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Diversity among the primate eosinophil-derived neurotoxin genes: a specific C-terminal sequence is necessary for enhanced ribonuclease activity

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ABSTRACT

The human eosinophil-derived neurotoxin (hEDN) is a secretory effector protein from eosinophilic leukocytes that is a member of the ribonuclease A (RNase A) family of ribonucleases. EDN is a rapidly evolving protein, accumulating non-silent mutations at a rate exceeding those of most other functional coding sequences studied in primates. Although all primate EDNs retain the structural and functional residues known to be prerequisites for ribonuclease activity, we have shown previously that recombinant EDN derived from a New World monkey sequence (*Saguinus oedipus*) had significantly less catalytic activity than the human (hEDN) ortholog. In this work, we have prepared recombinant proteins from EDN from sequences derived from orangutan (*Pongo pygmaeus*, oEDN) and Old World monkey (*Macaca fascicularis*, mcEDN) genomic DNAs, and from a second New World monkey sequence (*Aotus trivirgatus,* omEDN) as well. The catalytic efficiencies [k_{cat}/K_m (M^{-1} s^{-1})] determined for both oEDN and mcEDN were similar to that determined previously for hEDN, while omEDN displayed ~100-fold less catalytic activity. The relative ribonuclease activities of hEDN/omEDN chimeras pointed to a C-terminal segment as crucial to the enhanced catalytic activity hEDN, and substitution of Arg 132–Ile 133 of hEDN with the Thr–Thr pair at the analogous position in omEDN resulted in an ~10-fold reduction in hEDN's catalytic efficiency. However, the reverse substitution, Arg–Ile for Thr–Thr in omEDN, did not enhance the catalytic efficiency of this relatively inactive protein. These results indicate that the Arg and/or Ile residues adjacent to the C-terminus are necessary (but not sufficient) for enhanced ribonuclease activity among the primate EDNs, and will permit prediction of the relative ribonuclease activities based on differences in primary structure.

INTRODUCTION

The eosinophil-derived neurotoxin (EDN) is a small, glycosylated protein found in the large specific granules of eosinophilic leukocytes. Durack and colleagues (1) were first to report the isolation of EDN, and to determine that eosinophil-related neurotoxicity—a syndrome of ataxia and paralysis associated with Purkinje cell degeneration (the Gordon phenomenon, 2)—was mediated in part by the activity of this secretory protein (1,3). Gleich and colleagues (4) reported the N-terminal sequence of purified EDN, and noted the similarity between this peptide and the N-terminal sequence of bovine ribonuclease A (RNase A). EDN's membership in the RNase A family of ribonuclease genes was later confirmed by molecular cloning (5,6). In terms of enzymatic activity, EDN is a catalytically efficient ribonuclease (7–9) and exhibits some degree of preference among experimental substrates (10). Both Sorrentino and colleagues (10) and Newton and colleagues (11) have shown that EDN's neurotoxic effects are directly dependent on ribonuclease activity.

In an earlier study, we traced the evolutionary history of EDN and the closely related ribonuclease/toxin, eosinophil cationic protein (ECP), and found that both genes accumulated non-silent mutations at rates exceeding those of all other functional coding sequences studied in primates while retaining all the structural and catalytic components known to be prerequisites for ribonuclease activity (12). With this in mind, we were surprised to find that one of the novel isolates, EDN encoded by the New World monkey *Saguinus oedipus*, was 100-fold less catalytically active than its human ortholog (13). In the work presented here, we have examined the ribonuclease activity of recombinant proteins derived from three additional non-human primate species and compared them to the activities of recombinant human EDN and ECP. Information derived from these experiments has permitted us to create interspecies chimeras, and to identify amino acid sequence elements that support the enhanced level of ribonuclease activity characteristic of human EDN.

MATERIALS AND METHODS

Isolation of EDN gene from owl monkey (*Aotus trivirgatus*)

The intronless coding sequence of owl monkey EDN (omEDN) was isolated by polymerase chain reaction (PCR) as described (11); in this case, a 3′ primer encoding a segment of the 3′ untranslated region of human EDN was used in order to identify precise sequence at the 3′ end of the coding sequence. The source of PCR template was genomic DNA isolated from the owl monkey

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kidney cell line OMK (637-69) from the American Type Culture Collection (cat. no. CRL-1556). All sequence analysis and comparisons were performed with the assistance of the Wisconsin Genetics Computer Group programs available on-line at the National Institutes of Health.

Preparation of chimeras

Chimeras were created by overlapping PCR mutagenesis as described (13,14), with amplification primers designed to facilitate direct cloning into the pFCTS bacterial expression vector (International Biotechnologies, Inc., New Haven, CT) as described below. All chimeras were confirmed by dideoxy-sequencing.

Expression constructs and isolation of recombinant protein

All EDN and ECP coding sequences were PCR-amplified with primers containing restriction sites facilitating direct cloning into the pFCTS bacterial expression vector (IBI); all constructs were confirmed by dideoxy-sequencing. The pFCTS vector adds the octapeptide DYKDDDK ('FLAG') to the recombinant protein which permits its isolation and detection using the M2 anti-FLAG monoclonal antibody (mAb). We have shown previously that the FLAG octapeptide does not interfere with the folding or the catalytic activity of recombinant ribonucleases (13,15). Recombinant proteins were isolated from 2–4 l of bacterial cultures after a 1 h induction with isopropyl-1-thio-β-galactoside (IPTG; 1 mM for EDNs, and 1 μM for hECP). After harvest and sucrose lysis (EDNs) or harvest and cell lysis by freeze–thaw and sonication (hECP), recombinant proteins were concentrated and isolated by M2 mAb–agarose affinity chromatography (IBI) as described in detail in reference 13. The concentration of recombinant protein was determined by comparison to serial dilutions of a known concentration of FLAG-conjugated standard as described (13).

Ribonuclease assay and double reciprocal plots

Reactions were carried out with varying concentrations of yeast tRNA (Sigma Chemical Co., St Louis, MO, cat. no. R-9001) added in separate reactions to 0.8 ml of 40 mM sodium phosphate, pH 7.0, containing recombinant EDN at concentrations indicated. The assay, solutions, conditions and t = 0 controls were as described in reference 13. The ribonuclease activity from sham isolates (M2-resin equilibration and glycine elution of periplasmic proteins isolated from equivalent volumes of pFCTS-vector alone bacterial transfectants) was determined; the sham isolates had levels of ribonuclease activity that were insignificant when compared to hEDN, oEDN and mcEDN, and represented no more than 20% of the experimentally determined initial rates for mEDN, omEDN and the lower-activity chimeras. All double-reciprocal plots were constructed from appropriately corrected initial rates. All time points represent the average of triplicate samples. Calculations included the following approximations: the average molecular weight (M_r) of tRNA as M_r = 28 100 (75–90 ribonucleotides/tRNA molecule × M_r = 341/ribonucleotide), with A_{260} of 1.0 corresponding to 40 μg of RNA (16). Bestfits and correlation coefficients (r^2) were determined with the assistance of Cricket Graph software on-line at the National Institutes of Health.

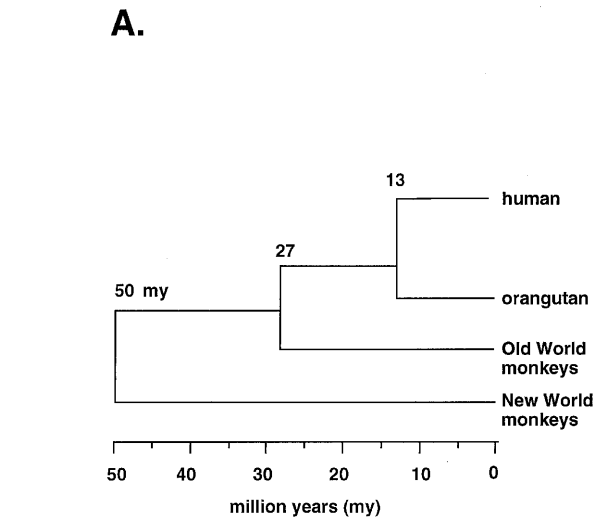
RESULTS

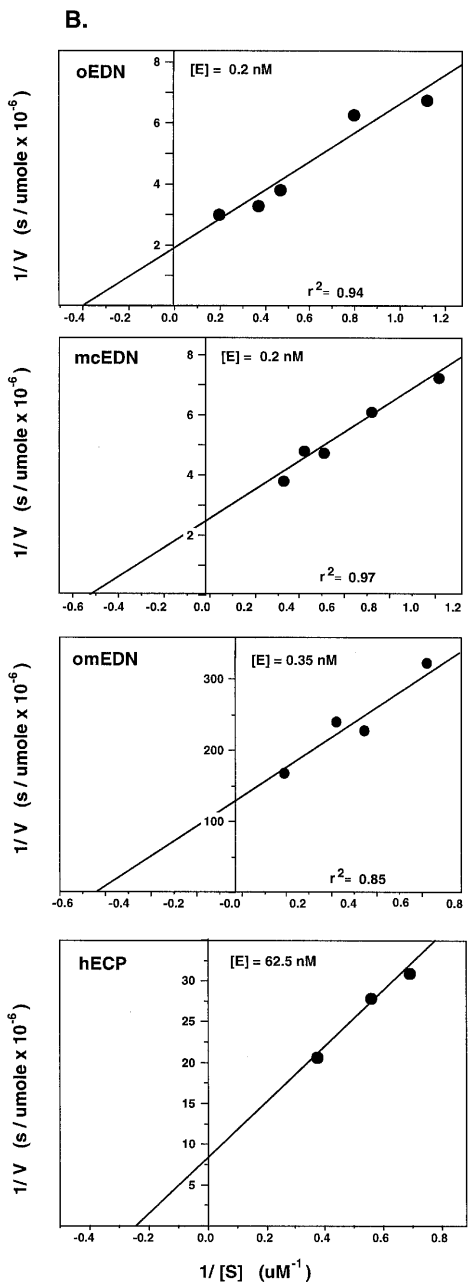
Ribonuclease activity of primate EDNs

The estimated evolutionary distances between the human and non-human primate species discussed in this work are shown in Figure 1A (13,17). The double reciprocal plots shown in Figure 1B demonstrate the relationship between substrate concentration and ribonuclease activity for recombinant hECP and EDNs prepared from several non-human primate sequences: orangutan (*Pongo pygmaeus*, oEDN), the Old World monkey, macaque (*Macaca fascicularis*, mcEDN), and the New World monkey, owl monkey (*Aotus trivirgatus*, omEDN). The values for K_m (μM), as determined from the x-intercepts, and k_{cat} (s^{-1}), as determined from the y-intercepts of these plots, are tabulated in Figure 1C and compared to those obtained previously for both human (*Homo sapiens*, hEDN)- and New World monkey (*S.oedipus*, mEDN)-derived recombinant proteins (13). Although the amino acid sequences of both oEDN and mcEDN differ significantly from that of hEDN (6.8 and 16.5% divergence, respectively), the catalytic constants and the calculated catalytic efficiency (k_{cat}/K_m) remain unchanged; the catalytic efficiences of recombinant hEDN, oEDN and mcEDN are all on the order of 10^6 M^{-1} s^{-1}. In contrast, the catalytic constants determined for the two EDNs isolated from New World monkeys (mEDN and omEDN) show significant reductions in k_{cat}, and thus reductions in overall catalytic efficiencies (~100-fold), with k_{cat}/K_m calculated for each at 0.96×10^4 M^{-1} s^{-1} and 1.2×10^4 M^{-1} s^{-1} , respectively. The catalytic efficiency of recombinant hECP was even lower, with k_{cat}/K_m determined at 0.59×10^3 M^{-1} s^{-1} , ~2000-fold less activity than that observed for recombinant hEDN and ~20-fold less than for omEDN.

Human (hEDN) and owl monkey (omEDN) sequence chimeras

An alignment of the predicted amino acid sequences of hEDN and omEDN is shown in Figure 2A. The omEDN sequence retains the eight cysteines as well as the catalytic histidines and lysine that are characteristic of the RNase A gene family (18,19). Similarly, the omEDN sequence contains the 'CKXXNTF' motif (amino acids 37–44) also found to be invariant among these proteins (18–20). The calculated amino acid sequence divergence between hEDN and omEDN is 29.2% (Fig. 1C). The two sequences were found to be identical within the two bracketed areas shown, permitting construction of chimeras A and B (Fig. 2B). The ribonucleolytic activities of both chimera A (first part hEDN followed by second and third parts omEDN) and chimera B (first and second parts hEDN followed by third part omEDN) were determined as '+', reflecting their similarity to the lower level of activity observed for omEDN (and mEDN). These results suggested that a sequence element (or elements) present in the C-terminus of hEDN was necessary for full catalytic activity. There are only two regions of significant divergence within this final segment of EDN sequence: the gap in omEDN in place of Arg 117 of hEDN, and the penultimate Thr–Thr in omEDN in place of Arg 132–Ile 133 of hEDN (Fig. 1A). Interestingly, both oEDN and mcEDN are more closely related to hEDN at these sites; oEDN is identical to hEDN, and in mcEDN, a Val replaces Arg 117.





C.

isolate	% divergence (aa)	K_m (uM)	k_{cat} (s^{-1})	k_{cat} / K_m (M^{-1} s^{-1})
hEDN[a]	0	0.70	0.91	1.3×10^6
oEDN	6.8	2.5	2.6	1.0×10^6
mcEDN	16.5	1.8	2.0	1.1×10^6
mEDN[a]	26.8	0.67	0.0064	0.96×10^4
omEDN	29.2	2.0	0.023	1.2×10^4
hECP	33.9	4.1	0.0024	0.59×10^3

Figure 1. (A) Estimated evolutionary distances between human and non-human primates (13,15). **(B)** Ribonuclease activities (substrate versus initial rates of reaction) presented as double reciprocal plots. Recombinant ECP (hECP) was prepared from the isolated human sequence [GenBank accession number X15161 (15)], and recombinant EDNs were prepared from sequences isolated from orangutan (*P.pygmaeus*, oEDN), the Old World monkey, macaque (*M.fascicularis*, mcEDN) and the New World monkey, owl monkey (*A.trivirgatus*, omEDN) genomic DNA, with GenBank accession numbers U24104, U24099 and U88827, respectively. **(C)** Characteristics of recombinant hECP and EDNs as listed in (B) as well as from human (*H.sapiens*, hEDN) and another New World monkey (*S.oedipus*, mEDN). Percent amino acid divergence for all but omEDN were as reported in reference 13. Values for K_m (μM) and k_{cat} (s^{-1}) were calculated from the data shown in (B) for oEDN, mcEDN and omEDN; [a]those for hEDN and mEDN were taken from reference 13.

C-terminal chimeras

As shown in Figure 3A, chimera C was created by exchanging the C-terminal Arg–Ile–Ile (132–134) of hEDN with Thr–Thr–Ile from omEDN, and chimera D, by exchanging the Thr–Thr–Ile of omEDN for Arg–Ile–Ile. Double reciprocal plots of substrate concentration versus initial rates yielded the catalytic constants listed in Figure 3B. Comparison of the values calculated for k_{cat}/K_m for hEDN (1.3×10^6 M^{-1} s^{-1}) and chimera C (1.6×10^5 M^{-1} s^{-1}) demonstrates that the C-terminal sequence exchange resulted in an ~8-fold reduction in catalytic activity. In contrast, k_{cat}/K_m determined for chimera D (1.3×10^4 M^{-1} s^{-1}) does not differ significantly from that determined for wild type omEDN. Taken together, these results indicate that the C-terminal sequence Arg 132–Ile 133 is necessary to sustain the full catalytic activity of hEDN, but at the same time it has no effect on the relatively inactive omEDN.

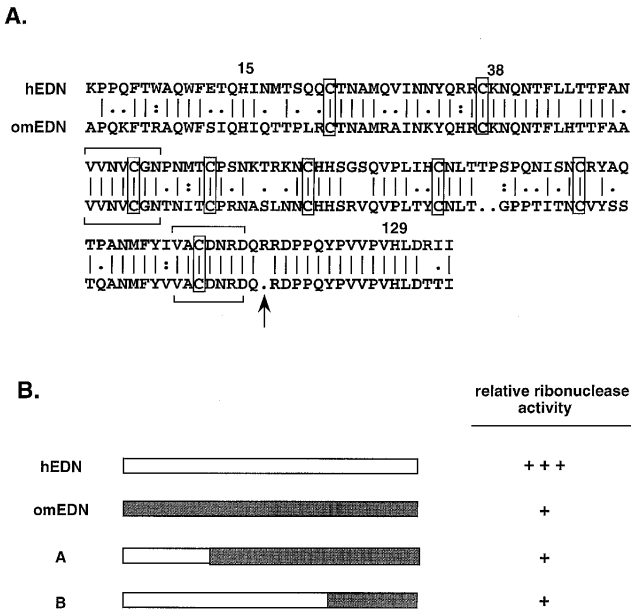


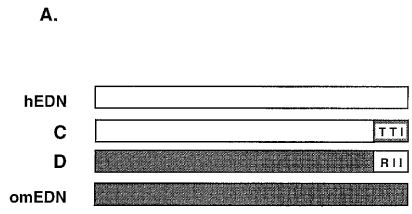
Figure 2. (A) Amino acid sequence comparison between human EDN and owl monkey EDN as predicted from their respective cDNA sequences. Boxes enclose each of the eight cysteines conserved in this gene family; numbering above the sequence denotes the position of the conserved catalytic residues His 15, Lys 38 and His 129, numbered as per hEDN sequence. The brackets indicate the division points within chimeras A and B, as shown in (B). Arrow indicates the gap in the omEDN sequence at position 117 (see Discussion). **(B)** Diagram of hEDN (open rectangle), omEDN (shaded rectangle) and chimeras A and B with portions of both sequences. The relative ribonuclease activities of the recombinant EDNs and chimeras are shown at the right.

DISCUSSION

In this work, we have compared the ribonuclease activity of five evolutionary variants of EDN, representing one branch of the ribonuclease A gene family (18). As we have reported previously, the EDN gene has an interesting structural and functional evolutionary history (12,13), with EDN and ECP emerging as distinct genes relatively recently, sometime after the divergence of the Old World from the New World monkeys (50 million years). The single EDN/ECP sequence found in the New World monkey, *S.oedipus* (mEDN), showed greater similarity to the EDN branch, in both primary structure (26.8% divergence from hEDN, 31.3% divergence from hECP) and isoelectric point (calculated pI = 8.25, comparable to all the other primate EDNs, with pIs ranging from 8.43 to 9.17, rather than primate ECPs, with pIs from 10.70 to 11.37). The additional single New World monkey sequence reported in this paper (omEDN), isolated from genomic DNA from *A.trivirgatus*, translates to a peptide sequence that is 90.8% identical to that from *S.oedipus* (with 29.2% divergence from hEDN and 31.5% divergence from hECP) with similar calculated isoelectric point (pI = 8.86). Moreover, we determined that recombinant EDN derived from *S.oedipus* sequence (mEDN) had only a fraction of the ribonuclease activity demonstrated by its human ortholog despite the presence of structural and catalytic elements known to be crucial to this function (13). Here we report that recombinant EDNs derived from orangutan (oEDN) and macaque (mcEDN) sequences retained full catalytic efficiency against the tRNA substrate used in these experiments, while recombinant EDN derived from *A.trivirgatus* was again ~100-fold less active. The relative ribonucleolytic activities of chimeras prepared from selected segments of hEDN and omEDN pointed to a C-terminal segment as crucial to the elevated catalytic rate, and further analysis identified the penultimate amino acids in hEDN, Arg 132–Ile 133 as necessary (but notably, not sufficient) for enhanced activity.

It is not immediately clear why the Arg–Ile pair in this position should play such a crucial role in mediating catalytic activity. In the crystal structure of human EDN recently reported by Mosimann and colleagues (21), Arg 132 and Ile 133 appear on the outer surface of the protein, within the sixth β-strand. They do not appear to interact directly with the active site residues (His 15, His 129 and Lys 38), nor are they included among the reported intermolecular contact points. Interestingly, Beintema (22) predicted the importance of a cationic residue in this position, suggesting that it might form electrostatic contacts with the negatively charged phosphate group situated immediately 5′ to the phosphodiester bond of the substrate undergoing ribonucleolytic cleavage; as such, Arg 132 would perform a function similar to that of Lys 66 in the p^0 subsite of bovine pancreatic ribonuclease (RNase A) (23). Consistent with this hypothesis, we have shown that exchanging the Arg 132–Ile 133 with Thr–Thr results in a significant reduction in the ribonucleolytic activity of recombinant hEDN. However, the reverse exchange, Arg 132–Ile 133 in place of the Thr–Thr, does nothing to enhance the relatively low catalytic activity of omEDN, indicating that Arg 132, while necessary, is not sufficient, and cannot act alone to augment the ribonucleolytic activity.

Another point to consider when evaluating the differential activities of the EDNs and hECP is the potential role played by the gap present in the hECP, mEDN and omEDN at position 117 relative to the hEDN sequence (Fig. 2A). Mosimann and colleagues (21) have shown that the region from Asp 115 to Arg 118 forms a helical loop that makes contact with both local (Asp 115 O



B.

	K_m (uM)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1}s^{-1}$)
hEDN	0.70	0.91	1.3×10^6
C	3.3	0.54	1.6×10^5
D	5.0	0.065	1.3×10^4
omEDN	2.0	0.023	1.2×10^4

Figure 3. (A) Diagram of chimeras. The three C-terminal amino acids of omEDN (Thr–Thr–Ile) replace those of hEDN (Arg–Ile–Ile) in chimera C, and the reverse in chimera D. **(B)** Values for K_m and k_{cat} were calculated from the intercepts of double reciprocal plots of substrate concentration versus initial rates for chimeras C and D; values for hEDN from reference 13 and omEDN, from Figure 1 were included for comparison.

hydrogen bonding to Asp 119 N) and distant (Arg 118 at 2.97 Å from Pro 3) sites within the protein. A gap within this segment could disrupt this helix, disturbing the aforementioned contact points and conceivably displacing the distal His 129 from its site within the catalytic crevice. Confirmation of this hypothesis awaits crystallographic analysis of hECP and/or the New World monkey EDN homologs.

The activity measured in the assay used here rests on the ability of each ribonuclease to create small, acid-soluble fragments from an acid-insoluble yeast tRNA polymer at pH 7.0. One limitation inherent in this assay is that variations in the way in which a ribonuclease processes substrate—removal of single, acid soluble nucleotides from one end, as opposed to creation of internal cuts, yielding larger, not necessarily soluble polymers—will be read inappropriately as differential activity. In addition, it is also important to recognize that we have no clear sense of the true physiologic substrates of these ribonucleases, nor do we understand the precise conditions under which these proteins work.

EDN is one of six human members of the RNase A family that have been characterized to date, and one of two ribonucleases present in large quantities in the human eosinophilic leukocyte. Although other members of this family have been characterized as agents of host defense (24,25) and of angiogenesis (26,27), the physiologic function of EDN remains unknown. Although EDN is among the most enzymatically active of the human ribonucleases (8), the overall biologic signficance of this observation awaits a clearer understanding of the role of EDN and its ribonuclease activity in eosinophil-mediated activites and eosinophil-related diseases.

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