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An Insertion in Loop L7 of Human Eosinophil-Derived Neurotoxin Is Crucial for its Antiviral Activity

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ABSTRACT

The human eosinophil granule ribonuclease, eosinophil-derived neurotoxin (EDN) has been shown to have antiviral activity against respiratory syncytial virus-B (RSV-B). Other closely related and more active RNases such as RNase A, onconase, and RNase k6 do not have any antiviral activity. A remarkable unique feature of EDN is a nine-residue insertion in its carboxy-terminal loop, L7 which is not present in RNase A, and differs in sequence from the corresponding loop in another eosinophil RNase, eosinophil cationic protein (ECP). ECP has a much lower antiviral activity as compared to EDN. The current study probed the role of loop L7 of EDN in its antiviral activity. Three residues in loop L7, Arg117, Pro120, and Gln122, which diverge between EDN, ECP, and RNase A, were mutated to alanine alone and in combination to generate single, double, and triple mutants. These mutants, despite having RNase activity had decreased antiviral activity towards RSV suggesting the involvement of loop L7 in the interaction of EDN with RSV. It appears that the mutations in loop L7 disrupt the interaction of protein with the viral capsid, thereby inhibiting its entry into the virions. The study demonstrates that besides the RNase activity, loop L7 is another important determinant for the antiviral activity of EDN. J. Cell. Biochem. 113: 3104–3112, 2012.

KEY WORDS: RIBONUCLEASE; EOSINOPHIL; ANTIVIRAL

Eosinophils play a crucial role in the pathophysiology of allergic respiratory diseases resulting mostly in detrimental effects; however, they have also emerged as the cells of host defense [Hogan et al., 2008]. Eosinophil granules contain a number of proteins including major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) [Gleich and Adolphson, 1986]. These granule proteins are the mediators of beneficial as well as detrimental functions of eosinophils [Rothenberg and Hogan, 2006]. Of these granule proteins, EDN and ECP possess ribonuclease activity and have been classified as the members of RNase A superfamily [Slifman et al., 1986]. The eosinophil ribonucleases, EDN and ECP manifest several special biological actions which include neurotoxicity, helminthotoxicity, antibacterial, and antiviral activity [Hamann et al., 1987; Sorrentino et al., 1992; Domachowske et al., 1998ab]. RNase A, a much more potent ribonuclease compared to EDN and ECP does not demonstrate neurotoxicity, helminthotoxicity, or antiviral activity [Hamann et al., 1987; Sorrentino et al., 1992; Domachowske et al., 1998bc]. A number of RNases of fungal, bacterial, plant, and animal origins are being investigated to explore their potential medicinal value in the treatment of cancer and AIDS as they demonstrate cytotoxicity to tumor cells and antiviral activity against HIV [Fang and Ng, 2011]. Among them, the most clinically promising RNases are mushroom RNases, Binase, and Barnase from bacteria, ginseng RNases, and Onconase from frog (*Rana pipiens*) [Fang and Ng, 2011]. EDN and ECP owing to their bioactions also belong to this interesting group of RNases, also referred to as RISBASES or "riboucleases with special biological actions (D'Alessio, 1993)." The special activities associated with these RNases, which are absent in many other more potent RNases, make them attractive as potential therapeutic agents.

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Domachowske et al. [1998a] have demonstrated that eosinophils in vitro can directly inhibit respiratory syncytial virus (RSV) and parainfluenza virus (PIV) infectivity. The inhibition of virus infectivity was dose-dependent and reversed by the addition of a placental ribonuclease inhibitor indicating the eosinophil antiviral activity to be through the RNase activity of EDN and ECP [Domachowske et al., 1998a]. EDN has been shown to be about ten-fold more effective as an antiviral agent than ECP, and a combination of EDN and ECP did not have any synergistic or additive effect [Domachowske et al., 1998b]. Using a mutant of EDN, and ribonuclease inhibitor it was confirmed that the ribonuclease activity is essential for the antiviral activity of EDN and ECP [Domachowske et al., 1998a].

The lack of special bioactions in RNase A indicates that besides the RNase activity there may be other determinants(s) in EDN and ECP that are involved in their anti-RSV activity, and ribonuclease activity may be necessary but not sufficient for the biological actions of EDN and ECP. A comparison of the three-dimensional structures of EDN, ECP, and RNase A reveals that, while the overall structure and the disulfide bridge pattern closely resemble, the regions that deviate most between these RNases involve the loops [Mosimann et al., 1996; Boix et al., 1999]. The loops, which differ significantly between the eosinophil RNases and RNase A, are L2 and L7. While L2 has a six-residue deletion corresponding to residues 20–25 of RNase A, L7 has a nine- and eight-residue insertion in EDN and ECP, respectively, as compared to that of RNase A (Fig. 1A). The loop L7 forms a single helical turn followed by a type III β-turn like structure, which corresponds to an omega loop, and the conformation of this loop is stabilized by various polar interactions [Mosimann et al., 1996]. The loop L7 interacts with, and influences the conformation of the N-terminus of EDN.

The present study aimed at analyzing the involvement of the C-terminal loop L7 of EDN in its antiviral activity. In both EDN and ECP, the loop L7 contains an insertion as compared to RNase A, however, the sequence of insertion differs between the two proteins. In the current study, the residues in loop L7 that differ between EDN and ECP were selected and mutated in EDN individually and in various combinations to investigate the role of this structural determinant in the antiviral activity of EDN against RSV. The single mutations were found to reduce the antiviral activity of EDN without affecting its RNase activity; combination of mutations reduced the antiviral activity further. The study demonstrates that the loop L7 in EDN interacts directly with RSV virions and facilitates protein's access to the viral genome.

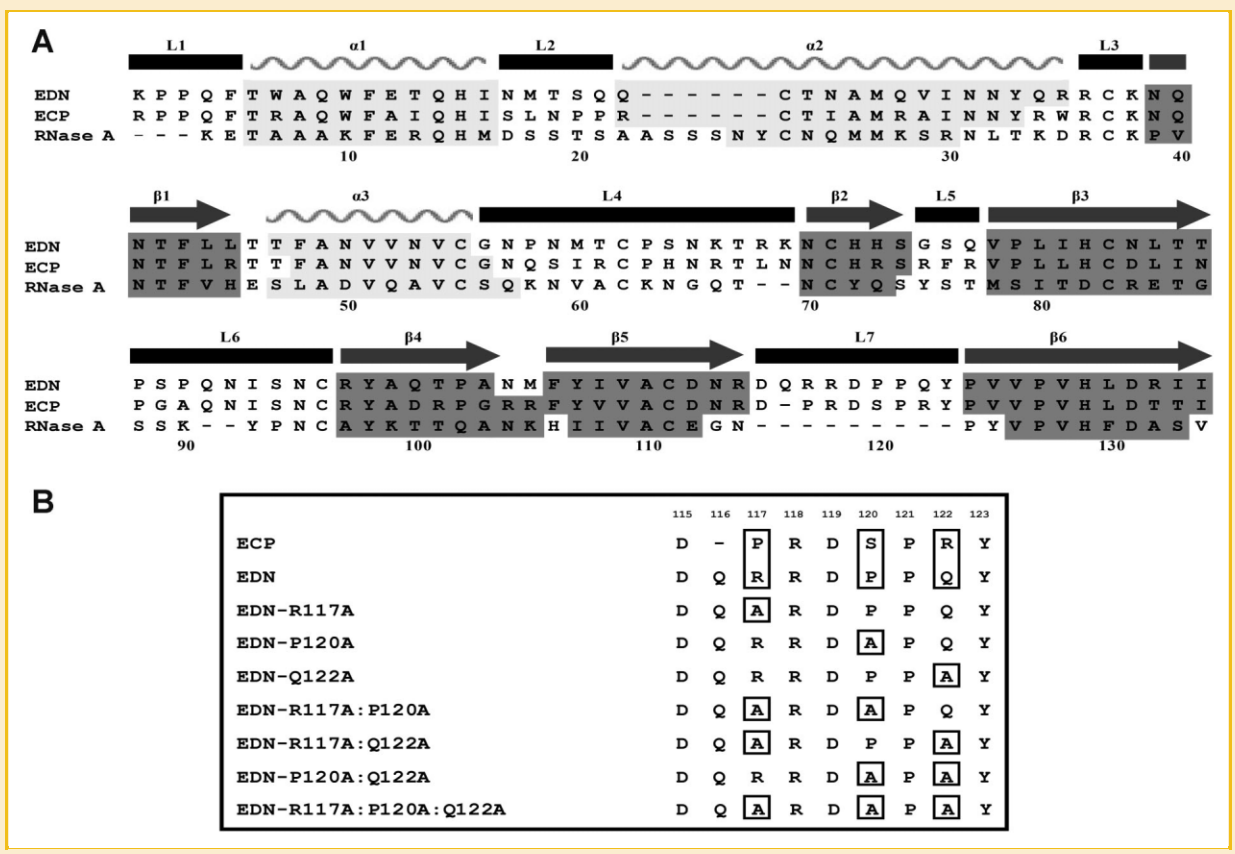

Fig. 1. A: Alignment of amino acid sequences of human eosinophil RNases and bovine pancreatic RNase A. The sequences were aligned using ClustalW software. The topologically equivalent secondary structure elements for EDN are from Mosimann et al., [1996], whereas that for ECP and RNase A are obtained from Mallorquí-Fernández et al. [2000]. The structural elements are shaded: α-helices with light gray background and β-strands with dark gray background. The number represents every tenth residue of EDN. B: Mutants of Loop L7 of EDN. Sequence comparison of insertion in loop L7 of EDN and ECP is shown. The dashes represent the absence of corresponding residues. The non-conserved residues between EDN and ECP, and mutations in various mutants are boxed.

EXPERIMENTAL PROCEDURES

CONSTRUCTION OF EDN MUTANTS

The plasmid pEDN [Sikriwal et al., 2007] was used to express the wild-type EDN protein, and also as the template to mutate the codons for amino acids Arg117, Pro120, Gln122, His15, and Gln14 to alanine. All mutations were carried out by oligonucleotide-mediated site-directed mutagenesis [Kunkel et al., 1987], and confirmed by DNA sequencing using dideoxy chain termination method [Sanger et al., 1977].

EXPRESSION AND PURIFICATION OF THE RECOMBINANT PROTEINS

EDN and its various mutants were expressed in *Escherichia coli* strain BL21 Codon Plus (λDE3)-RIL. Competent bacterial cells were transformed with the desired construct and grown in LB medium containing 0.1 mg/ml ampicillin and 0.05 mg/ml chloramphenicol at 37°C with shaking. The cultures were grown up to an A_{600} of 1.4–1.6, and induced with 0.5 mM IPTG for 8 h. All proteins were found to accumulate in bacteria in the form of inclusion bodies from where they were isolated and renatured as described by Buchner et al. [1992]. The proteins were purified as described earlier for EDN [Sikriwal et al., 2007]. Briefly, the inclusion bodies were dissolved in 6 M guanidine hydrochloride, reduced with dithioerythritol, and renatured by 100-fold dilution in a refolding buffer containing arginine and oxidized glutathione at 10°C for 60 h. The renatured material was dialyzed against 20 mM Tris–Cl buffer, pH 7.5 containing 100 mM urea. The dialyzed material was loaded onto a heparin-sepharose column, and bound proteins were eluted using 0–2 M gradient of NaCl. Relevant fractions, containing the desired protein were pooled, concentrated, and further purified to homogeneity by gel filtration chromatography on a Superose 12 column in TBS, pH 7.4. The protein concentrations were determined by Bradford's method using Coomassie Plus protein assay reagent from Pierce [Bradford, 1976].

CIRCULAR DICHROISM SPECTROSCOPY (CD)

For CD-spectral analysis, 150 μg of protein was taken in 3 ml of 10 mM Tris–Cl buffer, pH 7.5, and spectra were recorded in the far-UV range (190–250 nm) at room temperature, using a JASCO J710 spectropolarimeter. A cell with a 1 cm optical path was used to record the spectra at a scan speed of 50 nm/min with a sensitivity of 50 mdegree and a response time of 1 s. The sample compartment was purged with nitrogen, and spectra were averaged over 10 scans. Each spectrum was corrected for the blank contribution. The results are presented as mean residue ellipticity (MRE).

FLUORESCENCE SPECTROSCOPY

Fluorescence measurements were carried out using a Cary Eclipse spectrofluorimeter. The intrinsic tryptophan fluorescence at 280 nm excitation was recorded for emission from 290 to 450 nm at room temperature. The proteins were used at a concentration of 15 μg/ml in 10 mM Tris–Cl buffer pH 7.4.

STEADY STATE KINETICS OF EDN AND ITS MUTANTS FOR THE CLEAVAGE OF YEAST tRNA SUBSTRATE

RNase activity of proteins on yeast tRNA substrate was assayed by the method of Bond [1988] as described earlier [Gaur et al., 2001]. In a 200 μl reaction, respective proteins were incubated with different concentrations of yeast tRNA ranging from 0.178 to 17.8 μM in 10 mM Tris–Cl buffer, pH 7.4 at 37°C for 10 min. The concentrations of protein taken were 6.6 nM for EDN, R117A, P120A, Q122A, R117A:P120A, P120A:Q122A, and 66 nM for R117A:Q122A, and R117A:P120A:Q122A. The reaction was terminated by the addition of a stop solution containing 5% (v/v) perchloric acid and 0.25% (w/v) uranyl acetate. The undigested large molecular weight RNA was precipitated on ice for 30 min and removed by centrifugation at 15,000*g* for 10 min. The acid soluble product, present in the supernatant, was quantitated by measuring the absorbance at 260 nm. Linear regions were used to calculate V_{max} from which the K_m was calculated. For velocity constant calculations, $\Delta 260 = 0.013\ \mu M^{-1}\,cm^{-1}$ was used for ribonucleotides. Data were fitted using nonlinear regression in Sigma plot software (Version 8.0) to obtain kinetic parameters.

ANTIVIRAL ACTIVITY OF EDN AND ITS MUTANTS

Propagation of RSV-B. For propagation of RSV-B virus, Hep-2 cells were grown to a monolayer in a T-25 flask in DMEM, pH 7.2 containing 10% FCS. The monolayer was washed once with PBS to remove FCS, and inoculated with RSV-B virus stock in 0.5 ml DMEM, pH 7. The cells were incubated for 1 h at 37°C to allow viral adsorption with occasional tilting of flask so that the cells do not dry. The initial inoculum was then removed and 4 ml of DMEM; pH 7.0 supplemented with 2% FCS was added and the cells were grown till cytopathic effects were visible. The cells were lysed by freezing and thawing once. The lysed cell suspension containing the RSV-B virions was aliquoted and stored at −70°C.

Shell vial assay for antiviral activity of proteins. EDN and its mutants were assayed for their antiviral activity against RSV following the titration assay described by Domachowske and Bonville [1998], with some modifications. Briefly, 1.5×10^5 Hep-2 cells were seeded on cover slips placed in 24-well plates. The cells were allowed to grow for 48 h to form a monolayer. Various proteins at different concentrations were mixed with RSV-B suspension in DMEM pH 7 and incubated with gentle rotation at room temperature for 2 h. To infect Hep-2 cell monolayers, the culture medium was removed from the wells, cells were washed twice in PBS and 0.2 ml of the viral suspension treated or untreated with the protein was added. The plates were centrifuged at 1,000 rpm for 30 min to allow adsorption of virus to cells. Subsequently, 1 ml of DMEM pH 7 containing 2% FCS was added to each well and the plates were incubated at 37°C in 5% CO_2 for 48 h. At the end of 48 h, the medium was removed; cells were washed twice with PBS and fixed in chilled acetone. The infected cells were identified by indirect immunofluorescence staining using mouse anti-RSV monoclonal antibody as primary and goat anti-mouse-FITC conjugate as secondary antibody. The infected cells were counted under a fluorescence microscope. The percentage of infected cells in wells treated with proteins was compared to control where no protein was added. The

antiviral activity was expressed as ID_{50} values that represent the concentrations of proteins that inhibited viral infection by 50%.

Antiviral activity assay based on reverse transcriptase-polymerase chain reaction (RT-PCR). The damage to RSV-B RNA genome by EDN and its mutants was quantified by RT-PCR by detecting a 217 bp RSV G-protein fragment in control and virus samples treated with various proteins. RSV suspension (1 ml) was treated with different concentrations of EDN and its mutants for 30 min at room temperature, RNA was isolated using RNeasy columns (Qiagen) and eluted with 100 μl of autoclaved DEPC-treated water. Complementary DNA was prepared with equal volumes of RNA from control and each treatment sample using a RT-PCR kit from Applied Biosystems with random hexamer priming. The ABI PRISM 7500 sequence detection system was used to perform SYBR real-time PCR in triplicate with the following RSV G protein-specific 5′ and 3′ primers as described by Gottschalk et al. [1996] using equal volume of cDNA as template from each sample.

Fwd. 5′-ACTCATCCAAACAACCCACA-3′

Rev. 5′-GGAACAAAATTGAACACTTC-3′

The $2^{-\Delta C_t}$ method was applied to determine threshold values. Subsequently, the PCR products were also separated by 2.5% agarose gel electrophoresis, and the single amplification product of 217 bp was identified.

RESULTS

A remarkable unique feature of EDN is a nine-residue insertion in the carboxy-terminal loop, L7 compared to that in RNase A. ECP also contains an insertion in loop L7, however, there are differences in few amino acids compared to the corresponding insertion in EDN (Fig. 1A). This loop, located on the outer surface of the protein is highly charged, with an independent isoelectric point calculated to be 10.3. Earlier, EDN has been shown to be more active in inhibiting RSV infectivity than ECP. The current study probed the role of loop L7, particularly the unique residues within the insertion of EDN in its antiviral activity towards RSV. Three residues in loop L7, Arg117, Pro120, and Gln122, which differ between EDN and ECP, were mutated to alanine alone and in combination to generate single, double, and triple alanine mutants (Fig. 1B). These mutants were analyzed for their catalytic and antiviral activities.

CONSTRUCTION, EXPRESSION, AND PURIFICATION OF EDN MUTANTS

Three single amino acid mutants of EDN, R117A, P120A, and Q122A in its loop L7 were constructed by oligonucleotide-mediated site-directed mutagenesis. The double mutants, R117A:P120A, R117A:Q122A, P120A:Q122A, and a triple mutant, R117A:P120A:Q122A in loop L7 of EDN were similarly constructed. The active site residue mutants Q14A and H15A which were earlier constructed and characterized were used as controls in the current study [Sikriwal et al., 2009].

EDN and its mutants over-expressed and accumulated as inclusion bodies in *E. coli*. The inclusion bodies were purified, and proteins were refolded in vitro. The refolded proteins were purified by a two-step purification protocol, involving cation exchange and gel filtration chromatography. Analysis on SDS–polyacrylamide gels revealed a single band for each of the proteins (Fig. 2A). These homogenous protein preparations reacted as well with polyclonal antibodies raised against EDN as the wild-type EDN on Western blots (Fig. 2B).

STRUCTURAL CHARACTERIZATION OF EDN MUTANTS

The effect of mutations on the secondary structure of EDN was estimated by CD-spectroscopy in the far-UV range. The CD-spectrum of EDN was characteristic of an $\alpha + \beta$ structure, and the spectra for R117A, P120A, and Q122A were similar to that of the wild-type protein indicating that mutating these residues did not have any significant effect on the secondary structure (Fig. 2C). The CD-spectrum of P120A:Q122A was very similar to that of EDN, and spectrum of R117A:P120A was moderately altered (Fig. 2D). However, the spectra of R117A:Q122A and R117A:P120A:Q122A were significantly altered (Fig. 2D).

Fluorescence emission spectroscopy was used to probe the protein environment as a result of mutagenesis. The fluorescence spectra of single mutants of loop L7 were similar to that of native EDN, which correlated well with their CD spectra (Fig. 2E). The fluorescence spectra of R117A:P120A and P120A:Q122A were also very similar to that of EDN, whereas the spectra of R117A:Q122A and R117A:P120A:Q122A were drastically altered with a significant reduction in their amplitudes (Fig. 2F). The emission maxima for these proteins were not much different from that of the native EDN.

RIBONUCLEASE ACTIVITY OF EDN AND ITS MUTANTS

The kinetic parameters for the cleavage of yeast tRNA substrate by single, double, and triple mutants were evaluated by following their steady state kinetics. The K_m and k_{cat} values of all the single mutants were quite similar to that of EDN (Table I). Among the double mutants, the kinetic parameters of R117A:P120A and P120A:Q122A were similar to those of EDN (Table I). Although the K_m of R117A:Q122A and R117A:P120A:Q122A mutants were similar to that of EDN, they had decreased k_{cat} values, thereby resulting in a significant decrease in their specificity constants (Table I). The specificity constant for R117A:Q122A and R117A:P120A:Q122A decreased by fivefold for yeast tRNA (Table I). As reported earlier, the active site mutant Q14A had a 6- and 30-fold reduced K_m and k_{cat} values, respectively, resulting in a five-fold decrease in the specificity constant compared to that of EDN (Table I) [Sikriwal et al., 2009]. The other active site mutant, H15A had a 3.5- and 80-fold decrease in the K_m and k_{cat} values, respectively, thereby reducing the specificity constant by 15-fold compared to that of EDN (Table I) [Sikriwal et al., 2009].

ANTI-RSV ACTIVITY OF EDN AND ITS MUTANTS

The antiviral activity of EDN and its mutants against RSV was analyzed by two different ways. The effect of proteins on the infectivity of the virus was assayed by the shell vial assay, whereas the RT-PCR based assay analyzed the effect of proteins directly on the viral genome.

The ability of EDN to inhibit the infectivity of extracellular virions of single-stranded retrovirus, RSV-B was assayed by the Shell vial assay as described by Domachowske and Bonville [1998].

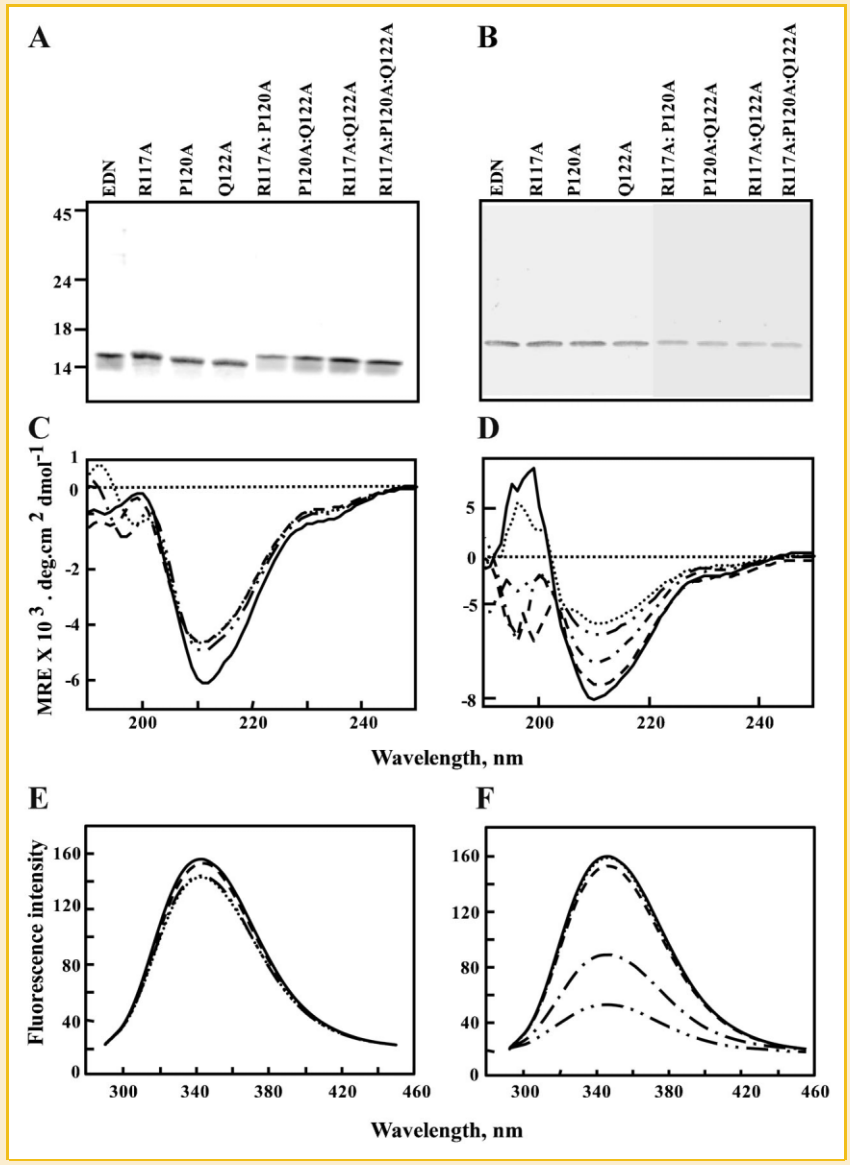

Fig. 2. Purification and structural characterization of EDN and its mutants. A: The purified recombinant EDN and its mutants were analyzed by 12.5% SDS–PAGE under reducing conditions, followed by Coomassie blue staining. B: Western blot analysis of mutants using a polyclonal antibody raised against EDN in rabbit. The molecular weight markers are shown in kDa on left. C: CD spectra of EDN and its single mutants; EDN (————), R117A (— — — —), P120A (·····), and Q122A (— · · — · · — · ·). D: CD spectra of EDN and its double and triple mutants, EDN (————), R117A:P120A (— · — · — · —), P120A:Q122A (— — — — —), R117A:Q122A (— · · — · · — · ·), and R117A:P120A:Q122A (·····). E: Fluorescence spectra of EDN and its single mutants, EDN (————), R117A (— — — — —), P120A (— · · — · · — · ·), and Q122A (— · — · — · —). F: Fluorescence spectra of EDN and its double and triple mutants, EDN (————), R117A:P120A (— — — — —), P120A:Q122A (·····), R117A:Q122A (— · — · — · —), and R117A:P120A:Q122A (— · · — · · — · ·).

The RSV virions were treated with EDN and its mutants and used to infect Hep-2 cells. EDN was found to inhibit the infectivity of RSV-B in a dose-dependent manner with an ID_{50} of 0.3 μM (Table II). As compared to EDN, the three single mutants in loop L7, R117A, P120A, and Q122A showed 50% decreased anti-RSV activity (Table II). The double mutants, R117A:P120A and R117A:Q122A showed a 12-fold reduced anti-RSV activity than that of EDN (Table II). The double mutant P120A:Q122A was three-fold less active than EDN, whereas the triple mutant R117A:P120A:Q122A did not show any activity up to 10 μM (Table II). The active site residue mutants Q14A and H15A also showed 10- and 14-fold decrease in antiviral activity, respectively. ECP, the other eosinophil granule ribonuclease was 22-fold less active than EDN in its activity against RSV (Table II). RNase A and human pancreatic ribonuclease (HPR), two much more active RNases than EDN were inactive even at concentrations as high as 10 μM (Table II). BSA, used as a control did not have any anti-viral activity up to 10 μM. EDN alone, up to 5 μM did not have any cytotoxic effect on Hep-2 cells after 48 h (data not shown).

To investigate if EDN directly interacts with RSV-B, a PCR-based assay was used in which viral particles were incubated with various concentrations of EDN and its mutants and subsequently the damage to viral RNA genome was quantitatively analyzed by amplifying a

TABLE I. Steady State Kinetic Parameters for the Cleavage of Yeast tRNA by Loop L7 Mutants of EDN

Protein	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$) × 10^6
EDN	3.36 ± 0.15 (1)	31.37 ± 0.96 (1)	9.34 ± 0.17 (1)
R117A	3.74 ± 0.02 (1)	50.57 ± 0.63 (1.6)[a]	13.52 ± 0.24 (1.4)[a]
P120A	3.15 ± 0.62 (0.9)	43.62 ± 1.94 (1.4)[a]	14.26 ± 2.21 (1.5)
Q122A	4.60 ± 0.15 (1.4)[a]	45.80 ± 0.80 (1.4)[a]	9.95 ± 0.20 (1)
R117A:P120A	2.09 ± 0.13 (0.6)[a]	25.51 ± 0.62 (0.8)[a]	12.22 ± 0.48 (1.3)[a]
P120A:Q122A	4.47 ± 0.20 (1.3)[a]	28.76 ± 0.70 (0.9)	6.43 ± 0.18 (0.7)[a]
R117A:Q122A	3.12 ± 0.17 (0.9)	5.60 ± 0.64 (0.17)[a]	1.80 ± 0.18 (0.2)[a]
R117A:P120A:Q122A	3.37 ± 0.19 (1)	4.80 ± 0.30 (0.15)[a]	1.42 ± 0.20 (0.15)[a]
Q14A[b]	0.56 ± 0.05 (0.16)[a]	1.07 ± 0.007 (0.03)[a]	1.94 ± 0.19 (0.2)[a]
H15A[b]	0.89 ± 0.19 (0.26)[a]	0.39 ± 0.04 (0.012)[a]	0.46 ± 0.074 (0.04)[a]

The fold differences as compared to EDN are given in parentheses. Data represent mean ± SE of three experiments. Data were analyzed statistically by employing students t test.
[a]Statistically significant at $P < 0.05$ as compared to EDN.
[b]From Sikriwal et al. [2009].

virus-specific 217 bp band, corresponding to the RSV G-protein by real-time PCR using cDNA prepared from the viral RNA as the template. The level of the 217 bp RSV-B specific fragment in the presence of EDN decreased in a dose-dependent manner (Fig. 3A,B). There was a 50% reduction in the viral genome with 2 nM EDN which was further reduced to 6% upon treatment with 20 nM EDN (Fig. 3A,B). ECP was much less effective and at 200 nM it was able to reduce the RSV genome only by 60% (Fig. 3B). All single mutants in loop L7 of EDN were about ten-fold less active than EDN as they produced 50% decrease in RSV genome at 20 nM concentration (Fig. 3A). The double mutant P120A:Q122A was similar to the single mutants in its anti-RSV activity, whereas the other double mutants R117A:P120A and R117A:Q122A, and the triple mutant R117A:P120A:Q122A were almost inactive (Fig. 3A). RNase A and HPR in spite of being much more active RNases did not show any antiviral activity against RSV (Fig. 3B). BSA did not produce any damage to the RSV genome (Fig. 3B). Q14A and H15A which had negligible ribonuclease activity did not affect the integrity of RSV genome corroborating that the RNase activity is required for the antiviral activity (Fig. 3B). Table III shows the data used in Figure 3. The products obtained after the real-time PCR were also analyzed by gel electrophoresis. Therefore, both, Shell vial assay and RT-PCR based assay qualitatively produced similar results.

TABLE II. Antiviral Activity of EDN and its Mutants Assayed by Shell Vial Assay

Protein	ID_{50} (μM)	Fold decrease
EDN	0.31 ± 0.02	1
R117A	0.78 ± 0.01[a]	2.5[a]
P120A	0.61 ± 0.04[a]	2[a]
Q122A	0.59 ± 0.06[a]	2[a]
R117A:P120A	3.90 ± 0.03[a]	12[a]
P120A:Q122A	0.99 ± 0.07[a]	3[a]
R117A:Q122A	3.84 ± 0.60[a]	12[a]
R117A:P120A:Q122A	>10.00[a]	>30[a]
Q14A	3.29 ± 0.003[a]	10.5[a]
H15A	4.37 ± 0.31[a]	14[a]
ECP	6.79 ± 0.72[a]	22[a]
HPR	>10.00[a]	>30[a]
RNase A	>10.00[a]	>30[a]
BSA	>10.00[a]	>30[a]

RSV-B suspension, treated with different concentrations of proteins were used to infect Hep-2 cells. The number of syncytia formed in each case was counted. The ID_{50} values were calculated as the amount of protein required to decrease RSV-B infectivity by 50%. Data represent mean ± SE of three experiments. Data were analyzed statistically by employing Student's *t*-test.
[a]Statistically significant at $P < 0.05$ as compared to EDN.

DISCUSSION

Human eosinophil granules contain predominantly four proteins, EDN, ECP, MBP, and EPO. The mechanism of action of eosinophil granule proteins and their role in eosinophil function is not clearly understood. Two of the eosinophil granule proteins, EDN and ECP are ribonucleases having antiviral activity against RSV, which is shown to be dependent on their enzymatic activity [Domachowske et al., 1998a]. EDN is ribonucleolytically more active than ECP and has a higher anti-RSV activity compared to ECP possibly due to its higher RNase activity. Other closely related and more active RNases such as RNase A, onconase and RNase k6 do not have any antiviral activity [Domachowske et al., 1998c], suggesting the existence of specific region(s) in EDN and ECP, different from those required for the RNase activity, that may be involved in their special biological activity. EDN and ECP have, respectively, a nine and eight amino acid insertion in their loop L7 as compared to the corresponding region in RNase A. Between EDN and ECP there are differences at three positions within the unique insertion in loop L7 (Fig. 1B). The current study investigated the role of loop L7 of EDN in its antiviral activity. Three residues in loop L7, Arg117, Pro120, and Gln122, which diverge between EDN and ECP, were individually and in combination mutated to alanine. The individual mutations of Arg117, Pro120, and Gln122, and combined mutations of Pro120/Gln122 and Arg117/Pro120 did not affect the overall conformation of EDN, however simultaneous mutations of Arg117/Gln122 and Arg117/Pro120/Gln122 did affect the EDN conformation. The mutants having intact overall conformation also had full RNase activity, however, they had decreased antiviral activity towards RSV both by the shell vial assay which measured the infectivity of the virus and by RT-PCR based assay which measured direct damage of the viral genome. The mutations in loop L7 are detrimental for the interaction of protein with viral capsid in turn compromising protein's entry into the virion reflecting in a loss in the antiviral activity.

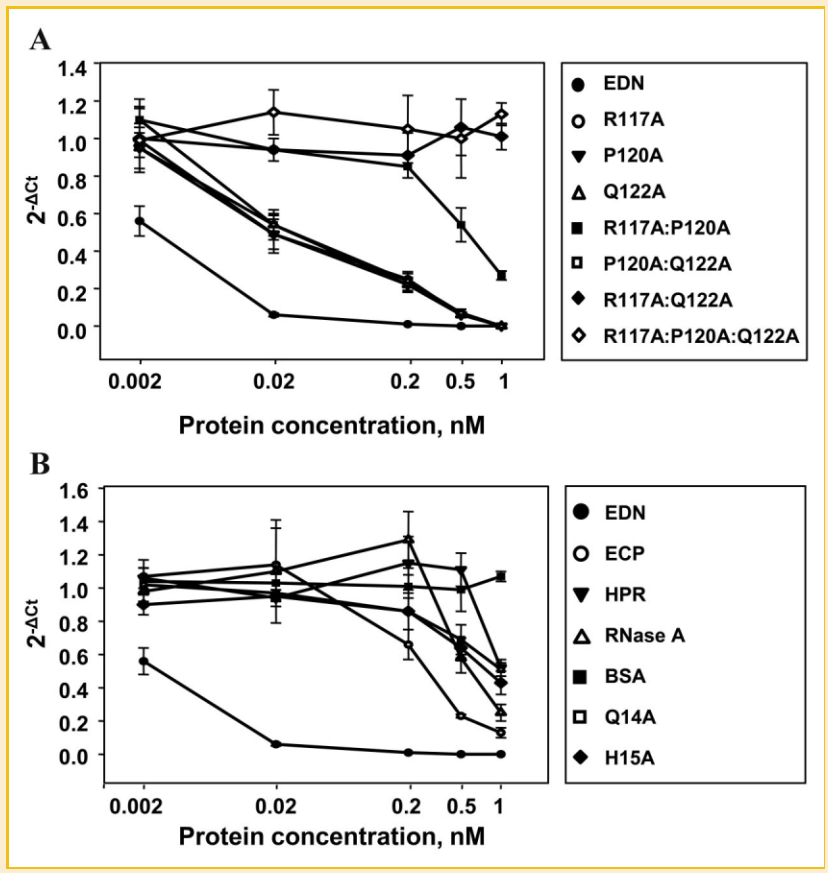

Fig. 3. Analysis of antiviral activity of EDN and its mutants by RT-PCR. cDNAs prepared from RNA isolated from virions treated with different concentrations of various proteins were used as templates for amplification of a 217-bp segment of RSV G protein. Comparisons are made with untreated virus samples. Values <0.01 are considered as 0. Data represent mean ± SE of three experiments. A: EDN and its loop L7 mutants. B: EDN and control proteins.

EDN also displays antiviral activity against clinical isolates of RSV-A and PIV-1, -2, and -3. The virions of these paramyxoviruses display similar but not identical ribonuclease-dependent sensitivity to isolated human eosinophils. PIV displays somewhat less sensitivity than the RSV isolates [Domachowske et al., 1998a]. EDN has also been found to be effective against Hepatitis B virus (HBV) as part of a novel antiviral approach, "capsid-targeted viral inactivation" or CTVI [Natsoulis and Boeke, 1991]. The anti-HBV activity of EDN is also dependent on its ribonuclease activity [Liu et al., 2003]. EDN is responsible, in part, for the HIV-1-inhibitory

TABLE III. Real-Time PCR Analysis of Antiviral Activity of EDN and its Mutants

	$2^{-\Delta C_t}$				
Protein	2 nM	20 nM	200 nM	500 nM	1,000 nM
EDN	0.56 ± 0.08	0.06 ± 0.01	0.01 ± 0.001	0	0
R117A	0.99 ± 0.09	0.49 ± 0.10	0.25 ± 0.03	0.06 ± 0.01	0
P120A	0.95 ± 0.11	0.49 ± 0.08	0.22 ± 0.03	0.06 ± 0.01	0
Q122A	0.95 ± 0.13	0.54 ± 0.06	0.22 ± 0.04	0.06 ± 0.01	0
R117A:P120A	1.10 ± 0.07	0.94 ± 0.06	0.85 ± 0.06	0.54 ± 0.09	0.27 ± 0.024
P120A:Q122A	1.10 ± 0.11	0.54 ± 0.08	0.24 ± 0.05	0.07 ± 0.02	0
R117A:Q122A	1.00 ± 0.03	0.94 ± 0.06	0.91 ± 0.12	1.06 ± 0.15	1.01 ± 0.07
R117A:P120A:Q122A	0.99 ± 0.17	1.14 ± 0.12	1.05 ± 0.18	1.00 ± 0.21	1.13 ± 0.06
Q14A	1.02 ± 0.10	0.97 ± 0.02	0.86 ± 0.11	0.69 ± 0.09	0.51 ± 0.04
H15A	0.90 ± 0.06	0.95 ± 0.02	0.86 ± 0.01	0.64 ± 0.06	0.43 ± 0.07
ECP	1.07 ± 0.10	1.14 ± 0.22	0.66 ± 0.09	0.23 ± 0.01	0.13 ± 0.03
HPR	1.06 ± 0.062	0.94 ± 0.05	1.15 ± 0.16	1.11 ± 0.10	0.52 ± 0.05
RNase A	0.98 ± .081	1.10 ± 0.31	1.29 ± 0.17	0.58 ± 0.09	0.25 ± 0.05
BSA	1.04 ± 0.08	1.03 ± 0.07	1.01 ± 0.07	0.99 ± 0.13	1.07 ± 0.03

cDNAs prepared from RNA isolated from virions treated with different concentrations of various proteins were used as templates for amplification of a 217-bp segment of RSV G protein. Comparisons are made with untreated virus samples. Values <0.01 are considered as 0. Data represent mean ± SE of three experiments.

activities in the supernatant of allogeneic mixed lymphocyte reaction [Rugeles et al., 2003]. Apparently, the loop regions of different ribonucleases contribute significantly to the specific biological actions they possess. Besides the variations due to the differences in length of the loops L2, L3, and L7 of EDN, they also have significantly different conformation from those in RNase A [Mosimann et al., 1996]. In EDN, the loop regions lie on the outer surface, and its molecular surface differs from RNase A [Mosimann et al., 1996]. The loop L7 forms a single helical turn followed by a type III β-turn like structure, corresponding to an omega loop [Mosimann et al., 1996]. The region Pro120-Tyr123 breaks the helix and adopts a type III turn like conformation that changes the direction of the polypeptide chain. Thus, mutation of Pro120 and Gln122 within this region could be responsible for an alteration in the structure of the loop resulting in a poor interaction of EDN with the virus. The RNase activity of EDN would come into effect only once the protein comes in contact with the viral RNA as a consequence of a productive protein–virus interaction. Arg117 per se appears to be crucial for the protein–virus interaction and thus replacing it with alanine showed a decrease in antiviral activity of the mutant in spite of it possessing the complete RNase activity. The current study also explains comparatively lower anti-RSV activity of ECP which lacks Arg117, Pro120, and Gln122 residues in its insertion in loop L7. The entire surface in EDN is essentially positively charged as opposed to RNase A where the positive regions are mainly restricted to the active site cleft (Fig. 4). This difference in the molecular surface may also be a contributor to the unique bioactions of EDN. The basic amino acids in ECP have been shown to play a crucial role in its antibacterial activity [Carreras et al., 2003; Singh and Batra, 2011]. Arginines 101 and 104 on the surface were found to be associated with ECP's membrane destabilization effects, while the region from residues 115–122 was identified to be involved in the binding of ECP with bacteria [Carreras et al., 2003]. Arg117 may be playing a similar role in EDN's interaction with RSV-B.

The antiviral activity of EDN has been found to be saturable and specific indicating that it interacts with a specific target on the virus [Domachowske et al., 1998c]. When antiviral activity of rhEDN was determined in the presence of increasing concentrations of a ribonucleolytically inactivated point mutant, rhEDNdK^{38}, a dose-dependent inhibition of antiviral activity was observed, however, when the identical experiment was done in the presence of increasing concentrations of rhRNase k6 which has ribonuclease activity but lacks antiviral activity, no such inhibition of antiviral effect was observed [Domachowske et al., 1998c]. These results suggest that EDN interacts specifically with one or more as-yet unidentified target molecule(s) with ribonuclease activity as necessary but not sufficient to explain all features of the antiviral effect [Domachowske et al., 1998c; Rosenberg and Domachowske, 2001]. The current study demonstrates that the unique insertion in loop L7 of EDN is involved in this virus–protein interaction. The inactivation of virions would depend on the ability of the RNase to penetrate the viral capsid and come in contact with the viral RNA. Such a dual site model has been hypothesized for another RNase, angiogenin, where a segment encompassing residues 60–68, and Asn109 though not essential for the RNase activity was found to be a candidate for a receptor or cell-binding domain [Hallahan et al., 1991, 1992]. The crystal structure of angiogenin revealed that the segments of the primary structure of angiogenin which comprise this site lie on adjacent loops and are among those regions that differ most markedly from RNase A [Acharya et al., 1994].

In conclusion, the current study demonstrates that the loop L7 in EDN is involved in its interaction with RSV virions, and disruption of this interaction affects the antiviral activity of protein.

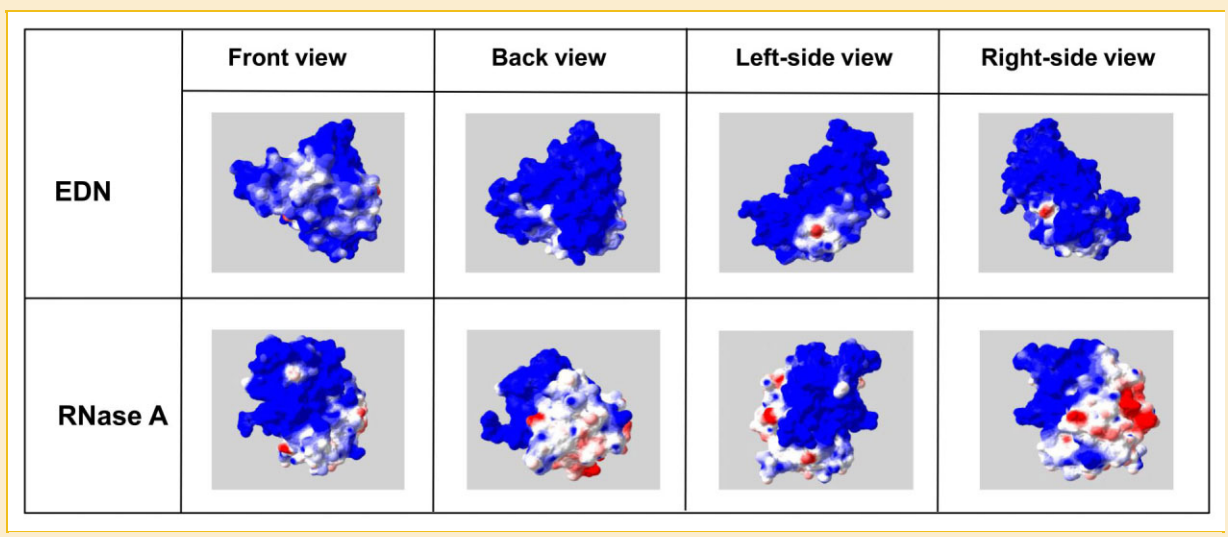

Fig. 4. Molecular surface comparison of EDN and RNase A. The coordinates of the crystal structure of EDN (1K2A) and RNase A (1RSA) from protein data bank were used for modeling. Blue color represents positive charge potential, red represents negative charge potential, white represents neutral charge potential and shades represent intermediate charges.

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