MINIREVIEW

Eosinophil Granule Proteins: Form and Function

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Experimental and clinical data strongly support a role for the eosinophil in the pathogenesis of asthma, allergic and parasitic diseases, and hypereosinophilic syndromes, in addition to more recently identified immunomodulatory roles in shaping innate host defense, adaptive immunity, tissue repair/remodeling, and maintenance of normal tissue homeostasis. A seminal finding was the dependence of allergic airway inflammation on eosinophil-induced recruitment of Th2-polarized effector T-cells to the lung, providing a missing link between these innate immune effectors (eosinophils) and adaptive T-cell responses. Eosinophils come equipped with preformed enzymatic and nonenzymatic cationic proteins, stored in and selectively secreted from their large secondary (specific) granules. These proteins contribute to the functions of the eosinophil in airway inflammation, tissue damage, and remodeling in the asthmatic diathesis. Studies using eosinophil-deficient mouse models, including eosinophil-derived granule protein double knock-out mice (major basic protein-1/eosinophil peroxidase dual gene deletion) show that eosinophils are required for all major hallmarks of asthma pathophysiology: airway epithelial damage and hyperreactivity, and airway remodeling including smooth muscle hyperplasia and subepithelial fibrosis. Here we review key molecular aspects of these eosinophil-derived granule proteins in terms of structure-function relationships to advance understanding of their roles in eosinophil cell biology, molecular biology, and immunobiology in health and disease.

Over the past three decades, the role of the eosinophil in human health and disease has received considerable attention (1, 2). The eosinophil has a vital role in allergic inflammatory processes that include asthma (3, 4). Evidence implicates the eosinophil and its granule proteins in host resistance to parasites, particularly helminths, but also antimicrobial activities toward bacterial, viral, and protozoan pathogens, and as mediators of hypersensitivity diseases. This evidence consists of associations between elevated levels of eosinophils in blood and the occurrence of disease; correlations between disease severity and degree of eosinophilia; findings that the cationic eosino-

phil-derived granule proteins (EDGPs)[3] are toxic to cells and human tissues, producing changes mimicking those associated with disease (*e.g.* in bronchial asthma); deposition of the toxic EDGPs in diseased tissue; and observations that glucocorticoids suppress eosinophilia as part of their therapeutic effect (5).

The eosinophil is rich in cationic granule proteins, shows a striking respiratory burst with the production of toxic oxygen radicals, brominates tissue and proteins, presents antigen to T-cells, and expresses both hematopoietic and inflammatory cytokines (2). These findings support the eosinophil as a key player in allergic inflammation and tissue homeostasis in hypersensitivity diseases (1, 2). Eosinophil-deficient mouse models (3, 4, 6), including EDGP gene-deleted mice (7–9), provide unique insights into the role of the eosinophil, in both tissue damage/repair/remodeling and novel immunomodulatory roles that bridge host innate and adaptive immune responses in allergic and parasitic diseases.

Initial views of the eosinophil as providing direct antipathogen (helminth) activity are being supplanted by more nuanced views of the immunomodulatory functions of the eosinophil, including roles in supporting parasitic nematode survival (10, 11); Appleton and colleagues (10, 11) show that growth and survival of the muscle stage larvae of *Trichinella spiralis* require eosinophils, which promote accumulation of Th2 cells and inhibit the induction of inducible NOS by macrophages and neutrophils. Studies using a conditional eosinophil-deficient mouse strain (*iPHIL*) show that eosinophils modulate the immune and inflammatory character of inducible allergic responses in the lung (6). Clinical trials using anti-interleukin-5 (IL-5) antibodies to ablate eosinophils in the bone marrow and blood, as well as reduce tissue eosinophils in patients with the eosinophilic but not neutrophilic phenotype of asthma, show efficacy in reversing eosinophil-mediated tissue damage, remodeling, fibrosis, and airway dysfunction (12), as well as end-organ damage in the hypereosinophilic syndrome (13), highlighting the complex proinflammatory and immunomodulatory activities of the eosinophil in shaping the pathogenesis of these diseases.

Eosinophil-derived Granule Proteins

With expectations that understanding the properties, activities, and secretion of eosinophil proteins in disease states would provide insights into cellular function, the cationic components of the large crystalloid-containing specific (secondary) granule of the eosinophil (Fig. 1) were extensively studied. These proteins include major basic protein-1 and -2 (MBP-1, MBP-2), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Fig. 1). The Charcot-Leyden crystal protein/Galectin-10 (CLC/Gal-

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[3] The abbreviations used are: EDGP, eosinophil-derived granule protein; MBP, major basic protein; EPX, eosinophil peroxidase; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; CLC/Gal-10, Charcot-Leyden crystal protein/Galectin-10; Treg, regulatory T cell; EETs, eosinophil extracellular DNA traps; EAR, eosinophil-associated ribonuclease; NMS, *N*-methyl scopolamine; DC, dendritic cell; LPLase, lysophospholipase.

MBP-1:
2° granule core, toxic to cells, tissues & helminth parasites, disrupts membranes, binds heparan sulfates, inhibits M2 muscarinic receptor, basophil/mast cell and neutrophil activation/secretion, epithelial activation & expression of tissue remodeling factors, antibacterial, component of eosinophil extracellular DNA traps.

EDN/RNase2:
2° granule matrix, RNase-2, potent ribonuclease, c-mannosylated, CNS – Purkinje cell toxicity (Gordon Phenomenon), dendritic cell chemotaxis, maturation & activation, anti-ssRNA virus activity.

EPX/EPO:
2° granule matrix, forms hypohalous acids and HOSCN-, tyrosine nitration of EDGPs, toxic to helminth parasites, bactericidal, induces epithelial activation & expression of tissue remodeling factors, lipid peroxidation.

CLC/Gal-10:
Cytosolic and 1° granule, hydrophobic and autocrystallization, galectin superfamily but glycan ligand has not been identified, binds to but is not a lysophospholipase as originally characterized.

ECP/RNase3:
2° granule matrix, weak ribonuclease, CNS – Purkinje cell toxicity (Gordon Phenomenon), membrane disruption (non-ion selective membrane pores), toxic to cells, helminth parasites, antibacterial, component of eosinophil extracellular DNA traps, amyloid-like aggregation.
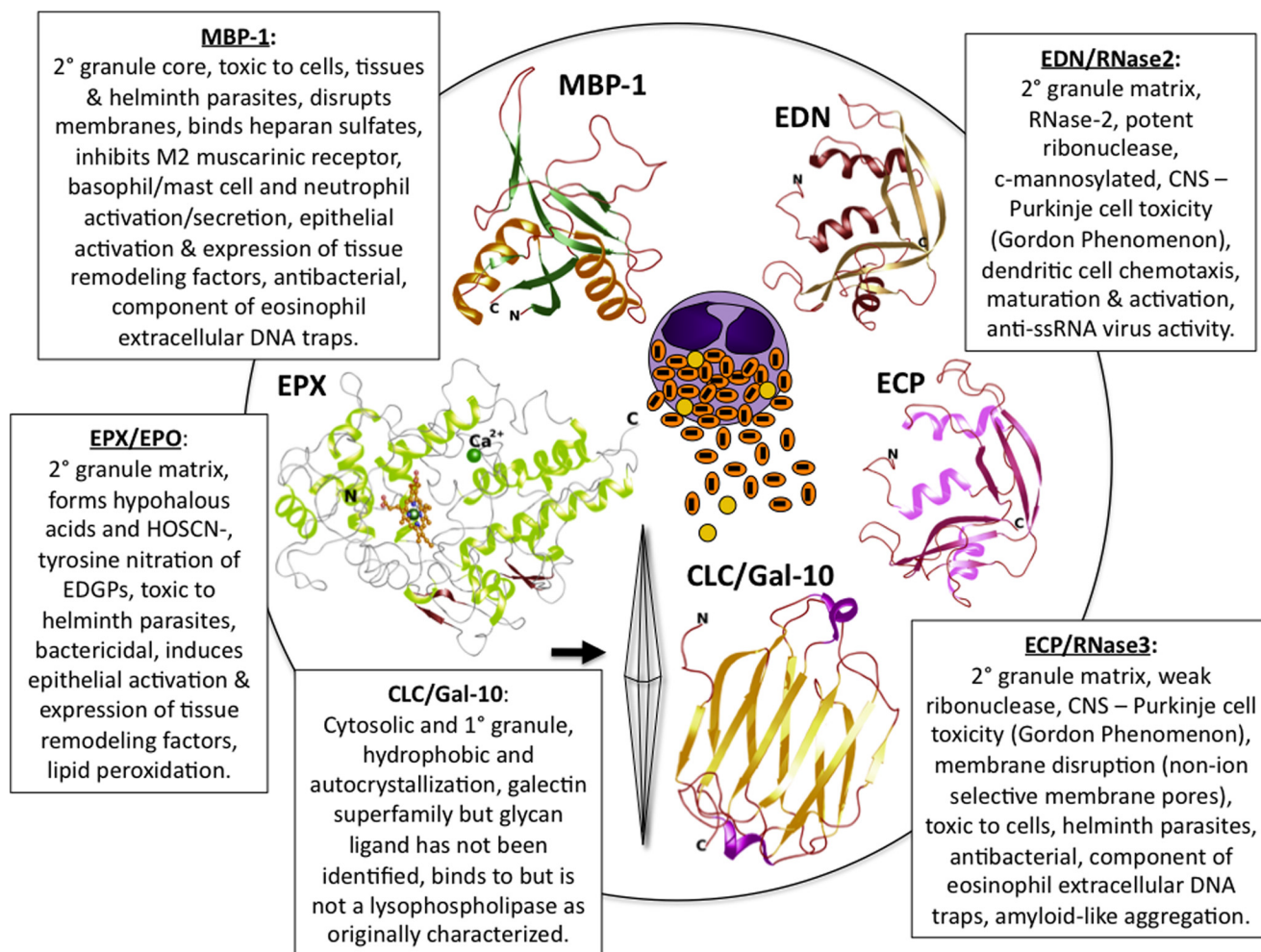
FIGURE 1. **Structural representations of the human eosinophil granule proteins showing their location and known functions.** The cationic granule proteins shown are: MBP-1 (Protein Data Bank (PDB) code 1H8U); EDN (RNase-2) (PDB code 1HI2); ECP (RNase-3) (PDB code 1QMT); and EPX/EPO) (based on molecular modeling using a myeloperoxidase structure, PDB code 1D2V). Also shown is CLC/Gal-10 (PDB code 1LCL), which is mainly cytosolic but also present in a small residual population of primary large core-less granules in the mature eosinophil. CLC/Gal-10 forms the hexagonal bipyramidal crystals (*arrow*) considered a hallmark of eosinophilic inflammation in tissues and body fluids in eosinophil-associated diseases.

10), although not cationic, is a hydrophobic autocrystallizing protein comprising ~7–10% of total eosinophil protein (Fig. 1). EPX and MBP-2 are the only EDGPs uniquely expressed by the eosinophil and not other cells; the other EDGPs are variably expressed in ~10–100-fold lesser amounts by other blood leukocytes, tissues, and cells including basophils (MBP-1 (14, 15), EDN (16), CLC/Gal-10 (17, 18)), neutrophils (EDN, ECP) (19–21), liver (EDN) (22), and regulatory T cells (Tregs) (23, 24) and T_H2 central memory T cells (CLC/Gal-10) (25). Although these EDGPs are expressed and may be secreted by these other cell types at sites of host innate and adaptive immune responses and inflammation, aside from expression of CLC/Gal-10 by regulatory T cells (23), the roles and functions of these proteins beyond those of eosinophils have not been investigated, other than their potential use as biomarkers.

Eosinophils form extracellular DNA traps (EETs), a component of innate antibacterial immune responses in a number of eosinophil-associated infectious, allergic, and autoimmune diseases (26, 27). EETs consist of a meshwork of DNA fibers (formed from mitochondrial rather than nuclear DNA), and a number of EDGPs, including MBP-1 and ECP, co-localize in the EETs, suggesting that they participate in trapping and killing of bacteria by this mechanism (28). EETs were identified in the lung in allergic asthma (29) and in a number of allergic/reactive skin diseases including allergic contact dermatitis, ectoparasitoses, and larva migrans (30). Multiple mechanisms of eosinophil priming and activation that include signaling through toll-like, cytokine, chemokine, and adhesion receptors initiate the formation of EETs containing the EDGPs (26, 31) in a process requiring activation of NADPH oxidase. Although the specific role of the EDGPs in EETs has not been determined, their formation may provide a mechanism for bringing together these cationic toxins with their pathogen targets, or alternatively limit collateral tissue damage by the EDGPs (26).

Eosinophil Major Basic Protein-1

MBP-1 (13.8 kDa, extremely basic, pI = 11.4) is an abundant granule protein localized to the electron-dense crystalloid core of the secondary granule. The protein is initially expressed as a 25.2-kDa polypeptide (pre-pro form), comprising a highly acidic "pro-domain" and highly basic MBP-1 protein. The pro-domain is thought to neutralize the basic nature of MBP-1 dur-

ing its synthesis, transport, and packaging in the eosinophil granule, but structural or functional studies are lacking. MBP-1 is highly toxic to mammalian cells *in vitro* and can damage helminths, bacteria, and mammalian cells (32). MBP-1 damages cells by disrupting the lipid bilayer membrane (32) or altering the activity of enzymes within tissues. MBP-1 stimulates mediator (histamine) release from basophils and mast cells, activates neutrophils and platelets, and augments superoxide generation by alveolar macrophages. MBP-1 levels are elevated in biological fluids (*e.g.* sputa and bronchoalveolar lavage fluids) from patients with asthma and other eosinophil-associated diseases. MBP-1 induces bronchoconstriction when administered into monkey lung and induces transient airway hyperreactivity when administered into rat, rabbit, or monkey trachea (33). These effects could be neutralized by polyanions such as heparin or polyglutamic acids. MBP-1 has been implicated in tissue damage associated with eosinophil infiltration, notably to the respiratory epithelium in asthma. MBP-1 binds lactoferrin (34) and is a potent inhibitor of recombinant and eosinophil heparanase (35). Direct instillation of MBP-1 into the mouse airway induces airway remodeling, including induction of epithelial TGF-β and MMP1 expression and subepithelial fibrosis (36). In a model of Duchenne muscular dystrophy and the *mdx* mouse model of Duchenne muscular dystrophy, eosinophils were shown to lyse muscle cells *in vitro* through release of MBP-1, and MBP-1 promoted fibrosis of dystrophin-deficient muscle and attenuated the cellular immune response to these cells (37).

The role of MBP-1 in the development of allergen-induced pulmonary pathologies in asthma has been studied in mouse asthma models in which the MBP-1 gene was knocked out (MBP-1$^{-/-}$) (38); MBP-1 deficiency had no effect on the development of allergen-induced airway histopathologies or inflammatory cell recruitment, nor any effects on airway hyperresponsiveness, which still developed in the absence of mouse MBP-1. Knock-out of the EPX gene (EPX$^{-/-}$) and resulting EPX deficiency had no impact on asthma-associated pulmonary pathologies induced by allergen sensitization and provocation (39), suggesting that the contributions of MBP-1 and EPX to disease pathology in allergic diseases likely occurred via their combined activities. Crossing MBP-1$^{-/-}$ and EPX$^{-/-}$ knock-out mice to address this issue in double knockouts unexpectedly generated a novel strain of mice with a highly specific deficiency in eosinophilopoiesis, and therefore eosinophils (9). Although the mechanism for the eosinophil deficiency in these double knock-out mice remains to be determined, results suggest that: (i) granule protein gene expression and/or defective granulogenesis may be a checkpoint for survival of eosinophil progenitors, (ii) loss of concurrent expression of MBP-1 and EPX disrupts lineage-instructive gene regulatory mechanisms required for self-renewal or eosinophil progenitor survival, or (iii) most likely, dysfunctional granulogenesis leads to aberrant intracellular release of a toxicant such as mouse eosinophil-associated ribonucleases (EARs) capable of degrading intracellular RNAs, leading to the observed cell-autonomous defect (39).

A homologue of MBP-1, called MBP-2, was identified and shown expressed exclusively by eosinophils. MBP-2 has ~66% amino acid sequence identity with MBP-1 but is significantly less basic (pI = 8.7) (40). *In vitro* activities of MBP-1 and MBP-2 appear similar, *e.g.* cell destruction, induction of superoxide anion, IL-8 release from neutrophils, and induction of histamine and leukotriene C4 release from basophils, but human MBP-1 is more potent than MBP-2 in these activities (52). MBP-2, present only in eosinophils, may be a useful biomarker for eosinophil-associated diseases, but its utility has not been evaluated.

MBP-1 is a monomer under physiologic conditions, but readily polymerizes in solution, forming insoluble aggregates due to the presence of five reactive thiol groups (in addition to four cysteines involved in disulfide bond formation) (41). MBP-1 is synthesized as a precursor that is proteolytically processed to the mature granule form during packaging into the crystalloid core of the granule. The pro-domain removed in this process is heavily glycosylated with *N*-glycans, *O*-glycans, and glycosaminoglycans, raising the molecular mass to ~30–50 kDa. MBP-1 does not exhibit high sequence similarity to other proteins aside from weak similarity (23–28%) to C-type lectin domains and the low affinity IgE receptor FcϵRII (42).

The three-dimensional structure of MBP-1 (Fig. 1) (43) shows that its overall topology is similar to that of C-type lectin domains, in particular to lithostathine, a glycoprotein expressed by exocrine pancreas. We showed that none of the amino acid residues involved in calcium binding in classical C-type lectins is conserved in MBP-1. The region corresponding to the carbohydrate-binding site in MBP-1 is highly basic and thus differs in structure from that of the other C-type lectins. The crystal structure of MBP-1 in complex with heparin disaccharide (Fig. 2*A*) showed that heparan sulfate may be a ligand (in agreement with data showing MBP-1 binding to heparin) (44). It is likely that heparan sulfate is not the sole physiologic ligand for MBP-1, and it may have the capacity to recognize a wide variety of sulfated ligands.

MBP-1 has been shown to function *in vitro* and *in vivo* as an endogenous allosteric antagonist of the inhibitory muscarinic M2 receptor (45). Fryer and colleagues (45) showed that MBP-1 potently inhibits binding of *N*-methyl scopolamine (NMS) to guinea pig M2, but not M3, receptors. MBP-1 was found to inhibit atropine-induced dissociation of NMS-receptor complexes, showing that MBP-1 interaction with the M2 receptor is allosteric, suggesting that it may function as an endogenous allosteric inhibitor of agonist binding to this inhibitory receptor. Inhibition of NMS binding by MBP-1 was reversible by heparin, which binds and neutralizes MBP-1, consistent with structural findings. Because eosinophils secrete MBP-1 during allergen-induced airway inflammation, and treatment of allergen-challenged or ozone-challenged guinea pigs with heparin or a neutralizing antibody to MBP-1 restores M2 receptor function (46–48), eotaxin/CCR3-mediated eosinophil recruitment to and release of MBP-1 on airway nerves may contribute to M2 receptor dysfunction and vagally mediated bronchoconstriction in the asthmatic diathesis.

Eosinophil-derived Neurotoxin

EDN is a small, basic protein that belongs to the ribonuclease A (RNase A) superfamily (19). It is localized to the matrix of the secondary granule of the eosinophil (49) and is also known as
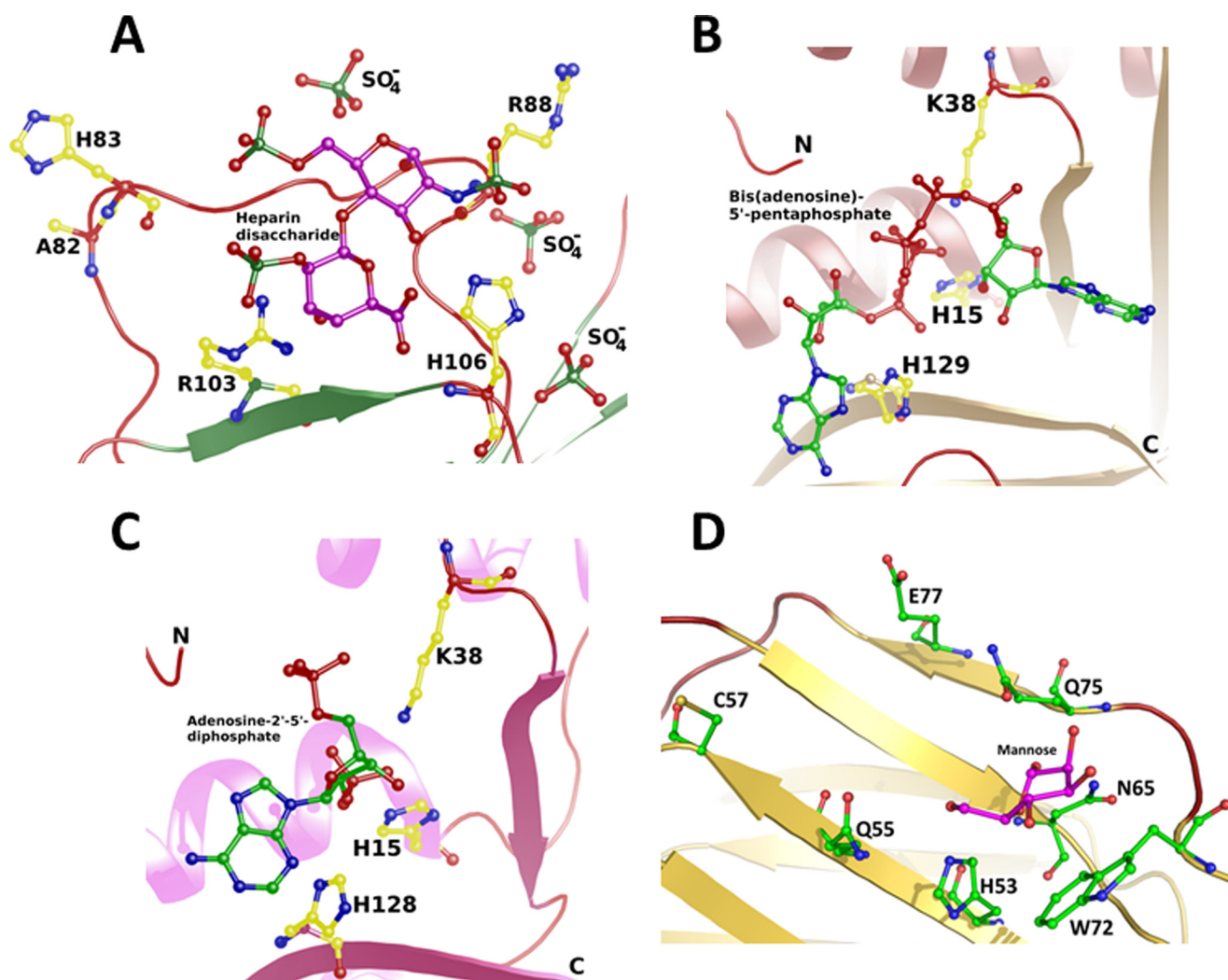
FIGURE 2. **Molecular details of the functional sites identified thus far.** *A*, MBP-1 carbohydrate-binding region with bound heparin disaccharide and sulfate ions (PDB code 2BRS). *B*, EDN ribonucleolytic active site with bound inhibitor bis(adenosine)-5′-pentaphosphate (PDB code 1HI5). *C*, ECP ribonucleolytic active site with bound adenosine-2′,5′-diphosphate (PDB code 1H1H). *D*, CLC/Gal-10 carbohydrate recognition domain with bound mannose (PDB code 1QKQ).

RNase-2, nonsecretory RNase, and RNase-Us, the latter based on its specificity toward uridine-containing nucleotides. EDN is one of the most abundant RNases in humans and has been isolated from a wide variety of sources including eosinophil, placenta, liver, and urine. There is ~3.3 μg of EDN/10^6 eosinophils (16). The initial identification of EDN was based on its induction of ataxia, incoordination, spasmodic paralysis, muscle stiffness, and killing of cerebellar Purkinje cells when injected intrathecally into rabbits, a paralytic syndrome termed the Gordon phenomenon (52, 53).

Although EDN shares 67% amino acid sequence identity with ECP, its sequence identity with RNase A is only 36%. The ribonucleolytic activity of EDN is ~3–30-fold lower than that of RNase A, depending on the substrate (22). This enzymatic activity is a prerequisite for its cytotoxic, neurotoxic, and antiviral activities (22, 51, 54). The primary sequence of EDN contains a Trp-*X-X*-Trp motif between residues 7 and 10, specifying an unusual *C*-mannosylation of Trp-7. This involves attachment of an *R*-mannosyl residue via a C–C link to the indole moiety of Trp-7, the first example of this post-translational modification (55). Unlike the other EDGPs, EDN is a

poor cationic toxin with limited toxicity for helminth parasites and mammalian cells at high concentrations (50, 56). However, as a ribonuclease, it is considerably more effective against single-stranded RNA viruses (51).

EDN activates human dendritic cells (DCs), leading to their expression of a variety of inflammatory chemokines, cytokines, growth factors, and soluble receptors (57). EDN also induces both phenotypic and functional maturation of DCs, as well as acts as an alarmin that activates the TLR2-MyD88 signaling pathway in DCs, enhancing Th2 immune responses (58).

The crystal structure of recombinant EDN (59) showed that the topology of the molecule includes the RNase A fold (Fig. 1) and that the core ribonucleolytic active site architecture (Fig. 2*B*) is conserved, although both ECP (60) and EDN exhibit significant differences at the peripheral substrate-binding sites (61). High-resolution crystal structures in complex with nucleotide inhibitors (59) present a detailed picture of differences and flexibility between EDN and RNase A in substrate recognition (Fig. 2*B*).

In the mouse, the related family of EARs, initially identified by Lee and colleagues (62), exhibits highly divergent properties

both from one another and from human EDN and ECP. Zhang *et al.* (63) showed that there is a striking similarity between the evolutionary patterns of the mouse EAR genes and those of the major histocompatibility complex, immunoglobulin, and T cell receptor genes, enabling them to hypothesize that host defense and generation of diversity are among the primary physiological functions of the murine EARs. The discovery of a large number of divergent EARs suggests the intriguing possibility that these proteins have been specifically tailored through evolution to fight against distinct mouse pathogens (63).

Eosinophil Cationic Protein

ECP also belongs to the RNase A superfamily and is known as RNase-3 (20, 64, 65). Mature ECP is a small cationic polypeptide of 133 residues. Similar to EDN, it is located in the matrix of the specific granule of the eosinophil, but as compared with EDN (pI = 8.9), ECP is considerably more cationic (pI = 10.8). There is ~5.3 μg of ECP/10^6 eosinophils (16). Like EDN, ECP induces the neurotoxic Gordon phenomenon (52). ECP has marked toxicity for a variety of helminth parasites, hemoflagellates, bacteria, single-stranded RNA viruses, and host tissues (66). Serum ECP levels can be used as a clinical tool for estimating eosinophil inflammatory activity in asthma and other allergic diseases, and levels are related to disease severity. The antibacterial activity and parasitic toxicity of ECP are greater than EDN (66). *In vitro*, ECP can function as an antiviral agent and may participate in host defense against the single-stranded RNA respiratory syncytial virus (67). The toxicity of ECP for bacteria and helminths does not appear related to its RNase activity (66), whereas RNase activity is required for its antiviral (67) and neurotoxic activities (52). The RNase activity of ECP is 100-fold lower than EDN for most RNA substrates, and their *in vivo* substrates have not been identified (20, 68).

The crystal structure of ECP (60) shows the "RNase fold" (Fig. 1), but with significant divergence from RNase A and EDN. The structure also shows how the cationic residues are distributed on the ECP surface, an observation that may have implications for understanding the considerable cytotoxicity of this enzyme. The structure of ECP in complex with adenosine-2′,5′-diphosphate revealed details of the active site (Fig. 2C) and a structural explanation for the lower substrate affinity and catalytic efficiency of ECP (69).

Although eosinophils and their EDGPs are associated with host defense responses against helminth parasites, a number of the EDGPs also possess antibacterial activity. ECP has antibacterial activities not shared by EDN (70, 71). Evidence is accumulating that eosinophils and the EDGPs may participate in host responses to certain bacterial infections (72). ECP is active *in vitro* against both Gram-negative and Gram-positive strains of bacteria, its mechanism of toxicity involving both the bacterial cell wall and the cytoplasmic membrane. Torrent *et al.* (73) propose and provide evidence for a novel molecular mechanism to explain the bacterial agglutinating activity of ECP, showing *in situ* formation of fibrillar, amyloid-like aggregates at the bacterial cell surface that bind amyloid diagnostic dyes. The agglutinating activity of ECP appears driven by the amyloid-like aggregation of the protein at the bacteria cell surface; elimination of the amyloidogenic behavior by a single point mutation

(I13A) abolished both its agglutinating and its antimicrobial activities, the mutant being defective in triggering leakage and lipid vesicle aggregation. These findings support the novel concept that the amyloidogenic behavior of ECP, and possibly other EDGPs, participates in antibacterial host responses to infection, and suggest that the biophysical properties of bactericidal N-terminal peptides of ECP (amino acids 1–45) (74) or other EDGPs might guide development of novel antimicrobials (75).

Native ECP purified to homogeneity from blood leukocytes or purified eosinophils shows considerable molecular heterogeneity, from multiple glycosylated isoforms to the nonglycosylated native protein, as well as functional heterogeneity of these glycoforms relative to nonglycosylated ECP with respect to its cytotoxic activity for mammalian cells (76). A gene polymorphism in the coding region, ECP 434(G>C), determines the cytotoxicity of ECP for mammalian cells but has minor effects on fibroblast-mediated gel contraction (measure of fibroblast activation) and no effect on ECP RNase activity (77). This polymorphism changes an arginine (base at 434 is G) at position 97 to threonine (base at 434 is C). The ECP 434(G>C) polymorphism correlates with the natural course of *Schistosoma mansoni* infection (78) and with inflammatory bowel disease in an age- and gender-dependent manner (79). These ECP genotypes also show associations with the symptoms of allergy and asthma (80, 81). Venge and colleagues (82) reported that the various ECP glycoforms are processed during eosinophil secretion; the modifications to secreted ECP by activated eosinophils is explained in part by differences in their degree of *N*-linked glycosylation, such that secreted ECP acquires the masses of the more cytotoxic, less glycosylated, isoforms, including the nonglycosylated species (82), explaining in part the structural and functional heterogeneity of ECP as reported in the literature.

Eosinophil Peroxidase (EPX/EPO)

During activation, eosinophils can generate potentially toxic reactive oxygen species, which unlike the neutrophil, are mainly directed extracellularly (83). Oxidant production begins with the generation of superoxide by the membrane bound NADPH oxidase of eosinophils, which dismutates into hydrogen peroxide (H_2O_2). EPX, the most abundant cationic protein of the matrix of the specific granule, uses this H_2O_2 as an oxidizing substrate to generate potent oxidizing species, including hypohalous acids. In addition to bromide and chloride, EPX preferentially uses thiocyanate (SCN^-) ions to generate $HOSCN^-$, shown by Wang *et al.* (84, 85) to exert considerable biologic activity, *e.g.* as a potent oxidant inducer of tissue factor activity in endothelial cells; the $HOSCN^-$ generated by EPX from activated tissue eosinophils may induce the prothrombotic and proinflammatory endothelial and endocardial phenotypes responsible for thrombotic complications seen in the hypereosinophilic syndrome. EPX is structurally distinguished from the other EDGPs, being a two-chain hemoprotein (68 kDa); it is initially synthesized as a single chain precursor that is proteolytically processed to a 55-kDa heavy chain and 12.5-kDa light chain. EPX is highly cationic and similar to ECP and MBP-1 in this regard. Biochemical evidence suggests that

Classical Exocytosis Compound Exocytosis Piecemeal Degranulation Cytolysis
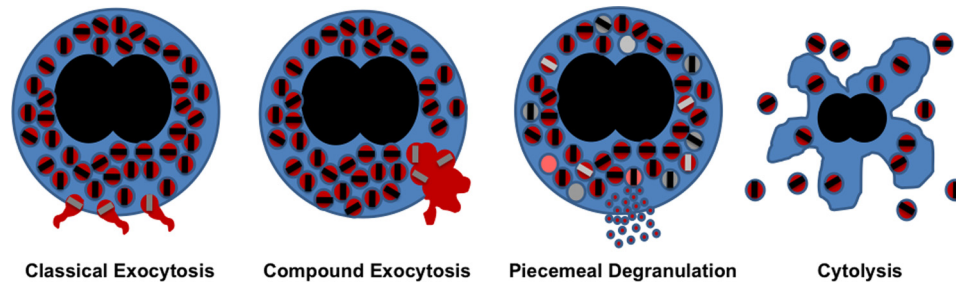
FIGURE 3. **Mechanisms of eosinophil degranulation.** Eosinophils release their secondary (specific) granule contents, including the granule cationic proteins and other immunomodulatory mediators, by four different mechanisms. In classical exocytosis, mainly seen at sites of bacterial infection, the contents of single granules are released by fusion of the granule membrane with the plasma membrane lipid bilayer. In compound exocytosis, mainly seen with eosinophil degranulation onto helminth parasites, a number of granules first coalesce and fuse, and the cationic protein contents are then released through a single fusion pore at the plasma membrane. In piecemeal degranulation, mainly seen in eosinophil inflammatory responses in human tissues, secretory vesicles bud from granules, capturing granule matrix and/or core contents, and are targeted to the plasma membrane in a fashion analogous to the release of neurotransmitters from neurons. Differential secretion of the cationic proteins from the matrix (EPX, EDN/RNase-2, ECP/RNase-3) or core (MBP-1, MBP-2) of the granule has been shown to occur, leaving coreless granules with intact matrix or intact cores with no matrix in the cell. In cytolysis, intact whole granules are deposited in tissues and body fluids after disruption of the plasma membrane due to eosinophil necrosis.

EPX is structurally related to myeloperoxidase (Fig. 1) present in neutrophil-specific granules. Patients with myeloperoxidase deficiency have normal levels of EPX, indicating independent expression mechanisms for these peroxidases. There is ∼12 μg of EPX/10^6 eosinophils (16). EPX exerts some cytotoxic effects as a cationic toxin, being able to kill parasites (86, 87) and mammalian cells in the absence of H_2O_2 and a halide co-factor. Furthermore, EPX exerts both anti-inflammatory (88) and pro-inflammatory (89) activities.

All of the human EDGPs including EPX itself, MBP-1, EDN, and ECP, are post-translationally modified by EPX via nitration at specific tyrosine residues during their synthesis and packaging in the developing eosinophil (90). High-resolution affinity-mass spectrometry showed single specific nitration sites at Tyr-349 in EPX and Tyr-33 in both EDN and ECP, and crystal structures of EDN and ECP, as well as structural models of EPX, suggest that these nitrated tyrosine residues are surface-exposed. Studies in EPX$^{-/-}$, gp91phox$^{-/-}$, and NOS$^{-/-}$ knock-out mice showed that tyrosine nitration of these cellular toxins and ribonucleases is mediated by EPX itself in the presence of H_2O_2 and small amounts of nitrogen oxide. Thus, EPX appears to nitrate itself via an autocatalytic mechanism. Tyrosine nitration of the EDGPs was shown to occur during eosinophil differentiation and was independent of inflammation. The specific roles of EPX-mediated nitrosylation of the EDGPs in eosinophil-mediated innate host immune defense mechanisms characterized by their secretion during cell activation, *e.g.* against parasites or during tissue damage in parasitic infections or allergic responses, have not been determined.

Charcot-Leyden Crystal Protein

CLC protein forms distinctive bipyramidal hexagonal crystals, hallmarks of eosinophil participation in allergic and related inflammatory reactions. These crystals, found at sites of eosinophil infiltration in tissues and in body fluids and secretions, were identified more than 150 years ago. CLC is a small, slightly acidic (pI ∼5.1–5.7) 142-amino acid protein of 16.5 kDa (91). It was initially identified as an eosinophil lysophospholipase (LPLase) (92) but has since been assigned to the galectin superfamily of S-type animal lectins as the 10th member (Galectin-10) based on amino acid sequence (91), three-dimensional

structure (CLC/Gal-10, Fig. 1) (93), and genomic organization (94). Importantly, we showed that CLC/Gal-10 lacks any LPLase activity and that the weak enzymatic activity initially associated with the purified protein was due to contamination by a highly active 75-kDa pancreatic LPLase also expressed by eosinophils (95).

The crystal structure of CLC/Gal-10 provides details of a carbohydrate recognition domain with both similarities to and important differences from other members of the galectin family (93). Structural studies showed that CLC/Gal-10 does not bind β-galactosides, but can bind mannose in the crystal in a unique manner different from the binding of lactosamine carbohydrates by other related galectins (Fig. 2D) (96). The physiologic significance of this mannose binding remains equivocal. The partial conservation of residues involved in carbohydrate binding leads to significant changes in the topology and chemical nature of the carbohydrate recognition domain and has implications for glycan recognition by CLC/Gal-10 *in vivo*. These findings are beginning to provide clues toward identifying a physiologically relevant ligand for CLC/Gal-10 and understanding its potential intracellular and extracellular roles in eosinophil biology.

Our recent experiments using Southwestern (ligand) blotting, co-purification, co-immunoprecipitation, and confocal microscopy show that CLC/Gal-10 interacts *in vitro* and intracellularly in activated eosinophils with the two glycosylated human eosinophil granule cationic ribonucleases, EDN and ECP.[4] Studies in human blood eosinophils using immunofluorescence confocal microscopy show that interferon-γ activation induces the rapid co-localization of CLC/Gal-10 with EDN and CD63.[4] Because CLC/Gal-10 does not inhibit the ribonuclease activity of EDN, it may function instead as a carrier for the sequestration and vesicular transport of these ribonucleases, during granulogenesis in the differentiating eosinophil, and during piecemeal degranulation (Fig. 3) in the activated eosinophil, enabling their extracellular functions in host defense and allergic inflammation without intracellular damage to the eosinophil itself during their secretion.

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[4] C. B. Doyle and S. J. Ackerman, unpublished results.

In addition to its considerable expression at both mRNA and protein levels in eosinophils and basophils, CLC/Gal-10 was identified as a constituent of human regulatory T cells. A global proteomics analysis of highly purified human $CD4^+CD25^+$ Tregs identified CLC/Gal-10 as a novel biomarker, shown by siRNA knockdown to be essential for maintaining Treg anergy and suppressive functions on T cell activation (23). The mechanism by which CLC/Gal-10 participates, through galectin-type interaction with a glycan ligand(s) or protein-protein interaction, in maintaining the $CD4^+CD25^+$ Treg phenotype has not been established.

A number of studies identify CLC/Gal-10 as a potentially useful biomarker of eosinophil involvement in asthma, allergic rhinitis, and other eosinophil-associated diseases. Elevated levels of CLC/Gal-10 have been measured by one of us, by ELISA, as a biomarker of active eosinophilic inflammation that is highly correlated with the number of tissue (esophageal) eosinophils in eosinophilic esophagitis (97), a rare immune-mediated food allergic disease of increasing incidence. CLC/Gal-10 was also identified as a potentially useful biomarker of eosinophilic airway inflammation in induced sputum for identifying the eosinophilic phenotype of asthma to guide treatment considerations (98). In celiac disease, CLC/Gal-10 expression was found related to both disease activity (histologic grade) and numbers of tissue eosinophils in intestinal lesions, suggesting it as a novel biomarker for evaluating tissue damage and eosinophil involvement in the pathogenesis of this gluten intolerance. Finally, genetic variations (SNPs) in the promoter region of the *CLC* gene were identified as potential susceptibility biomarkers for allergic rhinitis (99), with the pattern of variation compatible with a recessive inheritance model and observed increased levels of CLC/Gal-10 protein in the nasal fluid of patients with allergic rhinitis during the allergy season (100).

Perspectives

With a goal toward understanding the roles and specific functions of the EDGPs in the normal and pathologic activities of the eosinophil, significant progress has been made by determining the three-dimensional structures of four of these mediators using x-ray crystallographic approaches. These studies provided novel insights and vital clues toward understanding the structural basis for their unique biologic and enzymatic activities at a molecular level, paving the way for future "form and function" analyses to better define their unique biochemical properties, biologic activities, and pathologic contributions to eosinophil-mediated inflammatory responses, tissue damage and repair, remodeling, and fibrosis, as well as innate and adaptive host immune responses to infectious agents including parasitic helminths, bacteria, and viruses.

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