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Ribonuclease activity and substrate preference of human eosinophil cationic protein (ECP)

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The eosinophil cationic protein (ECP), a potent helminthotoxin with considerable neurotoxic activity, was recently shown to also have ribonucleolytic activity. In this work the substrate preference of ECP ribonuclease action was studied in detail. With single-stranded RNA or synthetic polyribonucleotide substrates ECP showed significant but low activity, 70- to 200-fold less than that of bovine RNase A. ECP hydrolyzed RNA more rapidly than it did any synthetic polynucleotide. Poly(U) was degraded more rapidly than poly(C), and poly(A) and double-stranded substrates were extremely resistant. Defined low molecular weight substrates in the form of the 16 dinucleoside phosphates (NpN') and uridine and cytidine 2',3'-cyclic phosphates were tested, and none showed hydrolysis by ECP at a significant rate. The results link ECP ribonucleolytic activity to the 'non-secretory' liver-type enzymes rather than to the 'secretory' pancreatic-type RNases.

Eosinophil protein; Helminthotoxin; Neurotoxin; Ribonuclease activity; Ribonuclease superfamily

1. INTRODUCTION

Human eosinophil granules contain several basic proteins with unusual biological activities. Two of these proteins, the eosinophil derived neurotoxin (EDN) and the eosinophil cationic protein (ECP), are particularly well characterized. Both proteins were initially isolated as neurotoxins [1], but their relationship to ribonucleases became obvious upon determination of partial amino acid sequences [1,2] and their comparison with the amino acid sequences of human 'secretory' and 'non-secretory' RNases isolated from human pancreas [3] or urine [4] and liver [5]. Full nucleotide sequences of cDNAs for EDN and ECP have now been determined [6–9] and it is clear that EDN is identical to liver RNase. In addition, the structure and chromosomal location of the EDN and ECP genes have been reported [10].

There is thus clear evidence that both EDN and ECP are products of very related genes in the RNase gene superfamily. Both proteins show similar neurotoxic potency [1], although the assay is not easily quantitated. ECP is the more potent helminthotoxin [11,12], while EDN possesses 50–100 times more ribonucleolytic activity than ECP [13,14]. The substrate specificity and preference of EDN and liver RNase have already been compared with each other and with bovine RNase A [15], and Iwama et al. [16] have studied these characteristics of the human urinary RNase Us which is probably the same protein. This work examines in detail the RNase activity of ECP with a variety of single- and double-stranded polynucleotides, dinucleoside phosphates, and 2',3'-cyclic nucleotides as substrates.

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2. MATERIALS AND METHODS

2.1. *Materials*

ECP, purified from human eosinophil granules as previously described [1], was the generous gift of Dr Gerald Gleich. Bovine pancreatic RNase A (type XII-A) and bovine serum albumin were purchased from Sigma. Wheat germ high molecular weight RNA and bacteriophage f2 RNA were prepared according to published procedures [17,18]. Yeast RNA, single- and double-stranded polynucleotides, dinucleoside monophosphates and nucleosides 2',3'-cyclic phosphates were all Sigma products.

2.2. *Enzyme assays*

Ribonuclease activity against wheat germ RNA was measured through the formation of perchloric acid-soluble nucleotides as described [18] except that the reaction buffer contained 0.05 M 4-morpholinopropanesulphonic acid (MOPS), pH 7.5, and bovine serum albumin (1 mg/ml). Activity with a yeast RNA substrate (further purified from the commercial product as described by Blackburn et al. [19]) was measured at pH 5.0 and 7.5 following the Kunitz [20] assay procedure. Degradation of single- and double-stranded synthetic polynucleotide substrates and of bacteriophage f2 RNA was followed spectrophotometrically in a variation of a previously described procedure [21,22]. Enzyme activity with dinucleoside phosphate substrates (NpN'), was measured by a modification of the method of Witzel and Barnard [23] while hydrolysis of cytidine and uridine 2',3'-cyclic phosphates was assayed by modifications of the procedures of Crook et al. [24] and Richards [25], respectively.

2.3. *Thermal transition profiles of nucleic acids*

Thermal transition profiles of poly(dA-dT):poly(dA-dT) were measured in the presence and absence of RNases. Measurements were made at 260 nm in thermostatically controlled stoppered cuvettes (Starna Ltd, London, type 29) with a Zeiss PM6 spectrophotometer. In all experiments the concentration of the polynucleotide was about 10 μg/ml (initial absorbance at 260 nm, 0.210); RNase, if present, was also 10 μg/ml (A_{260}, about 0.005–0.008).

3. RESULTS AND DISCUSSION

The major human ribonucleases, although part of the ribonuclease superfamily of related proteins, have been grouped into two broad classes [26] usually designated 'secretory' and 'non-secretory'. The former are most abundant in secretory organs and body fluids including pancreas, prostate, seminal fluid, milk, and saliva [27]; they are sometimes classed as 'pancreatic-type' enzymes. They show a marked substrate preference for poly(C) rather than poly(U) and, under specific conditions, a pH optimum near 8.0 for RNA degradation. The non-secretory or 'liver type' enzymes predominate in liver and spleen and are minor components of urine and serum [27]. These enzymes show a pH optimum near 7.0 with RNA as substrate, and hydrolyze poly(U) more readily than poly(C).

ECP is a secreted protein with a typical 27 amino acid putative signal peptide [7]. However, its deduced amino acid sequence shows 70% identity to human non-secretory RNase/EDN [4–7]. It is therefore of interest to determine whether the catalytic characteristics of ECP link it to either category of RNase.

As a measure of the substrate specificity of ECP as a ribonuclease, its action on three different natural RNA types and several different single- and double-stranded synthetic polyribonucleotide substrates was measured using different assay conditions and methods. As shown in Table I, ECP-catalyzed hydrolysis of all single-stranded polynucleotides was slow relative to that of bovine RNase A; the bovine enzyme was 70–200 times as active with RNA and poly(U) substrates, and more than 3000 times as active on poly(C). These data are in agreement with some previous results [13,14] obtained with RNA substrates. Beintema [28] and Barker et al. [7] have recently suggested that the low ribonucleolytic activity of ECP could be linked to its lack of a basic residue at sites equivalent to either position 66 or 122 of the protein (using the pancreatic RNase sequence numbering system).

ECP showed no measurable ribonuclease activity against poly(A) or the double-stranded substrates poly(A):poly(U) or poly(I):poly(C); EDN/liver RNase is also inactive with these polymers [15]. The inability of ECP to attack double-stranded substrates is correlated with its lack of helix-destabilizing activity, as shown in Fig. 1. Although ECP is a very basic protein [7], the data of Fig. 1 show that it is not able to disrupt the structure of double-stranded poly(dA-dT) under conditions of both high and low ionic strength. Helix destabilizing activity was considered to be one of the factors that favors RNase attack on double stranded polynucleotides [22]. For example, human seminal RNase, a basic secretory enzyme that is very similar if not identical to human pancreatic ribonuclease [29] and which degrades double-stranded polynucleotides 400–500 times faster than RNase A, shows marked helix-destabilizing activity [22,30].

The relative preference of human ECP for various macromolecular substrates is summarized and compared with other human RNases in Table II. It is clear that ECP RNase activity most resembles that of the non-secretory liver type enzymes. In particular, ECP is most active on an RNA substrate, and prefers poly(U) to poly(C) by a factor of four while the pancreatic type enzymes prefer poly(C) over all other substrates and poly(U) is degraded relatively slowly. It is worth noting that the poly(U) preference of ECP is due to a marked

Table I

Activity of human ECP on polymeric substrates compared with that of bovine RNase A

Substrate	Assay pH	Method	ECP (U/mg)	RNase A (U/mg)	RNase A / ECP
Wheat Germ RNA	7.5	[a]Acid soluble A_{260}	2170	356000	164
Yeast RNA	5.0	[b]Hypochromicity	1.3	95	71
	7.5	"	2.4	475	198
Phage f2 RNA	7.0	[c]Hyperchromicity	125	7940	63
Poly(U)	7.0	"	12.2	890	73
Poly(C)	7.0	"	3.1	13300	4290
Poly(A)	7.0	"	n.d.	0.13	–
Poly(A,U)	7.0	"	232	16600	71
Poly(A,C)	7.0	"	205	26000	126
Poly(A):Poly(U)	7.0	"	n.d.	2.9	–
Poly(I):Poly(C)	7.0	"	n.d.	30.2	–

[a] U defined in [18]. [b] U defined in [20]. [c] U calculated as: (change in A_{260}/min)/total measurable change in A_{260}; substrate (0.1 mM in phosphate) plus an appropriate amount of enzyme were mixed in 1 ml of 0.1 M 4-morpholinoethanesulphonic acid (MES), pH 7.0, 0.1 M NaCl, at 25°C. Each value is the average of duplicate determinations; n.d.: not detected.

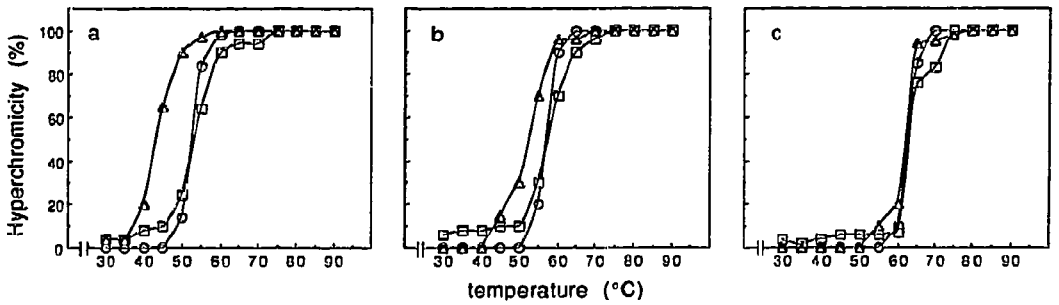

Fig. 1. Effect of human Eosinophil Cationic Protein or bovine RNase A on the thermal transition profile of double-stranded poly(dA-dT):poly(dA-dT) under different ionic conditions. In 1 ml of 10 mM MOPS buffer (pH 7) containing 20 mM NaCl (a), 50 mM NaCl (b), 100 mM NaCl (c), 10 μg of the double-stranded polymer were mixed with 10 μg of bovine RNase A (Δ), or ECP (□); (○), thermal transition profile of the nucleic acid in the absence of protein.

Table II

Substrate preference of human ECP compared with those of human pancreatic and liver ribonucleases

Enzyme	Ratio of substrate cleavage		
	RNA/Poly(C)	RNA/Poly(U)	Poly(C)/Poly(U)
ECP	40[a]	10[a]	0.25[a]
Pancreatic RNase	0.3[b]	8[b]	26[b]
Liver RNase	167[b]	12[b]	0.07[b]

[a] Calculated from Table I; [b] Calculated from [5]

decrease in the ability of the protein to degrade poly(C) rather than an absolute or relative increase in the rate of poly(U) hydrolysis; the ratio of RNA/poly(U) hydrolysis rates is similar for both enzyme types. These results also partially contradict the claim of Gullberg et al. [14] that ECP shows no substrate specificity. However, their paper does not present data to support this statement, and it is our observation that any such conclusion must be based on quantitation of initial cleavage rates of both polymeric and dinucleoside phosphate substrates.

With defined low molecular weight substrates ECP showed no measurable ribonuclease activity. None of the sixteen dinucleoside phosphates (NpN') and neither uridine and cytidine 2',3'-cyclic phosphates was degraded at an appreciable rate by ECP under the conditions used; in each assay bovine RNase A was included as a positive control on the method used. Again this is characteristic of the non secretory class of RNases which have been found to degrade dinucleoside phosphates very slowly and to be virtually inactive in the hydrolysis of nucleoside 2',3'-cyclic phosphates [15,16]. This inactivity with small substrates may be due in part to the absence of an aromatic residue (Phe or Tyr) at the site equivalent to position 120 of the bovine RNase A sequence; in human angiogenin the replacement of leucine by phenylalanine at this position increases the activity of this protein against small substrates up to 100-fold [31].

All of the nucleolytic characteristics of ECP presented here serve to link this protein to the 'non-secretory' or liver type RNases [26]. These observations further emphasize the confusion generated by use of the terms secretory and non-secretory when all members of the RNase gene superfamily encode signal peptides [10,32] which may lead to secretion or to localization in lysosomes. Perhaps designation of ECP as a liver type RNase or, as suggested by Maddalena et al. [33], as a member of the RNase IIu subclass is more logical.

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