Protein Chemistry and Structure:

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Eosinophil Cationic Protein and Eosinophil-derived Neurotoxin

EVOLUTION OF NOVEL FUNCTION IN A PRIMATE RIBONUCLEASE GENE FAMILY*

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Human eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are members of a unique subfamily of rapidly evolving primate ribonuclease genes that emerged via a gene duplication event occurring after the divergence of Old World from New World monkeys (Rosenberg, H. F., Dyer, K. D., Tiffany, H. L., and Gonzalez, M. (1995) *Nature Genet.* 10, 219–223). In this work, we studied the activity of the protein encoded by the EDN/ECP homolog of the New World monkey, *Saguinus oedipus* (marmoset), a representative of the "ancestral" single sequences. Although the nucleotide sequence of the single marmoset gene (mEDN) was equally homologous (82%) to both human genes, the encoded amino acid sequence, calculated isoelectric point, and immunoreactivity all suggested a closer relationship with EDN. Furthermore, mEDN (at 0.3–1.0 μM concentrations) had no measurable anti-staphylococcal activity, suggesting functional as well as structural similarity to EDN. However, with yeast tRNA as substrate, mEDN had significantly less ribonuclease activity than EDN; Michaelis constants were nearly identical (K_m (mEDN) = 0.67 μM; K_m (EDN) = 0.70 μM), while turnover numbers differed by a factor of 100 (k_{cat} (mEDN) = 0.91 s^{-1}; k_{cat} (EDN) = 0.64 × 10^{-2} s^{-1}). Thus, evolutionary constraints appear to have promoted two novel functions: increased cationicity/toxicity (ECP) and enhanced ribonuclease activity (EDN). The latter result is particularly intriguing, as it suggests a crucial role for ribonuclease activity in the (as yet to be determined) physiologic function of EDN.

Eosinophil-derived neurotoxin (EDN)[1] and eosinophil cationic protein (ECP) are small (15–16 kDa) cationic proteins found in the large specific granules of human eosinophilic leukocytes (1, 2). ECP has been characterized as a cytotoxin, helminthotoxin, and bacterial toxin with ribonuclease activity that appears to be unrelated to toxicity (3, 4). In contrast, EDN has ribonuclease (5, 6) and neurotoxic activity (7, 8), but no known physiologic function. The genes encoding EDN and ECP are striking in their similarity to one another. The two coding sequences are 85% identical, encoding polypeptides with structural and catalytic residues that are analogous to those of other members of the mammalian ribonuclease gene family (9–12). The genomic organization of EDN is likewise identical to that of ECP, and both genes have been mapped to indistinguishable loci on chromosome 14 (14q24q31) (13).

We have recently traced the rapid molecular evolution of the EDN/ECP gene family (14). While separate genes encoding EDN and ECP were found in Great Apes and Old World monkeys, only a single ECP/EDN sequence was detected in the more distant New World monkeys. These results suggested that ECP and EDN emerged as distinct sequences as a results of a gene duplication event that occurred after the divergence of the New World and Old World monkeys (Fig. 1). A representative of this single EDN/ECP sequence was isolated from the marmoset, *Saguinus oedipus* (New World monkey); its nucleotide sequence was found to be equally homologous to both human EDN and ECP (82% identity), and it encoded a polypeptide with the structural and catalytic residues analogous to those of other mammalian ribonucleases.

From calculations based on the sequences of these and other primate homologs, we demonstrated that the genes encoding EDN and ECP have accumulated non-silent mutations at rates exceeding those of all other functional coding sequences studied in primates (14). The results of this analysis suggested that EDN and ECP may be responding to unusual evolutionary constraints. In the work presented here, we have used the single marmoset EDN/ECP as the basis for the study of the functional evolution of EDN and ECP, in hopes of learning more about the nature of these constraints.

EXPERIMENTAL PROCEDURES

Preparation of Plasmid Constructs—The portion of the open reading frame encoding the mature EDN polypeptide (base pairs 127–528) (9) was PCR-amplified from cDNA and ligated in frame into the *Hin*dIII and *Eco*RI sites of the pFCTS bacterial expression vector (International Biotechnologies, Inc., New Haven, CT) to create hEDNS#1 (Fig. 2, *panel A*). Features of this vector include a tac promoter, amino-terminal bacterial secretion piece, and carboxyl-terminal FLAG polypeptide (DYKDDDK) detected by the M2 murine monoclonal antibody (IBI). The open reading frame encoding the mature ECP polypeptide (base pairs 136–534) (10) was amplified and subcloned as described above to create hECPS#12. The complete coding sequence of marmoset EDN was isolated directly from genomic DNA purified from an Epstein-Barr virus-transformed leukocyte cell line from the marmoset *S. oedipus* (ATCC no. CRL-1612, Rockville, MD) using primers as described elsewhere (14) (sequence identical to the marmoset isolate described in Rosenberg *et al.* (14); GenBank™ accession no. U24099); the original isolate was reamplified with compatible primers to facilitate insertion into the pFCTS vector, creating mEDNS#7. The mutant EDN construct encodes a single base pair mutation ($A^{239} \rightarrow G$) introduced by overlapping PCR mutagenesis (15); this results in the conversion of nucleophilic lysine (Lys^{38}) to arginine (Arg) (hEDNSΔLYS#6). All four constructs were confirmed by dideoxy DNA sequencing (U. S. Biochemical Corp., Cleveland, OH).

SDS-PAGE and Western Blotting—The samples to be described were subjected to gel electrophoresis in 14% Tris glycine gels (Novel Experimental Technologies, San Diego, CA). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with antibodies as per published procedures (16). Briefly, nonspecific binding was blocked with 5% non-fat dry milk (Safeway, Oakland, CA) in T-TBS

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[1] The abbreviations used are: EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; IPTG, isopropyl-1-thio-β-galactopyranoside; mEDN, marmoset EDN; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

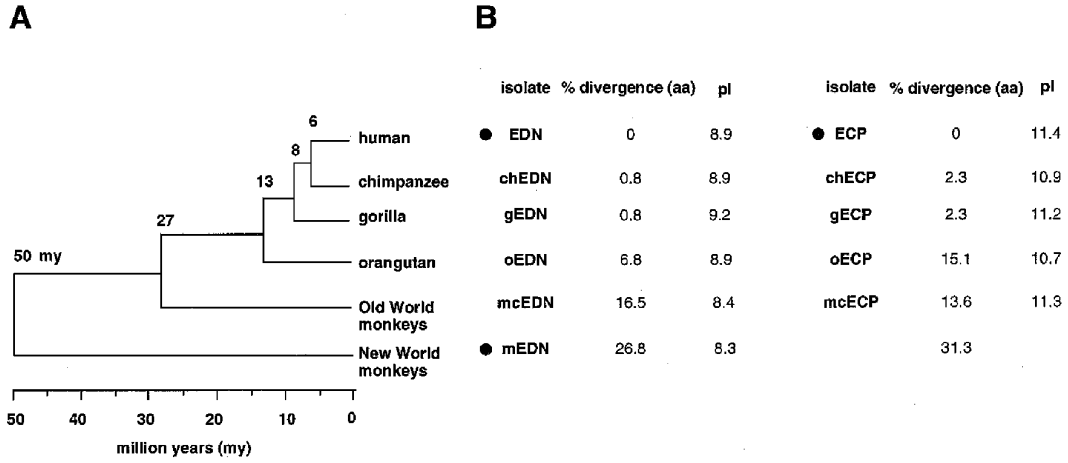

FIG. 1. *A*, estimated evolutionary distances between human and non-human primates (32, 33). *B*, calculated amino acid sequence divergences and isoelectric points of the individual EDN and ECP isolates are as described in Rosenberg *et al.* (14); *filled circles* designate the sequences evaluated in this work.

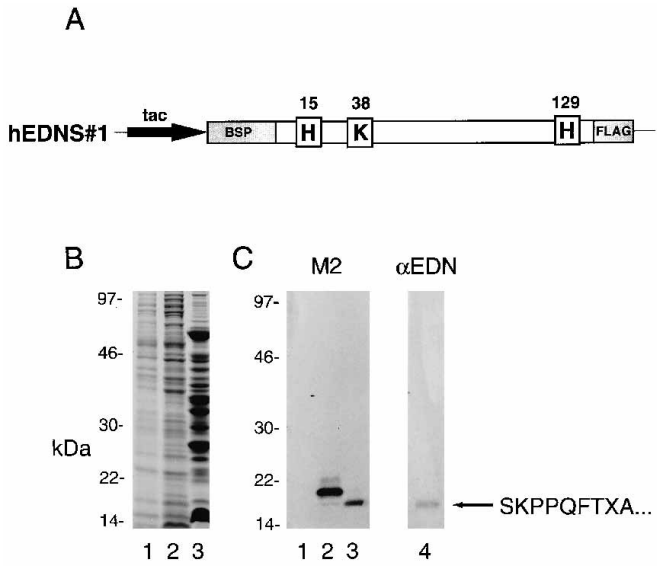

FIG. 2. *A*, schematic depicting the human EDN coding sequence in the prokaryotic expression vector, pFCTS. Features include the tac promoter, bacterial secretion piece (*BSP*), and the FLAG octapeptide (DYKDDDDK) detected by the M2 mAb. Conserved ribonuclease catalytic residues are highlighted in *boxes* with position noted above. *B*, Coomassie Blue-stained gel containing total bacterial contents before (*lane 1*) and 30 min after (*lane 2*) addition of IPTG. Periplasmic proteins isolated by heparin-Sepharose chromatography are shown in *lane 3*. *C*, Western blot of *lanes 1–3* described in *Panel B*, probed with the M2 mAb. *Lane 4* contains the periplasmic isolate shown in *lane 3*, probed with polyclonal rabbit anti-EDN antiserum. Amino-terminal sequence of this protein (at *arrow*) is as indicated. The first serine residue (*S*) remains after cleavage of the secretion piece; the remaining residues are those encoded by the EDN cDNA (*KPPQFTWA. . .*) (9, 11).

(50 mM Tris, pH 8.0, 150 mM sodium chloride, 0.05% Tween 20). The M2 anti-FLAG monoclonal antibody was used at a 1:200 dilution (14.5 μg/ml) in T-TBS with 1% gelatin. Polyclonal anti-EDN and anti-ECP antisera (preadsorbed to eliminate cross-reactivity) (17) were used at 1:300 dilutions in T-TBS with gelatin. Secondary antibodies included 1:1000 dilutions of alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgGs (Bio-Rad). Blots were developed in TM buffer (200 mM Tris, pH 9.5, with 10 mM $MgSO_4$) with 300 μg/ml nitro blue tetrazolium and 100 μg/ml 50-bromo-4-chloro-3-indolyl-phosphate (Bio-Rad).

Protein Preparations—One-ml overnight cultures of bacterial transfectants grown in LB broth with 50–100 μg/ml ampicillin were used to inoculate into 500-1500 ml of Super Broth (Biofluids, Inc., Rockville, MD) with 50–100 μg/ml ampicillin which were then grown overnight at 37 °C. Overnight cultures were diluted 1:1 with fresh Super Broth + ampicillin, and permitted to grow for an additional 30 min. Isopropyl-1-thio-β-galactopyranoside (IPTG) (Boehringer Mannheim) was added to a final concentration of 0.5–1.0 mM, and bacteria were harvested by centrifugation after a 30 min (ECP) or 2 h (EDN, EDNΔLys[38], and mEDN) induction period. SDS-PAGE and Western blot analysis of proteins were performed on bacteria harvested from 0.5-ml cultures resuspended directly in 2 × reducing sample buffer (16).

Periplasmic Isolates—The bacteria harvested as described above were washed twice with 10 mM Tris, pH 8.0, at room temperature, and once with 0.5 M sucrose in 30 mM Tris, pH 8.0 and 1 mM EDTA. The sucrose-washed pellet was resuspended in ice-cold distilled water (20 ml/liter bacteria), and the cellular debris removed by high speed centrifugation. Tris, pH 8.0, and sodium azide were added to the supernatants to final concentrations of 10 mM and 0.1%, respectively. Ten mg of heparin-Sepharose CL-6B (Pharmacia Biotech, Inc.) were added to the buffered isolate, followed by equilibration end-over-end overnight at 4 °C. The equilibrated resin was washed with 300 volumes of 10 mM Tris, pH 8.0, and the bound proteins were eluted either directly into 2 × reducing sample buffer for SDS-PAGE analysis, or into 0.3 ml of 10 mM sodium phosphate, pH 7.5, with 500 mM sodium chloride for ribonuclease assay. The concentration of periplasmic proteins eluted into the phosphate buffer was determined by BCA protein assay (Pierce, Rockford, IL) with spectrophotometric comparison (562 nm) to bovine serum albumin standards. Quantitation was confirmed by SDS-PAGE followed by Coomassie Blue staining. Quantitation of recombinant protein within an individual isolate was performed by comparison of serial dilutions of the periplasmic isolates to serial dilutions of a FLAG-containing protein standard of known molarity (recombinant FLAG-conjugated bacterial alkaline phosphatase) (IBI) on Western blots probed with the M2 anti-FLAG mAb. Control isolates (containing no recombinant protein) were prepared from IPTG-induced cultures of bacteria transfected with the pFCTS vector alone.

Isolation of Recombinant Protein on M2 Anti-FLAG Resin and Amino-terminal Sequencing—Phosphate-buffered saline and sodium azide were added to the supernatant recovered after resuspension in ice-cold distilled water and centrifugation as described above, to final concentrations of 1 × and 0.1%, respectively. M2 anti-FLAG-conjugated resin suspension (0.3 ml; IBI) was added to the supernatant, which was equilibrated end-over-end overnight at 4 °C. Resin was washed in 300 volumes of phosphate-buffered saline, and bound protein was eluted directly into 0.4 ml of 100 mM glycine, pH 3.0, and neutralized immediately with 50 μl of 2 M Tris, pH 7.5. For functional assays (ribonuclease, toxicity), the concentration of recombinant protein was determined by comparison to serial dilutions of a known concentration of FLAG-conjugated standard as described above; for amino-terminal sequencing, the sample was concentrated, subjected to SDS-PAGE, and transferred to an Immobilon P membrane as per the manufacturer's instructions (Millipore Corporation, Bedford, MA). The ~15.5-kDa band located by Coomassie Blue staining was cut from the membrane for determination of the amino-terminal sequence (performed at National Biological Resources Branch, NIAID, National Institutes of Health).

Ribonuclease Activity—The assay used was adapted from the procedure described by Slifman *et al.* (5) as described previously (4). Determination of ribonuclease activities of recombinant proteins in periplasmic isolates (Fig. 3) proceeded as follows: 5 μl (20 μg) of a 4 mg/ml

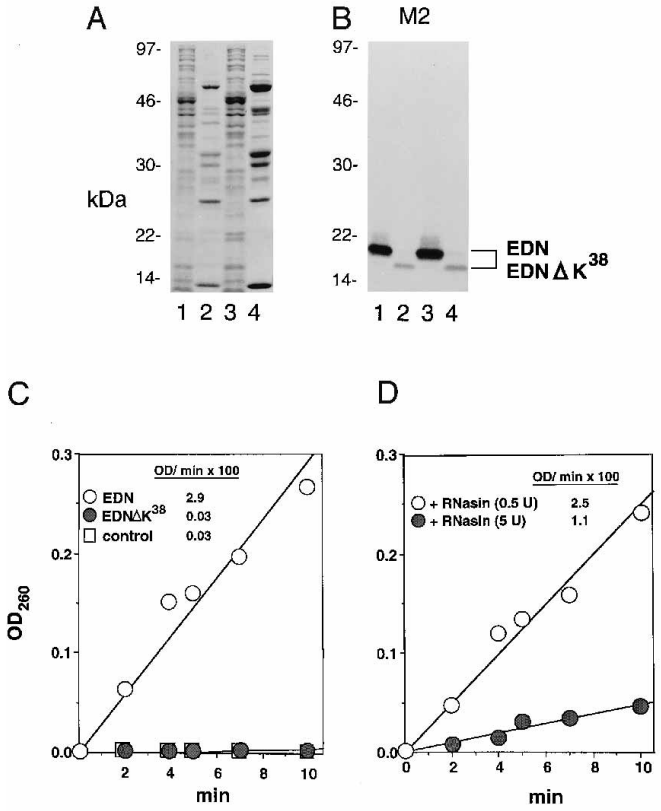

FIG. 3. *A*, Coomassie Blue-stained gel and *B*, Western blot probed with the M2 mAb containing total cell extracts (*lanes 1* and *3*) and periplasmic isolates (*lanes 2* and *4*) of IPTG-induced bacteria transfectants. *Lanes 1* and *2* contain wild type recombinant EDN (before and after removal of the secretion piece, respectively); *lanes 3* and *4*, recombinant EDN with a single base pair mutation ($A^{239} \rightarrow G$) converting $Lys^{38} \rightarrow Arg$ (before and after removal of the secretion piece, respectively). *C*, ribonuclease activity of 500 ng of periplasmic proteins containing recombinant EDN (*open circles*), containing mutant $EDN\Delta K_{38}$ (*filled circles*), and without recombinant protein (*open squares*). Initial rates (OD/min) are shown in the *inset*. *D*, ribonuclease activity of recombinant EDN preparation shown in *A* in the presence of 0.5 unit (0.0625 unit/ml; *open circles*) and 5 units (0.625 unit/ml; *filled circles*) of human placental ribonuclease inhibitor (*RNasin*). Initial rates (*OD/min*) are shown in the *inset*. Each time point represents the average of duplicate samples.

solution of yeast tRNA (catalog no. R-9001; Sigma) was added to 0.8 ml of 40 mM sodium phosphate, pH 7.0, containing 500 ng of periplasmic proteins eluted from heparin-Sepharose as described. At the given time points, the reaction was stopped by addition of 0.5 ml of an ice-cold fresh solution of 20 mM lanthanum nitrate with 3% perchloric acid. The $t = 0$ control was prepared by addition of stop solution to the phosphate and protein-containing reaction mixture prior to the addition of yeast tRNA. Stopped reactions were held on ice for 15 min, and insoluble tRNA was removed by centrifugation for 5–10 min at 12,000 × *g*. The amount of solubilized RNA was determined from the ultraviolet absorbance at 260 nm (A_{260}) of the supernatant fraction, with the $t = 0$ control used as the $A_{260} = 0.00$ standard (blank). 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 (18).

Determination of ribonuclease activities of recombinant proteins isolated by M2 anti-FLAG affinity chromatography proceeded as follows. Reactions as described above were carried out with varying concentrations (0.89–7.1 μM) of yeast tRNA added in separate reactions to 0.8 ml of 40 mM sodium phosphate, pH 7.0, containing either 0.3 pmol of recombinant EDN or 3.6 pmol of recombinant mEDN isolated on the M2 affinity resin as described. Equivalent volumes of sham isolations (M2-resin equilibration and glycine elution of periplasmic proteins isolated from equivalent volumes of pFCTS vector-alone bacterial transfectants) had levels of ribonuclease activity that were insignificant compared to human EDN and represented no more than 15–20% of the experimentally determined initial rates for mEDN; data presented were determined for appropriately corrected initial rates. Assay and calculations were as described above. Michaelis constants (K_m (M)) and turnover numbers (k_{cat} (s^{-1})) were determined from the appropriate intercepts of double reciprocal (Lineweaver-Burk) plots as shown.

RESULTS

Production and Secretion of Recombinant Human EDN—The coding sequence of the mature protein (without signal sequence) in the pFCTS bacterial expression vector is shown in Fig. 2, *panel A*, inserted in-frame with the vector-encoded amino-terminal OmpA bacterial secretion piece and carboxyl-terminal octapeptide (FLAG) recognized by the M2 murine mAb. In *Panel C*, a Western blot probed with the anti-FLAG M2 mAb demonstrates the absence of immunoreactive protein in total bacterial extracts prepared before the addition of IPTG (*lane 1*), and its presence 30 min after the addition of IPTG (*lane 2*). The predominant immunoreactive band in *lane 2* migrates with a molecular mass ~18 kDa, which is consistent with the size of EDN (15.5 kDa) with an intact secretion piece (2.5 kDa). Immunoreactive protein was also detected in the periplasmic protein isolate (prepared by osmotic shock followed by binding to heparin-Sepharose at pH 8.0, as described under "Experimental Procedures") shown in *lane 3*. Immunoreactive protein detected in the periplasm migrated with a molecular mass of ~15.5 kDa, suggesting cleavage of the 2.5-kDa bacterial secretion piece in conjunction with translocation of the recombinant protein to the periplasm. The 15.5-kDa protein was also detected by polyclonal anti-EDN antiserum (*lane 4*); amino-terminal sequencing confirmed the identity of this protein as recombinant EDN.

Ribonuclease Activity of Recombinant EDN—The Western blot probed with the M2 mAb (Fig. 3, *Panel B*) demonstrates that the production and secretion of the $Lys^{38} \rightarrow Arg$ mutant form of EDN ($EDN\Delta Lys^{38}$, *lanes 3* and *4*) proceeds in a manner identical to that of the wild type (*lanes 1* and *2*). The data in *Panel C* indicate that periplasmic isolate (500 ng) containing recombinant EDN has significant ribonuclease activity (100-fold) over the control (500 ng of periplasmic proteins isolated from an IPTG-induced vector-alone transfected culture). The conversion of the conserved $Lys^{38} \rightarrow Arg$ reduces the ribonuclease activity of recombinant EDN to control level, indicating that this residue is crucial for ribonuclease activity. The analogous lysine, Lys^{41} is the active-site nucleophile in RNA hydrolysis catalyzed by the prototype of this gene family, bovine ribonuclease A (19).

In *Panel D*, the effects of addition of human placental ribonuclease inhibitor (RNasin) on EDN-catalyzed RNA hydrolysis are examined. The results demonstrate a reduction in initial rate of reaction in proportion to the concentration of RNasin added, from 2.9 to 2.5 OD/min (0.067–0.058 nmol/min) with 0.0625 unit/ml RNasin, and to 1.1 OD/min (0.025 nmol/min) with 0.625 unit/ml RNasin.

Characterization of mEDN—In Fig. 4, *Panel A*, the amino acid sequence encoded by the EDN/ECP homolog of *S. oedipus* (marmoset) is compared to the sequences of human EDN and ECP. Although the nucleotide sequence of the marmoset gene is equally homologous (82.3%) to both human genes, the homologies of the encoded amino acid sequence (73.2% to EDN, 68.7% to ECP) and the calculated isoelectric point (pI = 8.3) suggest greater similarity to human EDN. As such, this EDN/ECP homolog has been designated marmoset EDN (mEDN).

In *Panels B*, *C*, and *D*, the cross-reactivity of recombinant mEDN is examined. *Panel B* shows a Western blot containing total cell extracts from IPTG-induced transfectants probed with the M2 mAb which detects all three recombinant proteins (EDN, ECP, and mEDN in *lanes 1* through *3*, respectively). Although the calculated molecular mass of ECP (15.6 kDa) is

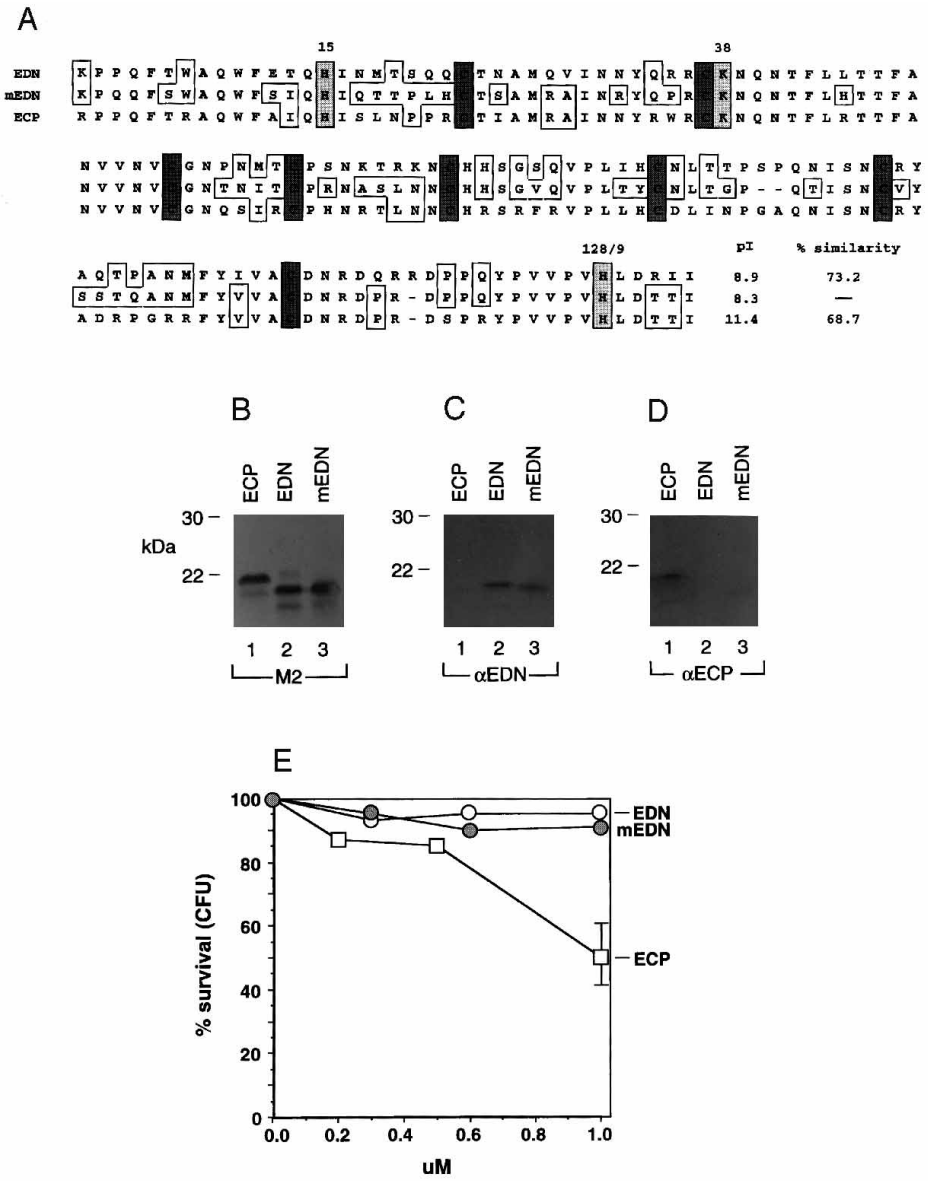

FIG. 4. *A*, amino acid sequence comparisons of human EDN, mEDN, and human ECP. *Lightly shaded boxes* enclose the conserved catalytic residues (His[15], Lys[38], His[128]-His[129]); *deeply shaded boxes* enclose the conserved cysteines. *Open rectangles* denote residues shared by EDN and mEDN but not by ECP and residues shared by ECP and mEDN but not by EDN; the *open squares* enclose those residues unique to mEDN. The percentage similarity of mEDN to both EDN and ECP, as well as the calculated isoelectric point of each sequence are listed in the final columns. *B*, Western blot containing total cell extracts of IPTG-induced bacterial transfectants probed with M2 mAb. *Lane 1*, recombinant ECP; *lane 2*, recombinant EDN; *lane 3*, recombinant mEDN. *C* and *D* are identical blots probed with polyclonal anti-EDN and anti-ECP antisera, respectively. *E*, percentage of colony-forming units of *S. aureus* surviving after 4 h incubation at 37 °C with recombinant EDN (*open circles*), recombinant mEDN (*filled circles*), and recombinant ECP (*open squares*). Each point represents the average of triplicate samples; *error bars* as indicated.

not significantly higher than those of EDN and mEDN, its cationicity results in the observed reduced mobility by SDS-PAGE. *Panel C* shows the identical blot probed with polyclonal anti-EDN antiserum, and *Panel D*, the identical blot probed with anti-ECP antiserum. The anti-EDN antiserum readily detects both EDN and mEDN, but not ECP; the anti-ECP antiserum detects ECP and (trace) mEDN, but not EDN. Thus, as predicted by amino acid sequence homology, mEDN shows cross-reactivity with antisera directed against both human EDN and human ECP.

The toxicity of mEDN for *Staphylococcus aureus* was examined in Fig. 4, *Panel E*. We have shown previously that micromolar concentrations of both granule-derived and recombinant human ECP were toxic to *S. aureus* (strain 502A); in contrast, neither granule-derived nor recombinant human EDN had any measurable antibacterial activity (4). The results presented here with purified recombinant ECP and EDN replicate these findings. The identical experiments were performed with purified recombinant mEDN (0.3–1 μM); no antibacterial activity was observed.

Comparative Ribonuclease Activity—Ribonuclease activities of EDN and mEDN were determined by evaluating the generation of acid-soluble ribonucleotide per unit time from varying concentrations (0.89–7.1 μM) of yeast tRNA substrate. The Lineweaver-Burk double reciprocal plots (1/v *versus* 1/[S]) derived from initial rates of these reactions is shown in Fig. 5; the Michaelis constants (K_m), turnover numbers (k_{cat}), and specificity constants (k_{cat}/K_m) calculated from these data are shown in the *insets*. As anticipated from the observed conservation of both structural and catalytic residues, mEDN has measurable ribonuclease activity. Interestingly, the Michaelis constants (K_m) for these two ribonucleases are nearly identical (0.70 μM for EDN; 0.67 μM for mEDN). In contrast, the turnover numbers (k_{cat}), and therefore, the specificity constants (k_{cat}/K_m), differ dramatically, with EDN ($k_{cat}/K_m = 1.3 \times 10^6$ M^{-1} s^{-1}) nearly 100-fold more effective at catalyzing the hydrolysis of yeast tRNA than the marmoset homolog, mEDN ($k_{cat}/K_m = 0.9 \times 10^4$ M^{-1} s^{-1}).

DISCUSSION

When expressed with an amino-terminal secretion piece, recombinant EDN could be isolated in biologically active form directly from the bacterial periplasm. A similar approach was used previously to prepare recombinant ECP (4). Newton *et al.* (20) reported purification and resolubilization of recombinant EDN from bacterial inclusion bodies; the approach presented

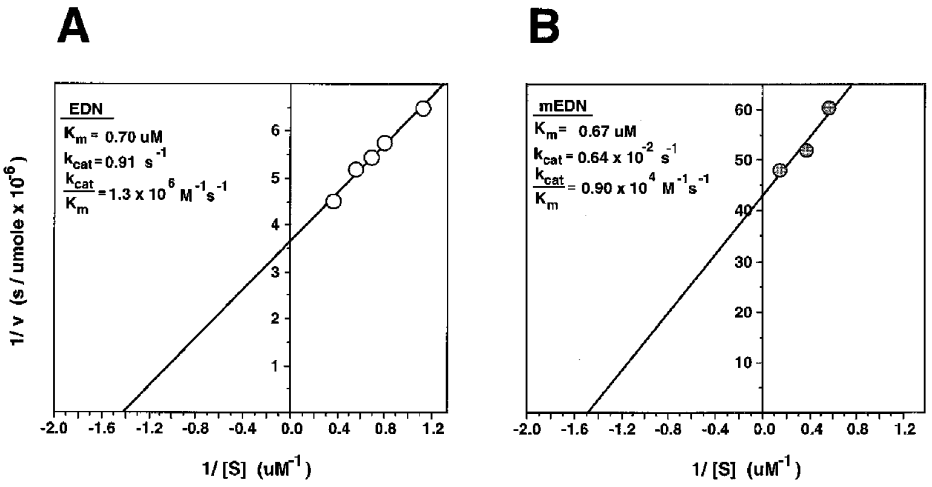

FIG. 5. Lineweaver-Burk plots (1/v *versus* 1/[S]) derived from initial rates of reactions containing *A*, 0.3 pmol of recombinant EDN and *B*, 3.6 pmol of recombinant mEDN isolated by M2 affinity chromotography with varying concentrations of a yeast tRNA substrate (0.89–7.1 μM). Values for K_m (μM), k_{cat} (s^{-1}), and K_m/k_{cat} ($M^{-1}\,s^{-1}$) for each enzyme are listed in the *insets*.

here results in the production of biologically active protein requiring no chemical refolding. As was found to be the case for recombinant ECP (4), the carboxyl-terminal FLAG peptide (DYKDDDK) aided in the detection of small amounts of protein and did not interfere with its transport, folding, or function.

We showed that Lys^{38} of recombinant EDN was functionally as well as structurally homologous to the active site nucleophile (Lys^{41}) of bovine RNase A, the prototype of the mammalian ribonuclease gene family. The conversion of $Lys^{38} \rightarrow Arg$ eliminated the ribonuclease activity of recombinant EDN; similar results were obtained previously with a $Lys^{38} \rightarrow Arg$ mutant of recombinant ECP (4). We also determined that the activity of recombinant EDN is reduced in a dose-dependent fashion in the presence of human placental ribonuclease inhibitor, analogous to results obtained with RNase A (19) as well as with other members of the mammalian ribonuclease gene family (21–23).

Using this expression system, we have prepared recombinant protein from a representative "ancestral" version of EDN. As shown in Fig. 1 (see also Ref. 14), the ECP/EDN gene pair originated from a gene duplication event that occurred after the divergence of the New World from the Old World monkeys. Although the single nucleotide sequence isolated from the marmoset (New World monkey) was found to be equally homologous to both human ECP and EDN (82%), the encoded amino acid sequence suggested a closer relationship with the latter protein. The immunoreactivity of the recombinant protein (mEDN) echoes this observation; while mEDN was detected readily by anti-EDN antiserum, it was only marginally detectable with anti-ECP. As such, we anticipated the functionality of mEDN to be more in line with that of EDN than of ECP.

As predicted, mEDN was similar to EDN in lacking antistaphylococcal activity at low micromolar concentrations. In an antibacterial assay initially described by Lehrer *et al.* (24), micromolar concentrations of granule-derived ECP were shown to be toxic to stationary phase cultures of staphylococci. Rosenberg (4) extended these findings, showing that granule-derived EDN was without effect at the same and at higher concentrations, and replicated the results with recombinant proteins. Although the mechanism by which ECP exerts its antibacterial (and other) toxicity has not been clarified, it has been proposed that its cationic residues might disrupt membrane phospholipid bilayers via a mechanism similar to that proposed for the cationic bee venom toxin, mellitin (10, 25, 26); similarly, Young *et al.* (27) have provided evidence suggesting pore formation. The benign natures of mEDN and EDN, both proteins with more neutral physiologic charges (pIs = 8.3 and 8.9, respectively) are consistent with these hypotheses. From an evolutionary perspective, these results, along with the structural data, suggest that the duplication and subsequent mutational events occurred under pressure to create a more cationic, more toxic protein, such as ECP.

With this in mind, we were quite surprised to find that mEDN was significantly less effective than EDN as a generalized ribonuclease. With yeast tRNA as substrate, the Michaelis constants (K_m) of the two proteins were about equal. The turnover numbers (k_{cat}), however, differed by a factor of 100, indicating that, mEDN is 100-fold less effective than EDN at converting a molecule of substrate present in the active site into product. From a structural point of view, it is not immediately clear why this should be the case, as both mEDN and EDN have the eight cysteine residues spaced appropriately for the formation of the four characteristic disulfide bonds, as well as the histidines and lysine analogous to those in the active site crevice of ribonuclease A (19); neither is mEDN excessively cationic, a feature which has been proposed as potentially damaging to the catalytic activity of ECP (10). A stepwise comparison of ribonuclease activity along evolutionarily informative pathways (28) is likely to provide information on the way in which this increase in observed ribonuclease activity was attained (gradually or at a result of a single transition), and will identify additional residue(s) crucial to this aspect of EDN's function. Interestingly, a similar study, focusing on pancreatic ribonucleases from the order Artiodactyla (cows, sheep, camels) was recently described by Jermann *et al.* (29); in this study, the conversion of a single residue ($Asp^{38} \rightarrow Gly$) that occurred in conjuction with the evolutionary emergence of the "true ruminants" resulted in a 5-fold enhancement of the hydrolysis of double-stranded RNA.

Thus, not only did the duplication and mutational events generating the EDN/ECP gene family yield a protein that was more cationic, and with greater antibacterial activity, they also yielded a protein with enhanced ribonuclease activity; evolutionary constraints appear to have favored the generation of not one, but two novel functions. Of the two, the latter function, enhanced ribonuclease activity, is more difficult to interpret. The physiologic function of EDN is not known; the role of ribonuclease activity is equally obscure. It is interesting to note, however, that the neurotoxicity of EDN (the induction of cerebellar dysfunction and Purkinje cell loss by introduction of EDN into the cerebrospinal fluid of rabbits, also known as the Gordon phenomenon) (7, 8) was shown to be blocked by ribonuclease inhibitors (30, 31). Although itself a nonphysiologic phenomenon, the observed dependence on ribonuclease activity suggests that a more careful analysis of the actions of EDN in the central nervous system may provide clues toward identify-

ing the true physiologic function of this protein.

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Additions and Corrections

Vol. 270 (1995) 21539–21544

Eosinophil cationic protein and eosinophil-derived neurotoxin. Evolution of novel function in a primate ribonuclease gene family.

Helene F. Rosenberg and Kimberly D. Dyer

Page 21542, Fig. 4: The reproduction of *panels B–D* was inadequate. An improved version is shown below with corrected figure legend.

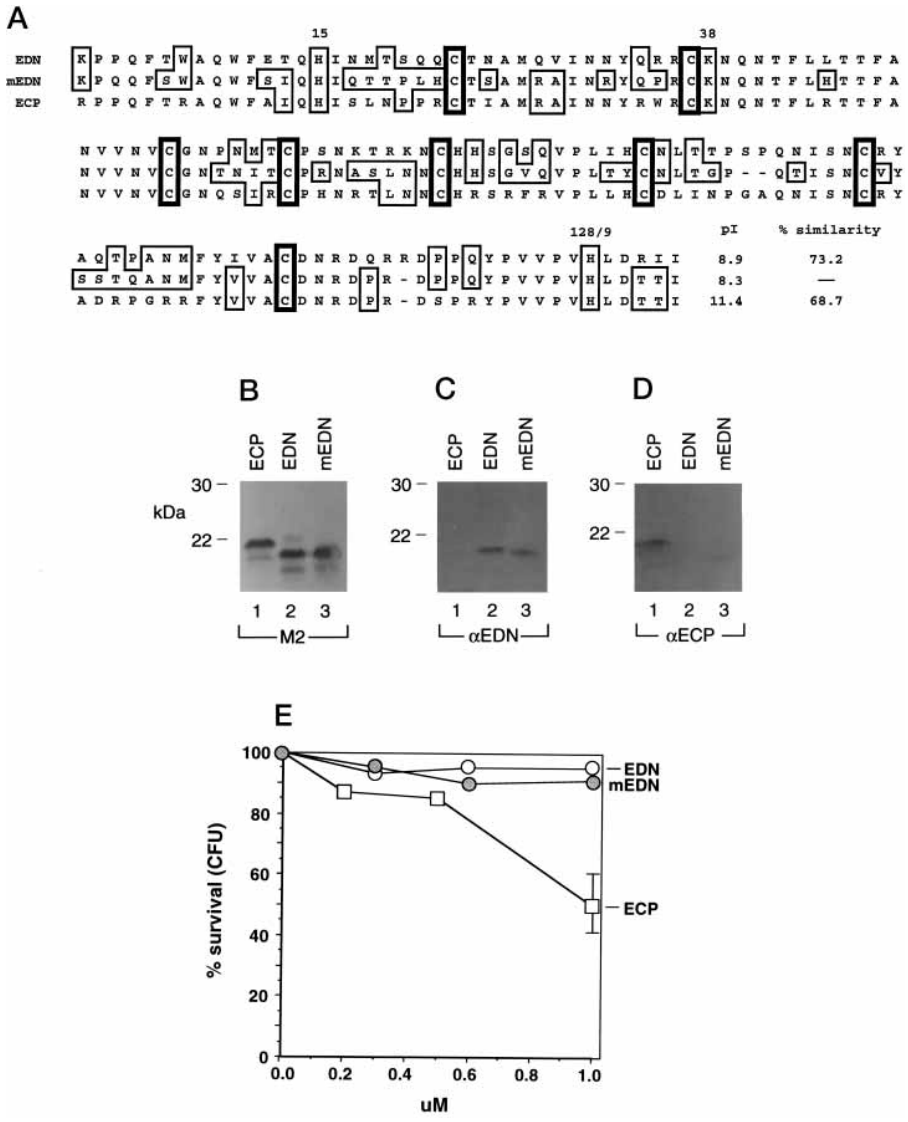

FIG. 4. *A*, amino acid sequence comparisons of human EDN, marmoset EDN (mEDN), and human ECP. *Numbered boxes* enclose the conserved catalytic residues (His[15], Lys[38], His[128/9]); *boxes* with *bold outline* enclose the conserved cysteines. *Open rectangles* denote residues shared by EDN and mEDN but not by ECP and residues shared by ECP and mEDN but not by EDN; *open squares* enclose those residues unique to mEDN. The percentage similarity of mEDN to both EDN and ECP, as well as the calculated isoelectric point of each sequence are listed in the final columns. *B*, Western blot containing total cell extracts of IPTG-induced bacterial transfectants probed with M2 mAb. *Lane 1*, recombinant ECP; *lane 2*, recombinant EDN; *lane 3*, recombinant mEDN. *C* and *D* are identical blots probed with polyclonal anti-EDN and anti-ECP antisera, respectively. *E*, percentage of CFUs of *S. aureus* surviving after a 4-h incubation at 37 °C with recombinant EDN (*open circles*), recombinant mEDN (*filled circles*), and recombinant ECP (*open squares*). Each point represents the average of triplicate samples; *error bars* as indicated.

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