CMLS, Cell. Mol. Life Sci. 54 (1998) 795–803
1420-682X/98/080795-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

The eosinophil ribonucleases

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Abstract. The eosinophil ribonucleases, eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic protein (ECP/RNase 3) are two closely related proteins with intriguing functional and evolutionary properties. While both EDN and ECP maintain the structural and catalytic residues typical of the RNase A superfamily, the role of ribonuclease activity in the physiologic function of these proteins remains unclear. The biochemistry and physiology of EDN, ECP and the recently discovered ribonuclease k6 (RNase 6) will be reviewed in this chapter.

Key words. Eosinophils; host defence; primates; rodents; evolution.

Introduction

The eosinophil ribonucleases, the eosinophil cationic protein (ECP) and the eosinophil-derived neurotoxin (EDN) were identified, isolated and functionally characterized well before their membership in the RNase A superfamily was apparent. Their identification as ribonucleases has introduced many interesting questions and opened several intriguing areas of research.

History

Paul Ehrlich (1854–1915), Nobel laureate and scientist extraordinaire, first noted the existence of eosinophilic leukocytes in an 1879 article entitled 'On the Specific Granulations of Blood,' in which he described 'cells that were so richly endowed with . . . granules that their entire protoplasm stained violet' [1]. Ehrlich went on to report that the 'eosinophil granulations' stained with virtually all acidic dyes, and he displayed remarkable prescience in speculating that these granules contained secretory components [2]. For more information on eosinophil biology and physiology, the reader is referred to several recent texts and reviews [3–8]. Anyone even mildly interested in the history of science will delight in reading the chapter 'Paul Ehrlich and the Discovery of the Eosinophil' by James G. Hirsch and Beate I. Hirsch in *The Eosinophil in Health and Disease* (edited by A. A. F. Mahmoud and K. F. Austen) [9], which clearly demonstrates that the social, political and personal pressures of academic research today have changed very little from what they were in 19th-century Europe.

In the early 1970s, several research groups embarked on the characterization of the protein components of the eosinophil granule (fig. 1). Interestingly, the association of ribonuclease activity with the granule fraction was first noted by Archer and Hirsch in 1963 [10], although the significance of this observation was not apparent at the time. Olsson and colleagues [11–14] were the first to report the separation of the granule cationic proteins, and to distinguish one of the major granule components, an arginine-rich protein of molecular mass 21 kDa, as eosinophil cationic protein (ECP). Shortly thereafter, Durack and colleagues [15, 16] published their studies on the neurotoxicity of eosinophils. As had been reported earlier by Gordon [17], eosinophils were known to contain a substance that, when injected intrathecally into laboratory animals, induced a syndrome of muscular rigidity, ataxia and paralysis associated with Purkinje cell loss; the etiologic agent identified by Durack and colleagues [16] was also a distinct granule

protein, the eosinophil-derived neurotoxin (EDN, molecular mass 18.4 kDa). Peterson and Venge [18] reported the purification and characterization of yet another granule cationic protein, eosinophil protein-X (EPX); more recently Slifman and colleagues [19] showed that EPX and EDN are functionally and immunologically indistinguishable proteins. Several biologic activities of ECP were characterized, including alterations in the coagulation cascade [20], augmentation of fibrinolysis [21] and regulation of components of the classical pathway of complement [22]. Other researchers focused on the association of blood eosinophilia with parasitic disease, and noted that ECP had toxicity towards schistosomula, trypanosomes and other human parasites [23–29]. ECP was also found to have antibacterial activity against both Gram-positive and Gram-negative species [30], and was reported as toxic to mammalian cells and tissues [31–35]. In contrast, EDN was observed to be relatively ineffective as an antiparasitic agent; the (nonphysiologic) neurotoxicity is EDN's only well-characterized activity at the present time.

Eosinophils, granule proteins and ribonucleases were joined in 1986 by the work of Gleich and colleagues [36], who isolated EDN and ECP from human eosinophils, reported their respective amino-terminal sequences and observed the similarity of these sequences to one another and to human pancreatic ribonuclease. Slifman and colleagues [37] and Gullberg and colleagues [38] demonstrated that both EDN and ECP were active ribonucleases, with EDN reported to be ~100-fold more catalytically active than ECP [37]. We have recently compared the ribonuclease activities of these proteins in recombinant form from bacteria and found recombinant human EDN (rhEDN) to be ~2000-fold more catalytically active than rhECP ($k_{cat}/K_m = 1.3 \times 10^6$ M^{-1} s^{-1} for rhEDN, 0.59×10^3 M^{-1} s^{-1} for rhECP) [39]. In contrast to the pancreatic-type ribonucleases (RNase A, human and bovine seminal ribonucleases), neither EDN nor ECP are capable of cleaving double-stranded RNA under any conditions tested [40–43].

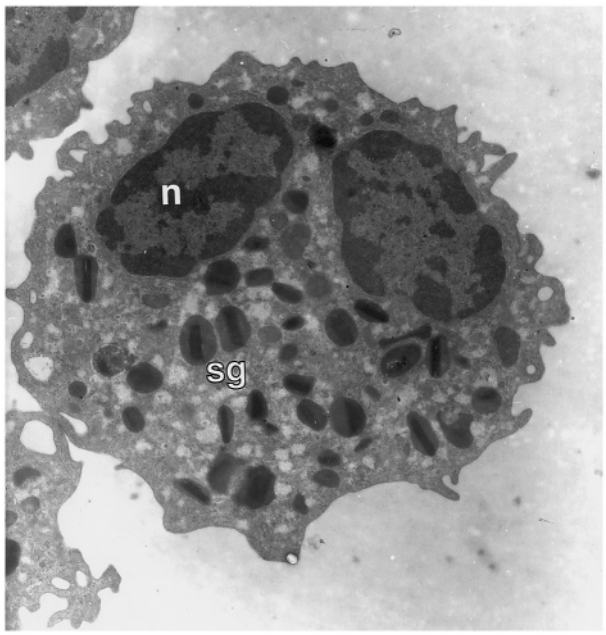

Figure 1. Electron micrograph of a human peripheral blood eosinophil with one lobe of the nucleus (n) and two of the many specific granules (sg) as noted. This electron micrograph was prepared by Dr. Arne Egesten, Lund University, Sweden.

Molecular cloning of both EDN [44, 45] and ECP [46, 47] defined the homology between these proteins (67% amino acid sequence identity) and confirmed the relationship of these proteins to those of the emerging ribonuclease A (RNase A) superfamily. Although the complete amino acid sequences encoded by the EDN and ECP complementary DNAs (cDNAs) were of only limited overall homology to human pancreatic ribonuclease (32 and 26%, respectively), each cDNA encoded eight cysteines analogous to those of human pancreatic ribonuclease [48, 49], as well as specific histidine and lysine residues corresponding to those in the active site defined for RNase A [50]. Molecular cloning also demonstrated that EDN was identical to ribonuclease Us, a protein previously isolated from human urine [51, 52] as well as to the amino terminal sequence of human liver ribonuclease [53]. The encoded amino acid sequences of both ECP and EDN include three and five potential sites for asparagine-linked glycosylation, respectively. The natural glycosylation patterns of both proteins have been described (see article by Sorrentino in this issue); hyperglycosylation of EDN and ECP derived from progenitor cells and cell lines has also been observed [54]. Expression of messenger RNA (mRNA) encoding EDN is widespread, primarily among phagocytic cells [44] and the organs associated with them [55], while ECP has been detected only in eosinophils and eosinophilic cell lines [54, 56, 57]. Another feature shared by EDN, ECP and all members of the RNase A superfamily characterized to date are intronless coding sequences [58]; EDN and ECP, together with all other human members of the RNase A superfamily characterized before [59] and since, [60, 61] have been mapped to chromosome 14 [58, 62]. An alignment of the amino acid sequences of the six known human ribonucleases is shown in fig. 2.

The X-ray crystallographic structure of EDN has recently been reported by Mosimann and colleagues [63].

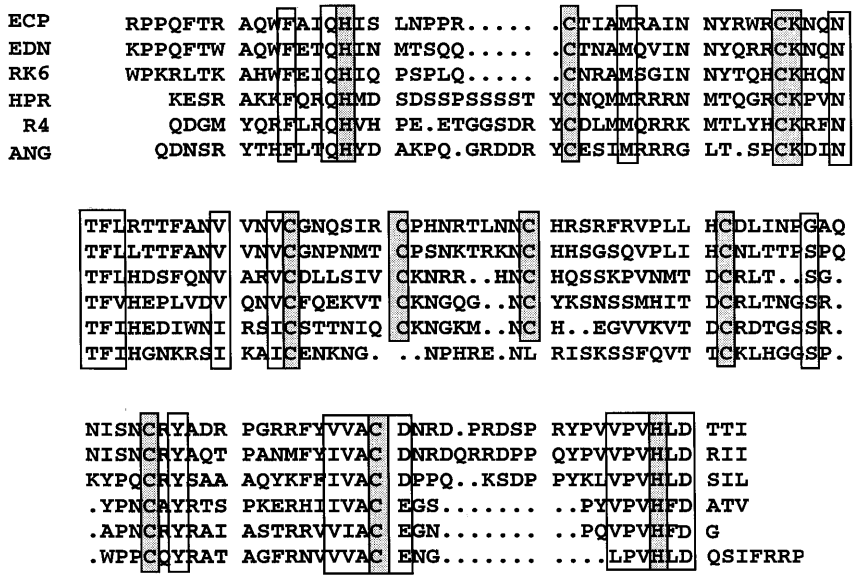

Figure 2. Alignment of the amino acid sequences of the six human members, defining five distinct lineages of the RNase A gene family. Shaded boxes enclose eight cysteines and catalytic histidines and lysine; open boxes enclose additional conserved residues. Amino acid sequences were translated from nucleotide sequences as reported to GenBank: pancreatic ribonuclease (HPR), X79235; eosinophil-derived neurotoxin (EDN), M24157; eosinophil cationic protein (ECP), X15161; RNase 4 (R4), U36775; angiogenin (ANG), M11567; RNase k6 (RK6), U64998. Reprinted with permission from: Rosenberg H. F. and Dyer K. D. (1996) Molecular cloning and characterization of a novel human ribonuclease (RNase k6): increasing diversity in the enlarging ribonuclease gene family. Nucleic Acids Res. **24:** 3507–3513, © 1998 Oxford University Press.

Interestingly, while the peptide fold of recombinant human EDN was clearly related to those observed for onconase, RNase A and angiogenin, its molecular surface differed significantly from those of the other ribonucleases. EDN is characterized by three loops that differ markedly in conformation from those of the aforementioned ribonucleases, and there is a sizable insert (Asp-115 to Tyr-123) that is unique to this protein (but shared with ECP and RNase k6). The biologic role(s) of the loops and insertion remain unclear, but it would be most interesting to determine if these structural differences provide any insight into the differential biologic activities of EDN and RNase A.

Ribonucleases and eosinophil physiology

With EDN and ECP established as full-fledged ribonucleases, an obvious question emerges: What does ribonuclease activity have to do with eosinophils and their effector functions? Is ribonuclease activity crucial to the known functions of either ECP or EDN? At current writing, the answer to this question is both 'yes' and 'no'. This was first tested with respect to EDN and its aforementioned neurotoxicity. Two independent groups have shown that alkylation directed at the active site simultaneously eliminated both the ribonuclease activity and the neurotoxic activity of human EDN [64, 65]. While these results link these two phenomena, the fact that the neurotoxicity is clearly nonphysiologic makes any conclusion with respect to ribonuclease activity and EDN physiology somewhat tentative at best. It is tempting to speculate that the interaction of EDN with a specific neuronal cell or cells mimics a physiologic process occurring at sites of eosinophil degranulation. As such, this phenomenon merits further investigation, with a particular eye towards developing a tissue culture model that will be more amenable to molecular dissection.

The situation with ECP is no less complex. Molina and colleagues [26] demonstrated that ECP's toxicity for trypomastigotes of *Trypanosoma cruzi* was unaffected by the addition of ribonuclease inhibitor (although the complete interpretation of these results awaits further understanding of the interaction of ECP with this inhibitor). More recently, in experiments involving wild-type and ribonucleolytically-inactivated forms of recombinant ECP, our group demonstrated that ribonuclease activity per se was not essential for ECP's antibacterial activity [66]. While these results are consistent with those reported earlier by Young and colleagues [34] suggesting that ECP functions by destabilizing lipid membranes, the finding that the cytoxicity of ECP was not necessarily linked to ribonu-

clease activity is peculiar and counterintuitive from an evolutionary perspective. If ribonuclease activity is unnecessary for function, what are the selective pressures permitting this activity (and underlying structure) to remain? Among several hypotheses, we proposed that the ribonuclease activity of ECP may be necessary for some functions but not for others, suggesting the possibility that there are biologic activities of ECP (and by extension, eosinophils) that have yet to be discovered.

Recent studies of the eosinophil ribonucleases

At this point in our studies, a number of colleagues asked us about the feasibility of using specific gene 'knockout' mice in order to get a clearer picture of the specific roles played by both EDN and ECP. Many colleagues thought it peculiar that we were not able to detect sequences homologous to either EDN or ECP in murine genomic DNA, particularly in light of the report by Murphy [67] noting that majority of human/murine coding sequence pairs diverged by no more than ~10–15%. By low stringency Southern analysis, we detected EDN/ECP sequence homologues among primates, but we were unable to detect homologous sequences in any nonprimate mammals, despite the fact that eosinophils have been detected in nearly all vertebrate species [68] (fig. 3). We successfully isolated genomic fragments of (intronless) EDN and ECP coding sequences from five nonhuman primate species. Our results suggest that the EDN/ECP gene pair arose from a duplication event that occurred some time after the divergence of the New World from the Old World monkeys, and, since duplication, the genes encoding EDN and ECP have accumulated nonsilent mutations at rates exceeding those of all other functional coding sequences studied in primates [nonsynonymous substitution rate ($K_a/2T$) = 1.9×10^{-9} and 2.0×10^{-9} substitutions/site/year, respectively]. Most intriguing is the fact that, despite the rapid rate at which these sequences have incorporated mutations, each sequence retains the eight structural cysteines and the catalytic histidines and lysine that are essential for ribonuclease activity [69]. Although the nature of the constraints promoting the rapid evolutionary rates remains unclear, the conclusion that ribonuclease activity is in some way essential to function would be difficult to contradict.

To take this conclusion one step further, we have compared the ribonuclease activities of human EDN and its most distant relative, the single EDN/ECP sequence identified in the New World monkey, *Saguinus oedipus* (cottontop tamarin) [70]. Much to our surprise, we found that recombinant protein prepared from the New World monkey sequence was not only without the bactericidal activity characteristic of ECP, it was significantly (~100-fold) less ribonucleolytically active than was human EDN, suggesting that evolutionary constraints may be in the process of promoting two novel functions, including increased ribonuclease activity. We have confirmed this finding with recombinant protein prepared from another New World monkey EDN/ECP sequence (*Aotus trivirgatus*, owl monkey), which also demonstrated ~100-fold less catalytic activity than rhEDN, while the ribonuclease activities of recombinant EDNs derived from the *Pongo pygmaeus* (orangutan) and *Macaca fascicularis* (Old World monkey, macaque) sequences were indistinguishable from that of recombinant human EDN [39]. Structure-function analysis has confirmed the '66–122' hypothesis proposed earlier by Beintema [71] relating the carboxy-terminal sequences of these proteins to ribonucleolytic activity. We have found that substituting the penultimate 'Arg-Ile-Ile' encoded by the gene for human EDN with 'Thr-Thr-Ile' encoded by both New World

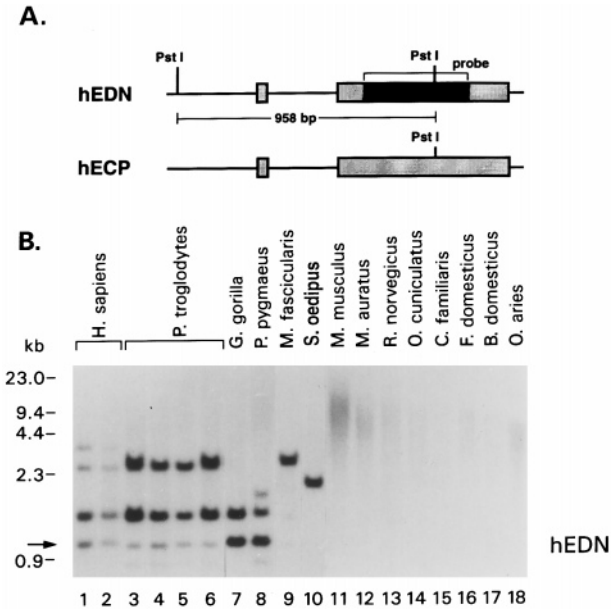

Figure 3. (*A*) Restriction maps of hEDN and hECP. The (intronless) EDN coding sequence (in black) was used to probe the blot shown in (*B*). (*B*) Pst I digested mammalian genomic DNAs probed with the EDN-coding sequence; the arrow denotes the hybridizing band corresponding to the first portion of the coding sequence of EDN. The genus/species identification is noted above each lane. Lanes 1–10 are from the order Primata; lanes 11–13, from the order Rodentia; lane 14, order Lagomorpha; lanes 15–16, order Carnivora; lanes 17–18, order Artiodactyla. Reprinted with permission from: Rosenberg H. F., Dyer K. D., Tiffany H. L. and Gonzalez M. (1995) Rapid evolution of a unique family of primate ribonuclease genes. Nature Genet. **10:** 219–233, © 1998 Nature Genetics.

monkey genes reduced the catalytic actvity of recombinant human EDN by ~10-fold; however, the reverse substitution – 'Arg-Ile-Ile' in place of 'Thr-Thr-Ile' in the owl monkey sequence – did not enhance the catalytic efficiency of this relatively inactive protein. While these studies are limited both by the nature of the assay [cleavage of acid-insoluble yeast transfer RNA (tRNA) into acid-soluble ribonucleotides] as well as by the fact that the true substrates of these ribonucleases are not known, the results suggest that the residues adjacent to the carboxy terminus are necessary but not sufficient for enhanced ribonuclease activity among the primate EDNs [39]. Interestingly, Russo and colleagues [72] demonstrated a pivotal role for C-terminal residues in effecting a conformational change that serves to regulate the ribonuclease activity of human angiogenin.

Another recent advance was the discovery of a novel RNase A superfamily lineage, as exemplified by human ribonuclease k6 [61]. In retrospect, the first member of this lineage to be discovered was a ribonuclease from bovine kidney (k2) described by Niwata and colleagues [73] and Irie and colleagues [74]. Bovine RNase k2 was initially thought to be an orthologue of human EDN on the basis of the 46% amino acid sequence identity observed between these two ribonucleases. In contrast, the sequence identity between bovine RNase k2 and the novel human RNase k6 sequence is 72%, and between human RNase k6 and human EDN, 47%, indicating k6 as EDN's nearest relative after ECP. Another RNase k6 orthologue from porcine kidney has also been described [75]. The amino acid sequence encoded by the RNase k6 genomic fragment is typical for an RNase A superfamily member; it includes the eight cysteines, catalytic histidines and lysine, and an amino terminal signal sequence. Interestingly, mRNA encoding RNase k6 is expressed in several human somatic tissues, and was detected in human neutrophils and monocytes, but not eosinophils. Recombinant RNase k6 was active against the standard yeast tRNA substrate, although with catalytic constants demonstrating ~40-fold less activity than observed for rhEDN. We have recently isolated several primate orthologues of RNase k6, which demonstrate a more conservative rate of nonsynonymous substitution ($K_a/2T = 0.45 \times 10^{-9}$ substitutions/site/year) than was observed for either EDN or ECP [76].

Also recently reported were sequences of murine [77] and rat [78, 79] ribonucleases, representing potential rodent orthologues of human EDN and/or ECP. The identification of cDNAs encoding both the murine and rat eosinophil ribonucleases were based on peptide sequences derived from eosinophil granule proteins, and as such, can be called eosinophil ribonucleases. However, the situation among the murine ribonucleases is rather complex and precludes definitive identification of orthologous pairs. Two murine eosinophil associated ribonucleases, mEAR-1 and mEAR-2 (both with 56 and 59% amino acid sequence similarity to EDN and ECP, respectively) were described by Larson and colleagues [77], who also noted that additional, closely related sequence homologues could be observed by Southern analysis of murine genomic DNA. We have isolated genomic fragments encoding four of these homologous sequences, including three novel ribonucleases and one pseudogene [80]. Together, these sequences form what we have called the 'mR cluster'. The amino acid sequence similarities among the six sequences of this cluster (including mEAR-1 and 2) vary from 60 to 94%, yet they are clearly more closely related to one another than any one is to any of the other RNase A superfamily members characterized at present; fig. 4 demonstrates the relationships of the ribonucleases of the mR cluster to one another, to murine angiogenin and pancreatic ribonuclease, and to human EDN, human ECP, and human, bovine and porcine RNase k6. Another interesting feature of the mR cluster is that its lineage with respect to the six known human RNase A-type genes cannot be determined on the basis of amino acid

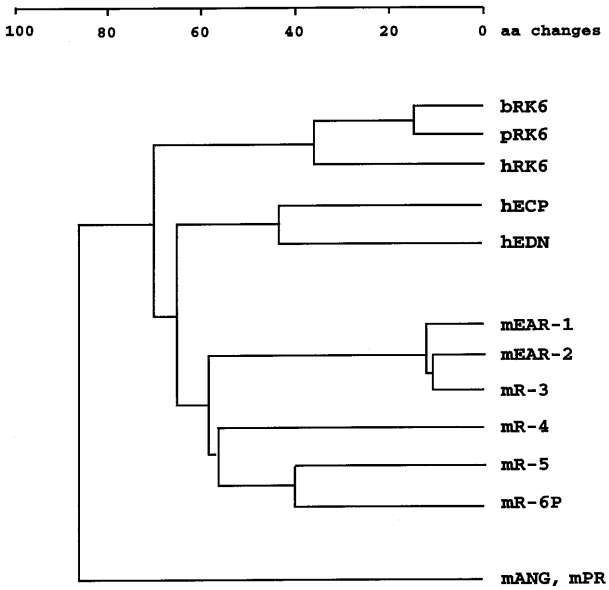

Figure 4. Dendrogram depicting relationships among the eight characterized murine ribonucleases, human EDN (hEDN), human ECP (hECP) and human, bovine [73, 74] and porcine [75] RNase k6 as determined by a modified UPGMA method. Abbreviations: mEAR-1 and -2, mouse eosinophil-associated ribonucleases 1 and 2; mR-3, -4 and -5, mouse ribonucleases 3, 4 and 5 and mR-6P; mPR (murine pancreatic ribonuclease, sw:rnp_mouse, [95]) and mANG (murine angiogenin, U72672.genpept). Reprinted with permission from: Batten D., Dyer K. D., Domachowske J. B. and Rosenberg H. F. (1997) Molecular cloning of four novel murine ribonuclease genes: unusual expansion within the Ribonuclease A gene family. Nucleic Acids Res. **25:** 4235–4239, © 1998 Oxford University Press.

sequence homology. Although as a group, they are more similar to EDN, ECP and RNase k6, and the sequences encoded by mEAR-1 and mEAR-2 match those of tryptic peptides isolated from eosinophils [77], the homology data do not stand in overwhelming support of a unique relationship between any of the mR cluster ribonculeases and either of the human eosinophil ribonucleases (table 1). The existence of an additional, as yet unidentified, ribonuclease lineage that includes the mR cluster cannot be ruled out.

Future trends

One of the most interesting developments with respect to EDN *is* the recent identification of a (potentially) physiologic function that is dependent on ribonuclease activity. In our laboratory we have focused on the intriguing associations among eosinophils, eosinophil granule proteins (particularly ECP) and the pathogenesis of respiratory disease caused by the enveloped single-stranded RNA virus, respiratory syncytial virus (RSV). While the detrimental features related to eosinophil influx (bronchospasm, tissue damage) have been described at length [81–83], we have entertained the possibility that eosinophils may also have beneficial features, and, via the actions of their secreted ribonucleases, may provide a form of host defence against this RNA viral pathogen. In our most recent work we have shown that eosinophils can mediate direct destruction of virions of RSV, an effect that is inhibited by placental ribonuclease inhibitor. Furthermore, we have shown that rhEDN, acting alone, also mediates the direct, ribonucleolytic destruction of these virions, an effect that is not shared by ribonucleolytically inactivated rhEDNdK[38] nor, strangely enough, by 1000-fold higher concentrations of RNase A [84]. While this finding has clear pharmacologic implications, confirmation of antiviral activity as EDN's true physiologic role awaits further clarification of the role of eosinophils in host defence against RSV and similar viral pathogens [85–88].

Other recent work has recognized the pharmacologic potential of EDN and its derivatives. Newton and colleagues [89] reported the expression and characterization of rhEDN fused to a single-chain antibody to the human transferrin receptor. While the enzymatic activity of the fusion protein was less than that of native EDN, the fusion protein bound to the transferrin receptor and mediated cytotoxicity against transferrin receptor-expressing leukaemic cells that was not observed with rhEDN alone. The possibility that this and related immunofusion proteins may mediate cell-specific cytotoxicity while eliciting little to no immune response is an appealing prospect meriting further investigaion. Another recent report details the expression and characterization of a cytotoxic chimera consisting of the amino-terminal sequence of EDN with the frog ribonuclease, onconase [90]. Finally, there are preliminary reports that EDN or its amino terminal fragment, representing a contaminant of preparations of urinary gonadotrophin [91], may mediate direct antiproliferative activity against cells of a Kaposi's sarcoma cell line (M. W. Szkudlinski, personal communication); Griffiths and colleagues [92] have recently reported that an 18-kDa ribonuclease isolated from urinary gonadotrophin preparations induces apoptosis in these cells.

As mentioned earlier, the specter of specific gene knockout mice looms on the horizon. While much can be learned from the careful and controlled analysis of the knockout phenotype, the evolution of the murine ribonucleases is clearly quite complex, and the divergence between the as yet to be definitively identified murine orthologues of EDN and ECP and their human counterparts is clearly quite large. Much caution will be required in interpreting the phenotype of any of these types of murine ribonuclease knockouts.

One aspect remaining to be developed is our understanding of the transcriptional and translational regulation of all the RNase A-type genes. Earlier, Hamann and colleagues [58] noted the presence of both CAAT and TATA boxes in the 5′ promoter regions of the genes encoding both EDN and ECP. We have recently shown that these 5′ regions function as active promoters, and that enhanced transcription of both EDN and ECP genes is mediated by a consensus-binding site for the transcription factor NFAT-1 present in the single intron [57, 93]. Interestingly, Carsana and colleagues [94] have reported on the existence of promoter-like elements in the single intron of the bovine pancreatic ribonuclease gene. The possibility that the transcription of all genes of the RNase A superfamily are regulated via intronic enhancer elements remains an intriguing point for future study.

Table 1. Amino acid sequence comparisons of murine and human ribonucleases.

	hEDN	hECP	hRK6	hPR	hR4	hANG
mEAR-1	56	**59**	58	47	50	49
mEAR-2	56	**59**	**59**	48	53	48
mR-3	54	57	**59**	49	51	51
mR-4	**60**	59	53	48	52	47
mR-5	**60**	58	56	57	54	47
mANG	43	49	49	55	57	**80**
mPR	53	52	59	**79**	66	60

Values are expressed as percentage similarity between pairs of amino acid sequences as determined by the BESTFIT algorithm of the Wisconsin Genetics Computer Group program on-line at the National Institutes of Health. Value representing the highest degree of sequence homology in each row is indicated in boldface. Abbreviations are as defined in figure 4 [80].

Acknowledgements. I would like to thank Dr. G. J. Gleich, Dr. J. J. Lee and Dr. M. W. Szkudlinski for sharing unpublished information on their work regarding these proteins, and Dr. J. B. Domachowske for critical reading of this manuscript. I would also like to thank all the members of my laboratory group for their enthusiastic participation in the experiments described, and Dr. H. L. Malech and Dr. J. I. Gallin for their ongoing support for the work in our laboratory.

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