Cytotoxic Activities of Eosinophil Cationic Protein and Eosinophil-derived Neurotoxin: *In Silico* Analysis

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Abstract. *Background: Eosinophil cationic protein (ECP) and eosinophil derived-neurotoxin (EDN) are homologous ribonuclease (RNAse) A family proteins. The objective of the present study was to in silico characterize ECP and EDN with respect to their cytotoxic activities. Materials and Methods: Structural, physicochemical, and conserved domain characterizations were carried-out using open-source software, such as InterProScan, NetOGlyc, NetPhos and Discovery Studio 3.1. Results: The proteins did not have atypical conserved domains. EDN had a greater number of glutamine amino acid residues, whereas ECP had a predominance of arginine. ECP had four possible N-glycosylation, three O-glycosylation and four phosphorylation sites. EDN had five putative N-glycosylation, three phosphorylation and no O-glycosylation sites. Conclusion: The greater cationicity of ECP may be related to its higher cytotoxicity and to the fact that the varying post-translational modification profiles can generate functional differences from structural alteration. In vivo and in vitro studies need to be performed in order to confirm these predictions.*

Eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are stored in human eosinophil cytoplasmic granules and have a close relationship with the immunoregulatory and cytotoxic activities of these leukocytes (1, 2). The RNS2 (*EDN*) and RNS3 (*ECP*) genes are localized to the q24-q31 region of chromosome 14, and it is likely that these two genes arose from a gene duplication event approximately 25-40 million years ago (3, 4).

ECP and EDN belong to the pancreatic-type RNase family, a vertebrate-specific enzyme family that includes eight human proteins with conserved active site amino acid residues (5). Although molecular cloning revealed similarities between EDN and ECP nucleotide and amino acid sequences (89% and 67%, respectively), these proteins exhibit distinct biological properties (2, 5). Interestingly, the RNase activity of ECP is lower than that of EDN, although ECP has stronger anti-bacterial and cytotoxic activities (6).

ECP has RNase-independent cytotoxic activity against bacteria, parasites, viruses and tumor cells (7, 8). This activity begins with protein binding and aggregation to the cell surface (5) and is followed by cell membrane permeability alteration, resulting in ionic intracellular equilibrium modification (9). These signals initiate specific morphological and biochemical cellular changes, such as chromatin condensation, reversion of membrane asymmetry, reactive oxygen species production, activation of caspase-3-like activity and, eventually, cell death (7, 10). The mechanism underlying the cytotoxic property of EDN remains unclear, but it appears to depend on the ribonucleolytic activity (8). Immunofluorescence staining obtained with anti-EDN antibodies suggests that this protein can be internalized by tumor cells (10).

Detailed characterization studies are necessary to elucidate the differential cytotoxic activity of these two proteins. Herein, we highlighted the importance of *in silico* studies, that provide important information gathered from DNA sequences and protein residues, enabling targeted experimental research.

Based on the analysis of sequences of computer EDN and ECP, the objectives of the present study were to provide details of the structure/function of these proteins in order to

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Key Words: Eosinophil cationic protein, eosinophil-derived neurotoxin, homology, bioinformatics, post-translational modifications.

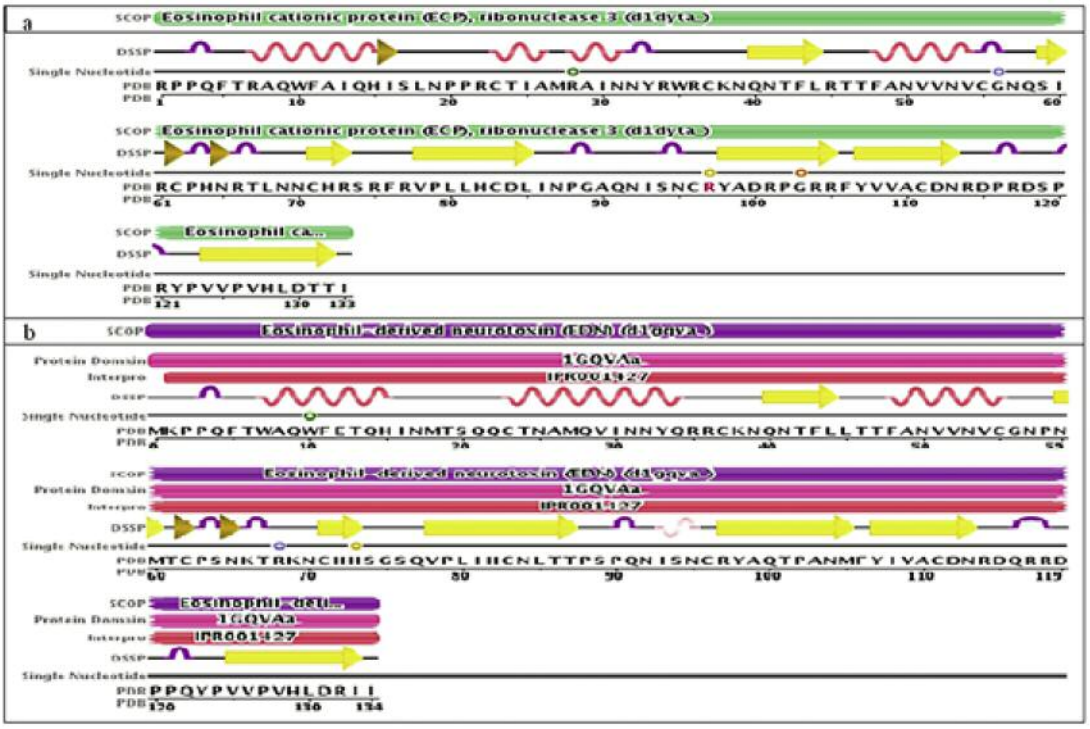

Figure 1. *Secondary structure of proteins: a. ECP and b. EDN.*

formulate hypotheses to help understand the factors responsible for differences in the cytotoxic function of these proteins.

Materials and Methods

The sequences of mature EDN and ECP were obtained from the Protein Database available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/).

Physicochemical and structural characterizations, as well as the search for conserved motifs, were conducted with the ExPASy Tools Portal (http://expasy.org/tools/), including *InterProScan* (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and *ProtParam* (http://web.expasy.org/protparam/).

The three-dimensional structures of ECP and EDN, obtained from the Protein Data Bank (PDB - http://www.rcsb.org/pdb/home/home.do), were superimposed, and select variant amino acids were marked with *Discovery Studio Visualizer 3.1* (Download: http://accelrys.com/resource-center/downloads/updates/discovery-studio/dstudio31/dstudio310.html).

Software available in the ExPASy Tools Portal, including *NetPhos* (http://www.cbs.dtu.dk/services/NetPhos/), *NetOGlyc* (http://www.cbs.dtu.dk/services/NetOGlyc/), *NetNGlyc* (http://www.cbs.dtu.dk/services/NetNGlyc/), *Myristoylator* (http://web.expasy.org/myristoylator/) and *NetAcet* (http://www.cbs.dtu.dk/services/NetAcet/), were used to predict post-translational modifications.

Results

The sequences of mature ECP and EDN were obtained from the Protein Database, National Center for Biotechnology Information (NCBI), under accession numbers NP_002926 and AAA50284.1, respectively. InterProScan analysis revealed that ECP and EDN possess the same conserved domains, except for a signal peptide in ECP. The amino acid analysis demonstrated that ECP contains many positive amino acids (arginine, in particular), whereas EDN has a predominance of glutamines.

Data obtained from the Protein Data Bank (PDB) reveals that amino acid slight variation causes differences in secondary and tertiary protein structure. Differences observed between ECP and EDN are the interruption of an alpha-helix near position 25, the formation of loops near amino acids 33 and 56 in ECP, and a loop (ECP) *versus* an alpha-helix (EDN) near amino acid 95 (Figure 1). Additionally, EDN presented an alpha-helix, which is not found in ECP (blue arrow - Figure 2 and black arrow – Figure 3), whereas only ECP has a loop at position 35 (green arrow - Figure 2).

ECP contains four possible N-glycosylation sites and EDN has five (Figure 4). *NetOGlyc* software indicated three

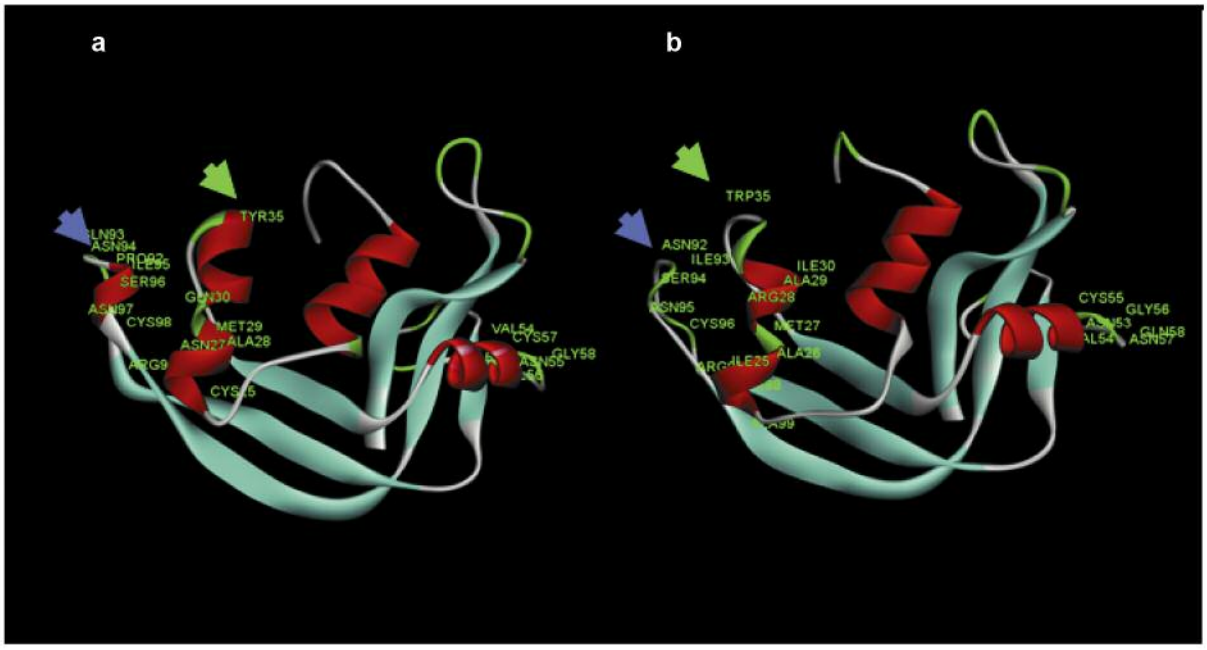

Figure 2. *Three-dimensional structure of a. EDN, b. ECP, illustrating key differences in amino acids and structure: an alpha-helix in EDN (blue arrow) and a lower alpha-helix in ECP beyond tryptophan 35 only in ECP (green arrow).*

potential sites for O-glycosylation in ECP and none in EDN. NetPhos 2.0 software revealed four potential phosphorylation sites in ECP and three in EDN. The positions of tyrosine phosphorylation are close in the two proteins. Myristoylation and acetylation sites were not found in these molecules using the Myristoylator and NetAcet softwares, respectively.

Discussion

Our results revealed that ECP and EDN possess the same conserved domains, except for a signal peptide in ECP. In contrast, Fang *et al*. (6) reported that both proteins present a short peptide that interacts with the cell membrane, known as the cell-penetrating peptide (CPP). Curiously, only one CPP corresponding to residues 32-41 of human ECP (ECP^{32-41}) was able to interact with cell-surface glycosaminoglycans and can serve as a drug delivery vehicle.

Although ECP^{32-41} and EDN^{32-41} possess heparin-binding sequences and differ only at two positions, they have dissimilar cell-binding and internalization activities. Residues R3 and W4 in ECP^{32-41} appear to be crucial for internalization. The two arginines adjacent to W4 in ECP^{32-41} possibly interact with negatively-charged cell-surface heparan sulfate proteoglycans, thereby promoting binding. Taken together, the positively-charged arginines and the aromatic tryptophan are necessary for ECP^{32-41} internalization and disruption of the membrane (6).

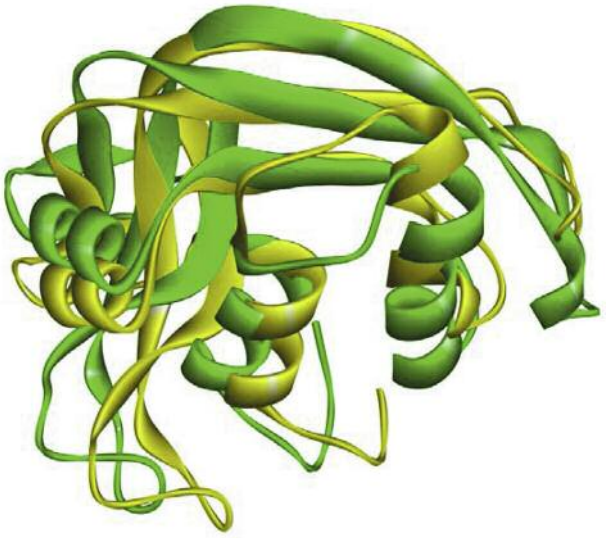

Figure 3. *Overlay of three-dimensional structures of EDN (in green) and ECP (in yellow) using Discovery Studio Visualizer 3.1 software. The black arrow indicates a differential region between the molecules.*

The exact cytotoxic mechanisms of ECP and EDN are not fully known (7, 10), although it is suggested this activity correlates with protein internalization (6). An *in vitro* study using colon carcinoma cells reported that EDN, but not ECP, was detected inside the target cells. In accordance with these

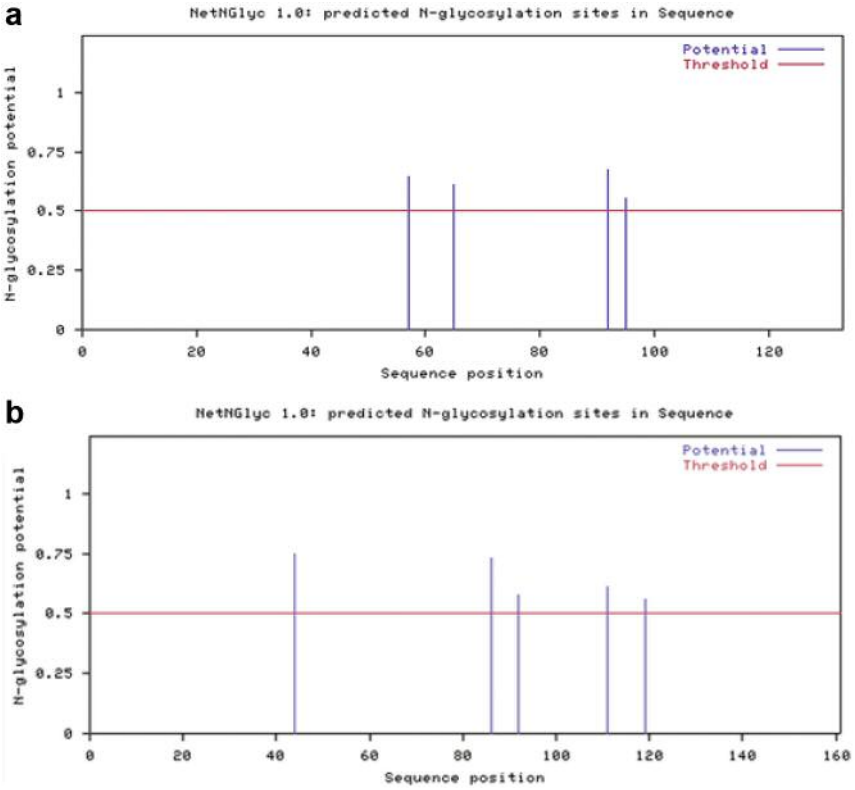

Figure 4. *Potential N-glycosylation sites and their positions: a. ECP, b. EDN. The result was obtained using the program NetNGlyc 1.0.*

results, *in vivo* confocal microscopy experiments indicate that ECP binds and aggregates to the cell surface without internalization of the protein (10).

One possible explanation for this discrepancy is that these studies (6, 10) were carried-out using different cell types. ECP and EDN may have different cell recognition mechanisms, that result in distinct consequences. Another relevant observation is that Fang *et al*. (6) used protein fragments, whereas Legrand *et al*. used complete recombinant proteins (10).

The amino acid analysis showed that ECP contains many positive amino acids, whereas EDN has a predominance of glutamines. As ECP and its CPP had a greater positive charge, the interaction and attachment with negatively charged cell surface molecules, such as heparin, are stronger (11, 12). This finding could explain the capacity of ECP to create pores in cell membranes and kill target cells more efficiently than EDN.

Differences in three-dimensional structure observed between EDN and ECP can constitute potential sites of interaction with the cell surface. Interestingly, this region contains W35, present only in ECP and its CPP (6). This emphasizes on the importance of amino acids not only for the differential substrate affinity but also for three-dimensional structure differences, making it essential to the cytotoxic activity of ECP, as described by Torrent *et al*. in 2007 (13).

ECP contains four possible N-glycosylation sites and EDN has five. N-glycosylation is the most common post-translational modification and may alter the conformational structure and stability of proteins (14). In contrast, other studies (15, 16) reported that the cDNA sequences encoding EDN and ECP have five and three potential N-glycosylation sites, respectively. Experimental studies are necessary to determine the actual numbers of glycosylation sites.

Glycosylation is related to cell interaction and signaling (14), as well as the cytotoxic activity of ECP (13). Glycosylation must be investigated to determine the role of the different N-glycosylation patterns in these proteins. Trulson *et al*. (13) reported that most native, purified ECP showed no cytotoxic activity, suggesting that post-translational modifications, along with genetic variations, are responsible for cytotoxicity and protein heterogeneity. In addition, Woschnagg *et al*. (17) observed that non-glycosylated ECP variants were more cytotoxic, suggesting that different degrees of glycosylation generated distinct

cytotoxic potential. These results indicate that ECP is stored in an inactive form and that activation occurs before secretion by eosinophils by removing these post-translational modifications.

Based on the results presented by these authors (13, 17), glycosylation could prevent the interaction of key ECP amino acids with the target cell, thus changing its conformation. Considering this hypothesis proposed by Woschnagg *et al*. (17), it is inferred that the activation process of ECP generates variants with different levels of cytotoxicity and results in differential interactions with the target cell.

This hypothesis would be valid only for studies with native proteins. In most studies that evaluate the cytotoxic effects of ECP and EDN on tumor cells, recombinant variants obtained from prokaryotic models are utilized (1, 8, 10). Under these experimental conditions, the proteins do not have post-translational modifications; however, several studies (1, 8, 10) have identified varying cytotoxic response profiles in different cell types. These data reinforce the need for additional studies to elucidate the role of post-translational modifications in these proteins and other molecular characteristics involved in the discrimination between cells.

The NetOGlyc software indicated three potential sites for O-glycosylation in ECP and none in EDN. Similarly, this modification was not found in EDN obtained from blood eosinophils (18). Helenius and Aebi (19) indicated that glycosylation is a way to add new features and to generate protein variations. These modifications allow for fine adjustment of the molecule's properties without modifying the amino acid sequence (19). If was confirmed experimentally, that O-glycosylation may be among the main differences between the two proteins.

The NetPhos 2.0 software revealed four potential phosphorylation sites in ECP and three in EDN. The positions of tyrosine phosphorylation are close in the two proteins. Considering that ECP has a potent cytotoxic effect in some cell types, phosphorylation could constitute a mechanism for regulation of secretion.

Conclusion

Our results, combined with literature reports, suggest that key amino acids contribute to the cytotoxic function of ECP and EDN proteins. The small differences between those would create in ECP small sites of interaction with the membrane and changes in the three-dimensional structure, that would be capable of influencing the interaction with the target cell. In this context, EDN would have less interaction with the membrane because it has a smaller number of arginines and does not have tryptophan 35. However, post-translational modifications cannot be ruled-out. N-glycosylation appears to protect important amino acids for protein function or to simply affect protein conformation. The existence of O-glycosylations and phosphorylations should be confirmed in experimental studies and could be responsible for functional differences between ECP and EDN. For the hypothesis confirmation, studies of the relationship between cell interaction and differential glycosylation need to be conducted to investigate a connection with the cytotoxicity mechanism of different cell types. In addition, phosphorylation may be a regulatory mechanism involved in various complex proteins.

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