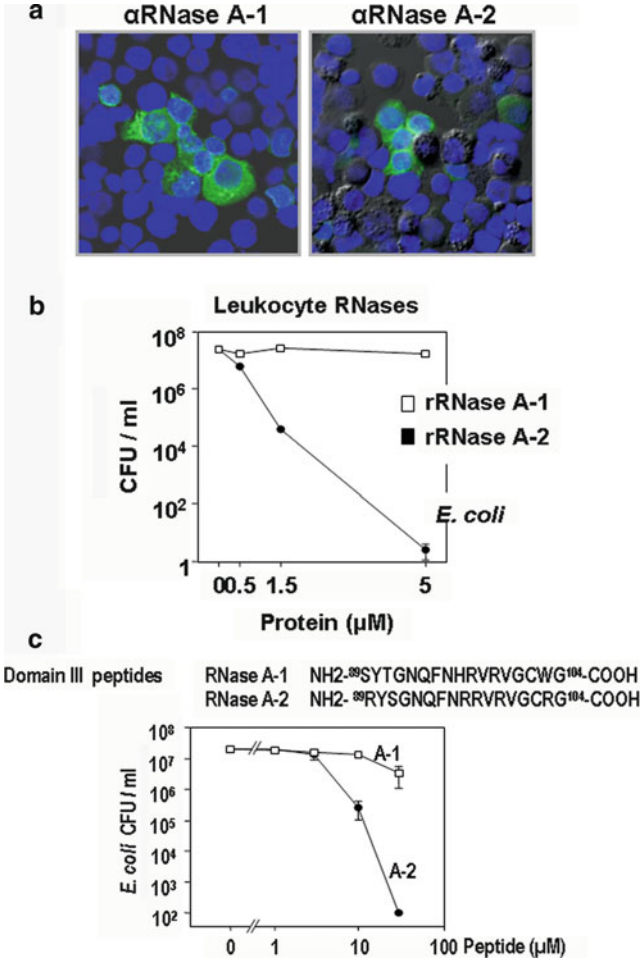

Fig. 2.3 (**a**) Detection of leukocyte RNase A-1 and A-2 in heterophils from bone marrow from chicken (*Gallus gallus*). (**b**) Challenge with low micromolar concentrations of leukocyte RNase A-2 (but not leukocyte RNase A-1) results in a 10^7-fold reduction in *E. coli* colony count. (**c**) Similar concentrations of unstructured domain III peptide, corresponding to amino acids 89–104 of RNase A-2, are nearly as effective at reducing colony counts of *E. coli*. (Reprinted from Nitto et al. 2006)

among ribonucleases of this family, the antimicrobial activity of ZF-RNase 3 was not dependent on enzymatic activity, nor was it dependent on tertiary structure. ZF-RNase 3 itself is cleaved at Arg30-Arg31 by the *E. coli* OmpT protease, thereby releasing an antimicrobially active C-terminal proteolytic fragment. Likewise, Pizzo and colleagues (2010) characterized ZF-RNase 5, which has activity against Gram-negative, but not Gram-positive bacterial species.

Of note, Balla and colleagues (2010) recently reported the isolation and characterization of zebrafish eosinophils, which are morphologically and functionally analogous to their mammalian counterparts. Although the zebrafish ribonuclease lineages are not directly related to the mammalian eosinophil ribonucleases, zebrafish eosinophils express transcripts encoding ZF-RNase 2, an antimicrobial protein with moderate activity against *E. coli* and *P. aeruginosa* in vitro. ZF-RNae 3 is expressed in liver, gut, and heart, and has relatively little enzymatic activity when compared to ZF/DR-RNases 1 and 3.

Given the relative ease with which gene manipulations are carried out in zebrafish, this may represent the most powerful resource available for exploring the role of ribonucleases in promoting host defense in vivo.

2.10 Conclusions

Vertebrate secretory ribonucleases have long been intriguing subjects for the study of protein structure and enzyme biochemistry. We proceed to build on this profound body of knowledge which provides the basis for our study of the contribution of the proteins to mucosal, innate and acquired immune function.

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