Minireview

Structure–function relationships in human ribonucleases: main distinctive features of the major RNase types

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Abstract Human extracellular ribonucleases (RNase), together with other members of the mammalian RNase superfamily, can be classified into four different enzyme types on the basis of their structural, catalytic and/or biological properties. Their occurrence and main distinctive features have been described, and catalytic differences (action on single- and double-stranded RNAs, dependence of enzyme activity on pH, ionic strength and cations, and hydrolysis of cyclic nucleotides) have been comparatively analyzed and discussed. In addition, some data considered here concerning human nonpancreatic-type RNases may support the suggestion [Chuchillo et al. (1993) *FEBS Lett.* 333, 207–210] that the enzyme 'ribonuclease', presently classified as 'hydrolase', should be reclassified as 'transferase'.

Key words: Human ribonuclease; RNase superfamily; Neurotoxin; Eosinophil-derived neurotoxin; Eosinophil cationic protein; Angiogenin

1. Introduction

The ribonucleases (RNase) of mammals and other vertebrates constitute a large superfamily of enzymes having greatly diverse functions other than a simple digestive role. Protein sequences from many species are known and their molecular evolution has been studied [1]. These enzymes are widely distributed in various organs and body fluids and, apart from pancreatic RNases, the physiological roles of nondigestive extracellular ribonucleases found in tissues other than pancreas are poorly understood. However, recent investigations have suggested that many members of this superfamily have important biological actions (reviewed in [1–3]) including neurotoxicity, angiogenic activity, immunosuppressivity, and antitumor activity. Some nondigestive extracellular RNases also serve as cytotoxic agents in host defence in higher plants and mammals [3] and their potential use as therapeutic agents for human disorders has recently been suggested [4,5].

In this review we will summarize the information available on the main structural and catalytic properties of the major human RNases (in comparison with other animal ribonucleases), and focus on the amino acid residues likely to be involved in determining some of their catalytic differences.

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Abbreviations: RNase(s), ribonuclease(s); pt, pancreatic-type; npt, nonpancreatic-type; EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; ang, angiogenin; dsRNA, double-stranded RNA

2. Classification, occurrence and features of human RNases

Human ribonucleases have been grouped (together with other mammalian RNases) into two broad classes [6] usually designated as 'secretory' and 'nonsecretory' RNases. However, it is now clear that these designations are not appropriate and might be confusing. Indeed, in some human 'nonsecretory' tissues (for example, brain [7] and kidney [8]) the only, or the major, expressed ribonuclease has been characterized as a 'secretory' RNase. In addition, it has been shown [9] that the human genomic DNA coding for eosinophil-derived neurotoxin (EDN), the so-called human 'nonsecretory' RNase [10–12], encodes a signal sequence typical of secreted proteins. Hence, for all mammalian extracellular RNases we prefer to use here the term 'pancreatic-type' (pt) instead of 'secretory' to classify the members of the RNase family (not only found in pancreas [13] but also in other human tissues and body fluids [14]) characterized by showing sequences as well as structural and catalytic properties similar to those of bovine [15] or human [13,16,17] pancreatic RNases. We also adopt here the term (previously suggested by us [17]) 'nonpancreatic-type' (npt) instead of 'nonsecretory' to categorize the members of the RNase family (expressed in tissues other than pancreas and also found in several fluids [14]) characterized by having sequence and catalytic properties similar to those of bovine kidney RNase K2 [18] or human EDN/liver RNase [11,12,17]. Other members of the RNase superfamily (for example, porcine liver RNase PL3 [19,20], and human plasma RNase 4 [21,22]) constitute a third distinct RNase family and could be referred to as 'pt/npt' RNases. These enzymes (characterized by having a blocked N-terminus, a unique two-residue deletion and other sequence features [19–22]) are indeed structurally more similar to ptRNases [16,19–22], but share some catalytic properties with both (pt) and (npt) ribonucleases [12,13,17,19–22].

Many human RNases have been isolated and characterized by several laboratories, and in different organs several variants of the same gene product have been shown to exist. However, at present, only five distinct proteins with RNase activity have been identified. Recently, the existence of a sixth human RNase has been reported [23]. So far, this novel human ribonuclease, named RNase K6, has been identified only in the genomic DNA, and a single mRNA transcript (1.5 kb) was detected in many human tissues. However, the deduced amino acid sequence of this RNase is 47% and 72% identical to the sequences of human nptRNase [9–12] and bovine kidney RNase K2 [18], respectively [23]. Thus, according to the genetic nomenclature recently introduced [9,22] and on the basis of the designations proposed above, the five structurally

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defined human ribonucleases shown in Fig. 1 can be classified as follows.

2.1. ptRNase 1

This enzyme [16] has been isolated mostly from pancreas [13], but enzymes which are products of the same gene (with different post-translational modifications) have also been purified from urine [24], seminal plasma [25], brain [7], and kidney [8]. In this enzyme, depending on the tissue origin, the glycosylation patterns of the three Asn–Xaa–Thr/Ser sites (Asn-34, Asn-76, Asn-88) are, indeed, quite different [16,26,27]. The primary structure of this enzyme (Fig. 1) shows 70% identity with that of bovine RNase A, most of the amino acid substitutions being conservative [16,26].

2.2. nptRNase 2

This enzyme [10,11] (also named EDN) occurs predominantly in spleen [28], eosinophils [29], liver [30], and placenta [31], but has been also isolated from urine [24], and kidney [8]; all these proteins (with quite similar glycosylation patterns [32]) are products of the same gene. In the sequence of this RNase, five amino acid positions (17, 59, 65, 84 and 92) with asparagine-linked carbohydrates have been identified [10] and their *N*-glycan structure [32] is very different from that reported for ptRNase 1 [27]. In addition, a novel post-translational modification (an α-mannopyranosyl residue C-glycosydically attached to the 2-position of the indole ring of Trp-7) has been recently demonstrated [33]. The primary structure of nptRNase 2 (Fig. 1) is only 35% identical to that of ptRNase 1. The major differences (RNase A numbering system) are: (i) a six-residue deletion (positions 17–22); (ii) the insertion of two, two and nine residues in three external loops of the molecule; (iii) the addition of three residues at the amino terminus. In particular, several amino acids that are considered important in mammalian ptRNases, like Lys-7, Arg-10 (P2 subsite), Lys-66 (Po subsite), and Phe-120 are replaced in a nonconservative way [10]. It is worth noticing that both the enzymes isolated from eosinophils [29] and liver [30] have been shown to possess a potent neurotoxicity linked to their RNase activity [12].

2.3. nptRNase 3

This protein [9,34], also named eosinophil cationic protein (ECP), isolated thus far only from granulocytes [29], is very basic (pI, 10.8) and highly homologous (70% identity) to nptRNase 2 [34]. In the sequence of this RNase (Fig. 1) there are three N-linked glycosylation sites [34] with complex oligosaccharides similar to those found in nptRNase 2 [29]. This protein is less neurotoxic than nptRNase 2 but is a more potent helmintotoxin also having antibacterial activity as well as showing cytotoxicity for mammalian cells in vitro [34]. While its neurotoxicity seems to be linked to the ribonucleolytic activity [12], RNase activity is not essential for cytotoxicity [35]. Its ribonucleolytic activity is much lower than that of nptRNase 2, but otherwise catalytic properties are similar [36].

2.4. pt/nptRNase 4

This enzyme [22] was first isolated from tumor-cell-conditioned medium [21] and later from normal human plasma [22]; recently the messenger RNA coding for this RNase was detected by Northern analysis in a number of human somatic tissues, including pancreas, liver, lung, skeletal muscle, heart, kidney and placenta, but not brain [37]. No N-linked glycosylation sites have been observed in the sequence of this protein, the only post-translational modification being the pyroglutamic acid found at its amino terminus [22]. Its sequence (Fig. 1) is more similar to that of human ptRNase 1 (43% identity) than to those of nptRNase 2 and RNase 3 (31% and 30% identity, respectively), but shows a unique deletion of two residues (positions 77 and 78 of RNase 1) [22]. In addition, this human RNase was shown to be highly homologous (about 90% identity) to porcine and bovine liver RNases [22].

2.5. angRNase 5

This protein [38] (called angiogenin), originally isolated from tumor-cell-conditioned medium [38] on the basis of its potent in vivo angiogenic activity, was also purified from normal human plasma [39]. Its principal biological role is to induce the formation of new blood vessels. Despite its remarkable structural similarity (Fig. 1) to pt/nptRNase 4, ptRNase 1, and nptRNase 2 (39%, 35%, and 27% sequence identity, respectively [10,11,22,38]), angRNase 5 shows an unusual ribonucleolytic activity [40,41], which differs markedly both in magnitude and specificity from RNase activity of the other human RNases. The extremely weak ribonucleolytic activity (toward standard RNase substrates [40]) of angRNase 5 which is, however, essential for angiogenicity, seems to be in part due to the obstruction of the pyrimidine binding site (as observed in the homologous RNase A structure) by Gln-117 [42].

3. Catalytic differences between human ribonucleases

The five structurally defined human RNases described above, despite their remarkable sequence similarities (Fig. 1) are catalytically quite different and, excluding angRNase 5 because of its unusual ribonucleolytic activity, the remaining four RNases show catalytic properties which are characteristic of one or both the two major RNase types, ptRNase 1 and nptRNase 2.

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                1         10        20        30        40
RNase A         KETAAAKFERQHMDSSTSAASSSNYCNQMMKSRNLTKDRCKPV
RNase 1      ---KESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPV
RNase 2      KPPQFTWAQWFETQHINMTSQQ------CTNAMQVINNYQRRCKNQ
RNase 3      RPPQFTRAQWFAIQHISLNPPR------CTIAMRAINNYRWRCKNQ
RNase 4      ---QDGMYQRFLRQHVHPEETGG-SDRYCNLMMQRRKMTLYHCKRF
RNase 5      --QDNSRYTHFLTQHYDAKPQGR-DDRYCESIMRRRGLTSP-CKDI

                50        60        70        80        90
RNase A      NTFVHESLADVQAVCSQKNVACKNGQT--NCYQSYSTMSITDCRETGSS
RNase 1      NTFVHEPLVDVQNVCFQEKVTCKNGQG--NCYKSNSSMHITDCRLTNGS
RNase 2      NTFLLTTFANVVNVCGNPNMTCPSNKTRKNCHHSGSQVPLIHCNLTTPS
RNase 3      NTFLRTTFANVVNVCGNQSIRCPHNRTLNNCHRSRFRVPLLHCDLINPG
RNase 4      NTFIHEDIWNIRSICSTTNIQCKNGKM--NCHEGV--VKVTDCRDTGSS
RNase 5      NTFIHGNKRSIKAICENKNGNPHRE----NLRISKSSFQVTTCKLHGGS

                100       110               120
RNase A      K--YPNCAYKTTQANKHIIVACEGN---------PYVPVHFDASV----
RNase 1      R--YPNCAYRTSPKERHIIVACEGS---------PYVPVHFDASVEDST
RNase 2      PQNISNCRYAQTPANMFYIVACDNRDQRRDPPQYPVVPVHLDRII----
RNase 3      AQNISNCRYADRPGRRFYVVACDNRD-PRDSPRYPVVPVHLDTTI----
RNase 4      R--APNCRYRAIASTRRVVIACEGN---------PQVPVHFDG------
RNase 5      P--WPPCQYRATAGFRNVVVACENG----------LPVHLDQSIFRRP
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Fig. 1. Alignment of the amino acid sequences of the five structurally defined human RNases with that of bovine pancreatic RNase A. Residues conserved in all six RNases are indicated (boldface type). The references for the sequences are given in the text.

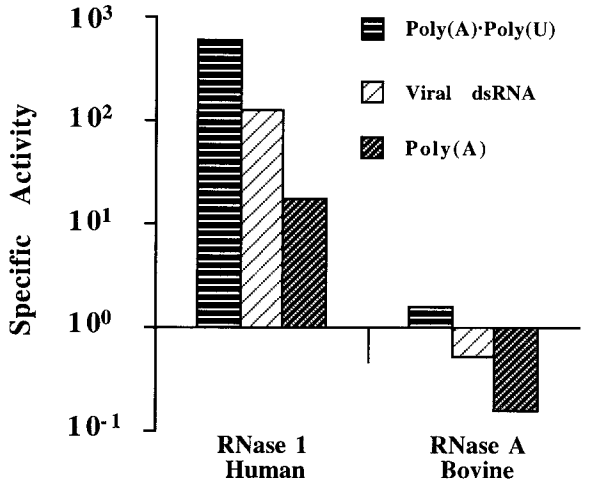

Fig. 2. Action of human ptRNase 1 and bovine pancreatic RNase A towards poly(A) and double-stranded polyribonucleotides. Figure prepared with data published in [17].

3.1. Activity on double-stranded RNA and poly(A)

The first notable difference between these two RNase types is that ptRNase 1 (Fig. 2) is active on poly(A) and double-stranded (ds) RNA (two orders of magnitude more than RNase A) while nptRNases 2 and 3 are totally inactive on those substrates [17,36]. Recently, it has been suggested that in bovine RNase A [43] (and very probably in all mammalian ptRNases) Asp-83 could play an important role in the activity towards poly(A). Interestingly, nptRNases at the corresponding position lack Asp-83. The enzymatic cleavage of dsRNA by ptRNases may occur, according to a mechanism already proposed by us [17,44], as the consequence of the preferential binding of the RNase molecule to short single-stranded sequences of the substrate transiently exposed by spontaneous thermal fluctuations. This model is supported by the observation that ptRNase 1 shows a remarkable DNA-helix-destabilizing action, while nptRNase 2 as well as nptRNase 3 have no such activity [17,36,44]. A complementary model, based on the binding of the enzyme to single nucleotides wound off the double-helix, was also advanced [45]. The DNA-unwinding activity of bovine RNase A has been related to the multiplicity of phosphate-binding subsites of the enzyme protein [46]. In this respect, the higher degrading activity shown by human ptRNase 1 towards dsRNA (Fig. 2) could be explained by a stronger local positive electrostatic potential developing on the enzyme because of the presence of three basic amino acid residues (Arg-4, Lys-6, Arg-32) at positions where in bovine RNase A three neutral residues are present instead [15,16]. In contrast, nptRNases 2 and 3, although higly basic proteins, do not show the extended multisite cationic region (nine basic residues) which characterizes mammalian ptRNases [46], i.e. many of their positive charges are located far from the enzyme active site.

In conclusion, while human nptRNases 2 and 3 (and possibly also other mammalian nptRNases) may be defined as true single-strand *specific* RNases, mammalian ptRNases might by now be classified as single-strand *preferring* RNases.

3.2. Substrate preference

As summarized in Fig. 3, in contrast to bovine RNase A and ptRNase 1 which degrade poly(C) faster than RNA and poly(U), human nptRNases 2 and 3 but also pt/nptRNase 4 strongly prefer poly(U) over poly(C). This preference seems to be due to a significant reduction of their affinity for poly(C), rather than to an increased preference for poly(U) [17,21,22,36] and could be attributed to the different microenvironment of the B1 subsite of both nptRNases and pt/nptRNase 4. In these enzymes, in fact, Thr-45 is conserved but Ser-123 is not, and this might prevent productive binding of the cytosine moiety of the substrate molecule. However, Ser-123 is replaced by other residues (Thr, Tyr) in several mammalian ptRNases [1]. Recently, it has been suggested [47] that in nptRNase 2 the side-chain of Gln-40, like Ser-123 of RNase A, can either accept or donate hydrogen bonds and could be responsible for binding the O-4 of uracil or N-4 of cytosine. In addition, in human pt/nptRNase 4 (and also in porcine 'pt/npt' RNase PL3 [20]) the phenylalanine residue at position 42 (Val-43 in ptRNase 1) has been suggested to play an important role through its possible interaction with uracil [22].

3.3. Influence of ionic strength and cations

The activity of the two major human RNase types towards yeast RNA has been shown to be differently influenced by ionic strength and divalent cations [17]. The increasing of the NaCl concentration from 50 to 300 mM enhances the activity of ptRNase 1, while the activity of nptRNase 2 decreases above 150 mM. This could be due to the stronger positive electrostatic potential of ptRNase 1 developing in the interaction of the enzyme protein with the polyanionic substrate. Another difference between the two RNase types is that while the activity of nptRNase 2 is not influenced by low zinc ion concentrations (0.5 mM), that of ptRNase 1 is strongly inhibited. Magnesium ions, instead, have no effect on the activity of both RNase types [17]. This could be partly ascribed to a stoichiometric bond possibly forming in ptRNase 1 between a zinc ion and His-12 and His-119, but not in nptRNase 2 [17] because of structural differences of its catalytic site [47].

Table 1
Structural and catalytic characteristics of human RNases compared with those of other members of RNase superfamily

Enzyme	Position 66	Phe-120	Asp-121	Position 122	pH optimum on yeast RNA	Activity on cyclic nucleotides
Bovine RNase A	Lys	+	+	Ala	8.0	+
ptRNase 1	Lys	+	+	Ala	8.0	+
pt/nptRNase 4	Lys	+	+	Ala	7.5	+
Bovine RNase K2	Lys	−	+	Lys	6.5	?
nptRNase 2	Pro	−	+	Arg	6.5–7.0	−
Turtle RNase	Ser	−	+	Lys	?	−
Onconase	deletion	+	−	Gly	6.0	+

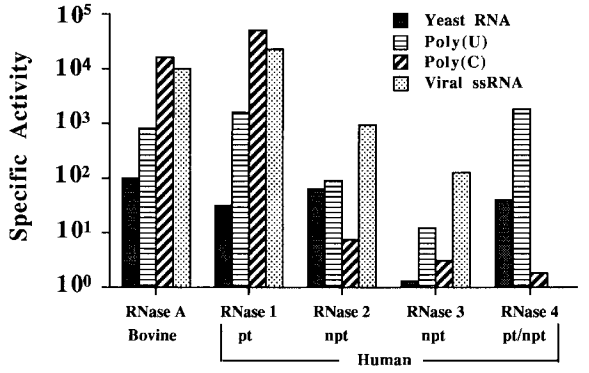

Fig. 3. Substrate preference of human ribonucleases compared with that of bovine pancreatic RNase A. Figure prepared with data published in [17,21,36]. The data concerning pt/nptRNase 4 (obtained by Shapiro et al. [21]) were transformed into the enzyme units defined in [17,36] and included in the figure only to allow a rough comparison.

3.4. *Influence of pH on RNase activity and hydrolysis of 2′,3′-cyclic nucleotides*

Two other remarkable catalytic differences of the two major human RNase types are their pH optima with RNA as substrate, and the so-called 'second step' of the RNase-catalyzed reaction [17]. Human ptRNase 1 shows indeed optimal activity with RNA as substrate at pH 8.0, and a hydrolytic activity towards 2′,3′-cyclic nucleotides which is comparable to that of bovine RNase A [7,8,13,17,24]. Human nptRNase 2, shows its pH optimum shifted to lower pH values (6.5–7.0) with yeast RNA as substrate [17,24,28], and is unable to catalyze the hydrolysis of cyclic nucleotides at a measurable rate [12,17,20]; this is also true for nptRNase 3 [36]. To understand these points, particularly important are the amino acid residues (RNase A numbering system) specified in Table 1.

In all mammalian ptRNases Lys-66 has an important role [46,48], and it has been suggested that a basic amino acid at either position 66 or 122 may be of primary importance for a 'normal' ribonucleolytic activity [48]. It is quite interesting that human nptRNase 2 and turtle pancreas RNase [1,48] lack Lys-66 (see Table 1) but have a basic residue at position 122, while angiogenin, nptRNase 3, and also onconase (an RNase from *Rana pipiens* oocytes) [49], lacking a basic residue at both those positions, show indeed a very low RNase activity [36,30,48,49].

The importance of Asp-121 in bovine RNase A has been demonstrated also by site-directed mutagenesis [50]. We think that, in all mammalian ptRNases, the possible interaction between Asp-121 and His-119 [51] could also serve to stabilize at pH 8.0 the protonated form of His-119, which is essential for catalytic activity. In human nptRNase 2 (Fig. 1), as well as in bovine kidney RNase K2 [18,52] and turtle RNase [1,48], while Asp-121 is a conserved residue, a basic amino acid is present (Table 1) at position 122 (where in ptRNases a neutral residue is found), which plays a crucial phosphate-binding role [48]. Now, a basic residue at position 122 may indeed influence the possible interaction between Asp-121 and His-119 and therefore be responsible for a lower pH value (6.5–7.0) necessary to maintain His-119 in its protonated form. In agreement with this idea, human nptRNase 2 and bovine RNase K2 (Table 1) show in fact such a lower pH optimum. Moreover, according to our hypothesis, turtle RNase although being a pancreatic enzyme should have a pH optimum similar to that of nptRNases. Unfortunately, for this enzyme no experimental data concerning this point are available thus far.

In conclusion, in all members of the vertebrate extracellular RNase superfamily a pH optimum of about 8.0 toward RNA seems to be ascribable to the presence of an aspartate residue at position 121. Its absence (as it occurs in onconase, Table 1) or the presence in some ribonucleases of a basic residue at position 122 could be responsible for the lower pH optimum of these enzymes.

As discussed above, human nptRNases 2 and 3 differ from human ptRNase 1 and pt/nptRNase 4 as well as from other mammalian ptRNases for many structural and catalytic properties. One of the most important difference consists in the fact that although nptRNases efficiently catalyze the depolymerization of RNA, they do not show any detectable (catalytically significant) hydrolytic activity towards 2′,3′-cyclic phosphodiesters [12,17,36], which are usually considered intermediates in the RNase A-catalyzed cleavage of RNA. Regarding this point, work from different laboratories [46,53,54] demonstrated that bovine RNase A and other pancreatic-type RNases release into solution cyclic phosphodiesters as true products of the transphosphorylation of RNA, which can be hydrolysed in a separate slower reaction. It is worth noticing here that (Table 1) while the ribonucleases capable of hydrolysing cyclic nucleotides have a phenylalanine at position 120, those lacking this residue are inactive on the same substrates. Accordingly, it has been observed [55] that while a substitution of leucine for phenylalanine at position 120 lowers the activity of bovine RNase A on cyclic nucleotides about 100-fold, the replacement of leucine by phenylalanine in human angRNase 5 (at a site equivalent to position 120 in RNase A) increases the activity of angRNase 5 against cyclic nucleotides up to 100-fold [56]. These observations seem to indicate that the absence in a RNase of the aromatic side-chain of Phe-120 while being responsible for the inability of the enzyme to catalyze efficiently the hydrolysis of cyclic phosphodiesters, does not influence RNA transphosphorylation.

On the basis of these data, human nptRNases might represent a proper indication that the enzyme *ribonuclease* does not require a water molecule as a substrate for RNA cleavage. This may support the suggestion already advanced [53,54] that *ribonuclease* (presently classified as *hydrolase*) has indeed evolved primarily to catalyze RNA transphosphorylation. If so, it should be reclassified as *transferase*.

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