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A Pulmonary Perspective on GASPIDs: Granule-Associated Serine Peptidases of Immune Defense

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Abstract

Airways are protected from pathogens by forces allied with innate and adaptive immunity. Recent investigations establish critical defensive roles for leukocyte and mast cell serine-class peptidases garrisoned in membrane-bound organelles—here termed Granule-Associated Serine Peptidases of Immune Defense, or GASPIDs. Some better characterized GASPIDs include neutrophil elastase and cathepsin G (which defend against bacteria), proteinase-3 (targeted by antineutrophil antibodies in Wegener’s vasculitis), mast cell β -tryptase and chymase (which promote allergic inflammation), granzymes A and B (which launch apoptosis pathways in infected host cells), and factor D (which activates complement’s alternative pathway). GASPIDs can defend against pathogens but can harm host cells in the process, and therefore become targets for pharmaceutical inhibition. They vary widely in specificity, yet are phylogenetically similar. Mammalian speciation supported a remarkable flowering of these enzymes as they co-evolved with specialized immune cells, including mast cells, basophils, eosinophils, cytolytic T-cells, natural killer cells, neutrophils, macrophages and dendritic cells. Many GASPIDs continue to evolve rapidly, providing some of the most conspicuous examples of divergent protein evolution. Consequently, students of GASPIDs are rewarded not only with insights into their roles in lung immune defense but also with clues to the origins of cellular specialization in vertebrate immunity.

Keywords

Neutrophil elastase; cathepsin G; tryptase; chymase; granzyme; factor D

WHERE DO GASPIDS FIT IN THE DEGRADOME?

In primates, the degradome comprises ~560 peptidase genes [1,2]. Of these, ~150 encode proteases of the serine class, all of which use a serine side chain hydroxyl group to attack peptide bonds and initiate hydrolysis. Slightly more than half these serine peptidases belong to the structurally related, ~one billion years-old trypsin family, which customizes the substrate binding site for each enzyme within a cleft formed by twin “ β -barrel” protein domains. The variation in substrate preference, target specificity and biological function achieved by varying the topography of amino acid side chains lining the binding site is remarkable. GASPIDs are a robust subset of this group, related in form but varied in function. In one way or another most GASPIDs are proven or presumed to serve immune defense (see below); however, several GASPIDs, although expressed in immune cells, lack an assigned function. In mammals, the number of GASPID genes is not well conserved. Comparisons of degradomes or humans, rats

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and mice, for example, reveal striking proliferation of mast cell chymase and granzyme B-like genes in rodent genomes compared to primate and canine genomes [1,3–7].

CLUSTERING AND EXPANSION OF GASPID GENES

Generating Diversity Through Gene Multiplication

Mammalian GASPID genes tend to congregate in clusters, which reveal clues about shared evolutionary origins and mechanisms by which these genes proliferated, diversified, and, in some cases, came to share patterns of expression. Human GASPID genes segregate into four clusters: 5q11.2 (*GZMA*, *GZMK*), 14q11.2 (*CMA1*, *CTSG*, *GZMH*, *GZMB*), 16p13.3 (*TPSG*, *TPS1*, *TPSB2*, *TPSD*), and 19p13.3 (*NE/ELA2*, *DF*, *PRTN3*, *AZU1*, *PRSSL1*, *GZMM*). These genes and the proteins they encode are listed and decoded in Table 1. As noted, all of these genes encode trypsin-family serine peptidases and are related through a shared ancestor (Fig. 1). However, tryptase GASPIDS of chromosome 16p 13.3 are more distantly related than any of the other GAS-PIDs. Most tryptases are soluble but one of them (γ -tryptase, product of the *TPSG* gene) is a type I transmembrane peptidase with a C-terminal peptide anchor. The closest non-GASPID relatives of mammalian tryptases, whether soluble or membrane-anchored, themselves are type I transmembrane peptidases (for example prostasin, *PRSS8*) [8]. Curiously, it thus appears that 16p13.3 tryptases evolved from membrane-anchored ancestors [9], with some losing their hydrophobic tails, thereby becoming free of membrane attachments. The remaining GASPIDS on 5q11.2, 14q11.2 and 19q13.3 are more closely related to each other, sharing idiosyncrasies of gene and protein structure [4,6,10–12]. Therefore, it is clear that these genes shared a common ancestor more recently than with the 16p13.3 tryptases. These hypotheses about shared ancestry also are supported by formal phylogenetic comparisons of primary structure [6,8,9,12–14]. Notably, the genes within each cluster are more closely related to each other than to genes in other clusters, suggesting that the multiplicity of genes in each cluster arose *via* duplications of once-solitary genes in isolated loci.

Gene Conversion and Specificity-Altering Mutations

In several cases, as in the human $\alpha/\beta/\delta$ -tryptases, gene conversion caused tandemly arrayed genes to become more similar with time rather than increasingly dissimilar [15,16]. In others, as in human chymase (*CMA1*)/cathepsin G (*CTSG*)/granzyme B (*GZMB*) [14,17] and mouse granzyme B-like clusters [13], similar genetic events created chimeric genes and proteins. Additional diversity was generated by traditional point mutations affecting amino acid determinants of activity and substrate specificity. In GASPIDS as in other peptidases, small changes in sequence in the vicinity of the catalytic center and substrate-binding site can cause large differences in behavior. Examples include a single amino acid mutation converting α -tryptase to a largely inactive enzyme [18,19] and a murine MCP5 mutation drastically altering specificity from chymotryptic to elastolytic [20,21]. The often dramatic differences in mammalian genomes with regard to GASPID number, type and specificity (Table 2) suggests that selective evolutionary pressures may have accelerated incorporation of changes into the genome. The driving force behind this putative hyper-evolution is unclear. One possibility is that GASPID genes are co-evolving with pathogen genes, such as those encoding peptidase inhibitors or GASPID-susceptible target peptides affecting virulence.

COMMONALITIES AMONG GASPIDS

Gene Superstructure

In addition to the fact that GASPIDS are trypsin-family serine peptidases, several other features are hallmarks of these enzymes as a group. One of these is shared gene organization. In most GASPID genes, the number, phase and placement of introns in relation to exons encoding propeptide and serine peptidase-defining residues is similar and highly characteristic of the

group [4,12,17,22,23]. Conservation of such patterns preserves evidence of shared ancestry despite hundreds of millions of years of evolution and much writing and rewriting of protein-coding sequence. The intron-exon organization of mast cell tryptase genes, however, is sufficiently different that this group of genes stands apart from (and likely diverged longer ago from) other GASPID genes [8,24,25].

Milieu and Mechanics of Activation

One of the most consistent shared features is an intracellular setting for activation. This characteristic, which provided some of the first hints of GASPID existence [26,27], is now known to involve proteolytic maturation by excision of a propeptide. Like most peptidases, GASPIDs are synthesized initially as nearly inactive zymogens (Fig. 2). Mature human chymase, for example, is 7,000-fold more active than its proenzyme zymogen [28]. Unlike classic serine peptidases such as pancreatic trypsinogen, which is activated to trypsin after secretion from the exocrine cells of the pancreas, GASPIDs mature in the endoplasmic reticulum, Golgi and specialized intracellular granules. GASPID propeptides are short compared to those of other types of serine peptidases--typically just two amino acids. The soluble tryptases again are outliers. They have a longer propeptide, which appears to be removed in two steps, the first of which is autolytic, leaving a residual dipeptide (see below). Many if not all GASPIDs, including the dipeptide form of protryptase, can be activated by removal of the pro-dipeptide by dipeptidylpeptidase I (cathepsin C), an enzyme that is expressed in a wide variety of cells, but unlike classical lysosomal "housekeeping" cathepsins, is particularly abundant in mast cells [29,30] and white blood cells [31,32]. The creation of dipeptidylpeptidase I-null mice has allowed more global explorations of GASPID contributions to immune defense and to collateral tissue damage during immune reactions. Activation of most murine GASPIDs, including neutrophil elastase, proteinase 3, cathepsin G, mast cell chymases, and granzymes A and B depend profoundly on dipeptidylpeptidase I for activation [32–34]. For some murine GASPIDs, such as granzyme C and MCP-6/tryptase, dipeptidylpeptidase I may not be the sole activating enzyme [32,33].

Papillon-Lefevre Syndrome: A Human Disease Associated with Defective GASPID Activation

Redundant or alternative processing machinery also appears to exist in humans, as suggested by examination of leukocytes from subjects with Papillon-Lefevre syndrome, a collection of rare, autosomal recessive diseases characterized by severe periodontal disease during tooth eruption, palmo-plantar hyperkeratosis, and inherited defects in DPPI [35–37]. Such subjects have severe reductions in neutrophil elastase and cathepsin G activity in circulating neutrophils, but substantial retained granzyme activity and lymphocyte-activated killer cell-mediated cytotoxicity in some families with the syndrome [37]. The nature of the alternative GASPID activation pathways suggested by these phenotypes remains to be determined. Possibly, other dipeptidases, or a mono-aminopeptidase acting processively, can substitute for dipeptidylpeptidase I in the activation of some GASPIDs. In the special case of human α -tryptase, dipeptidylpeptidase I and related exopeptidases may be unable to process the pro-form because a mutation in the pro-dodecapeptide prevents initial autolytic trimming to a dipeptidylpeptidase I-cleavable pro-dipeptide form of the zymogen [38]. With respect to lung disease, the most important observation is the apparent lack of a major lung phenotype. Although numerous *in vitro* and genetic deletion studies in mice suggest that dipeptidylpeptidase I-activated neutrophil serine peptidases like elastase are important for bacterial killing, the lack of unusually frequent pneumonias in humans with Papillon-Lefevre syndrome suggests that the availability of active neutrophil serine peptidases is not of prime importance to host antibacterial defense. By the same token, the characteristics of these subjects also hint that pharmacological inhibition of elastases and other neutrophil peptidases to prevent lung and airway destruction in diseases like emphysema and cystic fibrosis may not be as immunosuppressive as one might predict from mouse studies.

Post-Activation Control of Activity: Sequestration in Granules

Given that GASPIDs can be markedly destructive to proteins and deadly to targeted cells, failure to control GASPID activity after removal of the pro-peptide could have untoward consequences for the cells of origin, adjacent extracellular matrix, and neighboring cells. GASPIDs and the cells that make them have evolved several strategies to limit bystander effects. Most GASPIDs are sequestered within membrane-bound intracellular organelles, or “granules”. This sequestration serves several purposes: (i) it separates GASPIDs from cytosolic proteins, thereby minimizing the potential for autodigestion; (ii) it allows proteases to accumulate until needed; (iii) it minimizes constitutive or uncontrolled release outside of the cell, allowing cell-specific regulation of secretion and coupling to external stimuli; (iv) it allows GASPIDs to be stored under conditions that would be inconsistent with cellular function if created elsewhere in the cell of origin. For example, many GASPID-rich granules, such as those in mast cells and neutrophils, have a low pH compared to the cytosol. As a rule, serine peptidase activity is optimal at neutral to alkaline pH; therefore, the acidic environment within GASPID granules reduces activity. Most GASPIDs are packaged in granules with high concentrations of proteoglycans like heparins and chondroitin sulfates. One function of these macromolecular polyanions is as a kind of packing material, allowing the GASPIDs, many of which are cationic, to be stored at high density-even in crystalline form-without repelling or degrading each other. The granule proteoglycans also can influence GASPID activity following release. For example, the rat chymase MCP1 is released from peritoneal mast cells imbedded in a pellet of proteoglycan, which swells upon being exocytosed but is not readily soluble. Chymase within the swollen pellet is active and able to cleave small substrates, but is afforded a large measure of protection from circulating antipeptidases, such as serpins, which are too big to penetrate the interstices of the granule [39]. On the other hand, β -tryptases are released from human mast cells as part of a fully soluble, proteoglycan-bound complex [40]. In this particular example, proteoglycan stabilizes the enzyme’s active conformation (see below) and prolongs activity after release [41,42].

Sheltering at the Cell Surface: Targeting in Autoimmune Vasculitis and Avoidance of Inhibition

Another GASPID strategy for regulating activity is engagement with the plasma membrane on the cell of origin’s extracellular surface. For example, a fraction of elastase and cathepsin G in neutrophils binds to the cell surface after secretion [43,44]. This position affords protection from circulating inhibitors, while at the same time limiting targets to those at the immediate interface between the neutrophil and its extracellular milieu. The protective effect is less pronounced in free neutrophils in solution [45] and may be most important in regions of direct contact between cells or between a cell and matrix. Another somewhat unique example among GASPIDs is provided by γ -tryptase, which is synthesized and secreted with a transmembrane peptide anchoring the enzyme to the mast cell surface [8,46,47], although it may be exported in membrane-bound exosomes [48,49]. GASPID secretion and surface exposure may contribute to the development of pathological antibodies in autoimmune disease. In an example important in lung disease, autoantibodies raised against proteinase 3 are the major component of antineutrophil cytoplasmic antibodies, which are the serological hallmark of Wegener’s vasculitis. More than just markers of the disease, the antibodies targeting neutrophil antigens such as proteinase 3 on the cell surface [50] may worsen the disease by activating neutrophils; further, increased cell surface expression of proteinase 3 increases risk of relapse (reviewed in [51]).

Self-Control

Another major control strategy is self-inhibition. The GASPID complement factor D (product of the *DF* gene in the 19p13.3 cluster) provides the most dramatic example [52]. The biological

activity of factor D appears to be limited to hydrolysis of one bond in a single protein substrate (C3b-bound complement factor B). Unlike most other GASPIDs and serine peptidases in general, factor D has very little activity towards small synthetic substrates and circulates in a mature, uninhibited form. Factor D's inactivity towards most substrates and inhibitors appears to be explained by self-blockade of the active site; its extreme specificity may relate to an ability of its one substrate to displace residues blocking the active site [52,53]. The self-inhibition principle also applies to self-compartmentalizing GASPIDs, such as soluble mast cell tryptases and granzyme A, albeit by a different mechanism. Mature α/β tryptases and related enzymes form non-covalently linked tetramers with each of the four catalytic sites facing into a central cavity to which access is limited to substrates and inhibitors small enough to reach and engage an active site [19,54]. The canine mast cell tryptase-like GASPID, mastin, is similar, although it can form oligomers with more than four catalytic subunits, some of which appear to be stabilized by formation of disulfide links between subunits [9,55]. Granzyme A plays a variation on this theme by forming a disulfide-linked dimer, which extends the substrate-binding cleft beyond what would be available in a monomer granzyme A [56,57], thereby increasing specificity and selectivity towards macromolecular substrates and restricting access to inhibitors.

Control by Antipeptidases: Implications for Emphysema

In classical fashion, extracellular activity of several GASPIDs is controlled (and often irreversibly terminated) by serpins and other antipeptidases (Table 3). As noted, factor D and mast cell tryptase/mastin-like enzymes are exceptions because they block access of large proteinaceous inhibitors to their active sites. Thus, not all GASPID activity is subject to physiological control by antipeptidases. Arguably the most widely known and studied GASPID-antipeptidase interaction is between neutrophil elastase and the serpin-class inhibitor α_1 -antitrypsin (also known as α_1 -proteinase inhibitor; gene name *PI* or *SERPINA1*). Like most serpins, α_1 -antitrypsin inhibits a variety of GASPID and non-GASPID serine peptidases of varying specificity. However, neutrophil elastase may be its most important substrate. This has been argued on the basis that interreaction kinetics of α_1 -antitrypsin and elastase are particularly favorable [58] and also that severe α_1 -antitrypsin deficiency in cigarette smokers is linked with early-onset emphysema, a disease thought to result from lung destruction by proteases--and by elastases especially [59–61]. Unlike type I collagenases, which tend to be selective for triple helical collagen, elastases are not specific for elastin. Indeed, elastases tend to be omnivorous enzymes with high destructive potential--hence the need for tight control. The lung phenotype of humans with severe deficiency of α_1 -antitrypsin, which is a major plasma protein, hints that its major role is to prevent tissue destruction by GASPID elastases. The fact that α_1 -antitrypsin also is an acute phase reactant suggests that extra inhibitory capacity may be needed in stress and infections [62], which are usually associated with increased production of neutrophils with their burden of GASPID elastases.

α_1 -Antitrypsin may not be the only serpin protecting the lung from accelerated breakdown. For example, α_1 -antichymotrypsin (gene name *AACT* or *SERPINA3*) also is linked to inherited, premature emphysema [63,64]. Compared to α_1 -antitrypsin, α_1 -antichymotrypsin is a more effective inhibitor of GASPIDs with chymotryptic activity, such as cathepsin G and to a lesser extent chymases, which may be its major physiological targets [65]. Thus, these enzymes also may destroy lung alveolar interstitium over time. Not all GASPID-inhibitor interactions take place extracellularly. For example, proteinase inhibitor 9, an intracellular serpin, appears to be the most important human inhibitor of granzyme B, which is expressed by cytolytic T lymphocytes, natural killer and mast cells and launches apoptosis programs when injected into target cells (reviewed in [66]). Expression of proteinase inhibitor 9 by a given cell affords protection from self- or bystander targeting by granzyme B [67,68]. It should be emphasized that GASPIDs are not the only peptidases targeted by serpins (although GASPIDs are the

peptidases most often implicated in lung infections, immune rejection, and emphysema). By the same token, serpins are not the only class of inhibitor capable of GASPID inactivation. In the specific case of neutrophil elastase, secretory leukoprotease inhibitor, which is a major part of anti-elastase defenses in the upper respiratory tract [69,70], and α_2 -macroglobulin, which is a large non class-specific antipeptidase of plasma and epithelial lining fluid [71], are examples of non-serpin inactivators. It is important to realize that neutrophil elastase bound to α_2 -macroglobulin remains able to cleave short peptide substrates (and will be measured as active in commonly used activity assays) but is not “free” because it is trapped within the inhibitor’s cage and cannot cleave elastin or otherwise contribute to the destruction of macromolecular components of lung extracellular matrix [71].

GASPID DIVERSITY

Primary Substrate Specificity

Notwithstanding the likelihood that all GASPIDs arose in the course of vertebrate evolution from a shared ancestral peptidase, these enzymes as a group exhibit striking diversity of structure and function. GASPIDs in fact provide one of the more emphatic examples of divergent evolution in vertebrates. Among the most conspicuous and easily documented differences is primary specificity, i.e., the preference for particular side chains in the residue that becomes the new C-terminus of the cleaved peptide after hydrolysis (Table 1). Collectively, GASPIDs have evolved to cleave targets after amino acids with basic, aromatic, acidic, short or long aliphatic side chains. Individually, GASPIDs exhibit striking pickiness for particular substrates. This pickiness arises from interactions with the side chain of the amino acid at the site of hydrolysis within the enzyme’s so-called primary specificity pocket, and also from extended binding site interactions with side chains several residues removed from the actual site of hydrolysis. Although students of these enzymes sometimes can predict ability to cleave native protein targets by observing preferences towards libraries of small peptides [72], other factors can be paramount determinants of actual GASPID behavior. Such factors include active site accessibility (as in the oligomeric tryptases with active sites sequestered in a proteasome-like cavity [9,54]), the ability of a native protein target to displace residues engaged in self-blockade of the active site (as in factor D [53]), and substrate-specific exposure of cleavage-susceptible sequences (as in albumin’s chymase-vulnerable Tyr84, which, unlike most aromatic residues, is not buried in the protein’s hydrophobic interior [72]). On the other hand, some targets of GASPIDs are not proteins, but peptides, an example of which is angiotensin I, which is cleaved by cathepsin G and mast cell chymases to generate vasoactive angiotensin II [44,73–75]. The kinetic parameters (k_{cat}/K_m) for this substrate may be more favorable for some GASPIDs than for angiotensin converting enzyme, an ecto-metallopeptidase otherwise unrelated to serine peptidases. In lung, heart and vessels, these two angiotensin II-generating enzymes inhabit different compartments [76], with chymase and angiotensin converting enzyme being the major peptidases responsible for angiotensin II generation outside and inside of blood vessel lumens, respectively. Although some GASPIDs (such as factor D, as noted) are exquisitely specific, others are much less selective. The human GASPID with least selectivity appears to be cathepsin G, which has chymotryptic, tryptic and “met-ase” activity, meaning that it cleaves peptide targets containing basic (Arg, Lys), aromatic (Phe, Tyr) and long aliphatic (Met, Leu) side chains at the site of hydrolysis [77]. Cathepsin G is related functionally as well as phylogenetically to ruminant “duodenases”, which are broad-specificity peptidases expressed primarily in mast cells [78].

Correlating Structure and Function: Crystal and Mutants

Because evolution has crafted a great variety of functions out of one basic GASPID structural template (the serine protease double β -barrel)-and because GASPIDs are suspected to contribute to immune defense and lung pathology--a great deal of effort has been expended by

academic and industrial investigators to identify natural substrates and to relate form to function. As part of this effort, several GASPIDs have been crystallized and their tertiary (and in some cases, quaternary) structures solved by diffraction (Table 4). The first GASPID amino acid sequence and crystal-based structure to be solved was that of a mast cell chymase, rat mast cell protease II [79]. Thus, this enzyme may be considered the prototypical GASPID, although when first characterized it was not appreciated to be the product of an immune cell. This was followed by more detailed structures [80], and also by crystallographic analysis of the quite distinct human chymase [81,82], including its proenzyme form [28], revealing mechanisms of zymogen activation that are likely to be broadly applicable to other GASPIDs. These structures have helped to craft models of other chymases in the absence of diffraction data [20,83,84]. Related GASPID crystal-based structures (some solved with the help of the chymase template) include neutrophil elastase [85], cathepsin G [86], granzyme B [87] and granzyme K [88]. Comparison of these structures identifies particular regions of primary structure that are critical determinants of enzyme function. Consequently, it is increasingly possible to predict activity and specificity by examining primary structure alone. Granzyme A tertiary structure also is related closely to that of chymase-related GASPIDs, although crystal-based structures reveal that it extends and alters its substrate-binding site by dimerizing [56,57]. The use of oligomerization to limit activity and gain protection from anti-peptidases is even more dramatic in the β -tryptases and mastins [9]. This particular correlation between structure and function was immediately made clear by the structure of the β -tryptase tetramer [54], as was the basis for the limited activity of α -tryptase [19]. Insights of similar clarity were provided by structures of factor D [52,53], which provided a structural explanation for the enzyme's extreme substrate specificity as well as the resistance of the mature factor D to inhibition by circulating anti-peptidases. Additional insights have been gained by mutating GASPIDs to reveal contributions of particular amino acids to specialized functions. Examples include targeted alterations in chymases [20,84], tryptases [89], and granzymes [90]. Such studies establish the general principle that GASPID function can change and has in fact evolved very rapidly by combining gene duplication with changes in a few key residues or even a single amino acid in a vulnerable site.

Immune Cell Expression

The cell expression profile of each GASPID is distinct (Fig. 3). Most GASPIDs are synthesized predominantly in cells of bone marrow origin. Factor D may be an exception. In addition to expression in cells of monocytes/macrophage lineage, factor D is made and secreted by fat cells, which may contribute the lion's share of enzyme in the circulation. Indeed, when discovered in mice as a marker of adipocyte differentiation, the enzyme was first dubbed "adipsin" before it was determined to be the mouse ortholog of factor D [91,92]. Irrespective of its origin, it is clear that Factor D, like other GASPIDs, serves an immune function [91]. Most GASPIDs are expressed in more than one type of immune cell. For example, granzyme B is highly expressed in cytolytic T and natural killer cells, where it aids target cell killing, but also has been found in eosinophils and mast cells, where its function is less clear [68,93]. Mast cell tryptases, which are so abundant in human mast cell granules that they can comprise as much as 25% of all mast cell protein [94], are also found in basophils, though at much lower levels [95]. Neutrophil elastase, as its name implies, is found in greatest abundance in polymorphonuclear leukocytes, but also can be expressed in a more limited way in monocytes and macrophages [96]. Similarly, cathepsin G, which is traditionally associated with neutrophils, is expressed generously in the granules of the same subset of mast cells that express chymase [97]. In the lung, this subset tends to reside in bronchial submucosa, especially in the vicinity of glands [98], but not in alveolar interstitium [99]. Interestingly, shared membership in a particular gene cluster or phylogenetic predictions of recent evolutionary origins does not necessarily predict shared patterns of expression. The most obvious implication is that GASPIDs evolved in parallel with the immune cells in which they are now expressed; thus,

GASPID variations open a window onto the origins and development of mammalian immune cells in their current state of complexity and specialization.

Man or Mouse?

Overall, variations in GASPID expression profiles produce major phenotypic differences between immune cells. They also account for some overt differences in immune cell phenotype between mammals, as between humans and mice. Specifically, striking differences in the size of the gene clusters encoding chymases, group B granzymes, and (to a lesser extent) tryptases are responsible for differences in GASPID expression between humans and rodents [1,7] (Table 2). The multiplicity of chymase-like enzymes in mice and rats result in dominant expression of chymotryptic activity in all subsets of mast cells, whereas in humans chymotryptic activity is limited to a subset of mast cells most generously represented in the skin [6]. A gene dosage effect produced by triplication of human mast cell $\alpha/\beta/\delta$ tryptase genes may be responsible in part for the very high levels and broad range of tryptase expression in human mast cells [8,15]. In mice, the large number of seemingly redundant granzyme B-like genes may prevent the phenotype of *Gzmb* $-/-$ mice from being more severe than it is. On the other hand, humans, although they have far fewer granzyme genes than mice or rats, have a unique chymotryptic granzyme ("H") [14,100], which has no obvious counterpart in rodents, and is of uncertain function. These and other differences have made the results of GASPID-targeted gene deletion and pharmacological studies in rodents challenging to extrapolate to humans.

ROLES OF GASPID IN LUNG HOST DEFENSE: INSIGHTS FROM GENETICALLY MODIFIED MICE

Neutrophil Elastase

Results of a variety of targeted genetic modifications in mouse genomes highlight the overall importance of GASPID to mammalian host defense (Table 5). One of the most extensively examined GASPID in this regard is neutrophil elastase. *NE*-null mice are slow to kill Gram-negative bacteria (e.g., *Klebsiella pneumoniae* and *E. coli*) in models of peritonitis, and are more vulnerable to sepsis and death [101]. In the specific case of *E. coli*, killing appears to depend on elastase-mediated cleavage of an outer membrane protein [102]. Similarly, neutrophil elastase kills *Shigella* species by targeting virulence factors that facilitate escape from phagocytes [103]. Neutrophil elastase's role in preventing or limiting lung infection is less clear, especially in humans, as noted above in connection with the lack of a lung infection phenotype in elastase-deficient subjects with Papillon-Lefevre syndrome. In mice, neutrophil elastase also can damage tissues in non-infectious inflammation, as in a model of bullous pemphigoid [104], which is an autoimmune blistering skin disease, and in a model of arthritis induced by antibodies to type II collagen [34]. Of course, NE has long been suspected to be important in humans in the development of cigarette-associated emphysema, which is associated with chronic neutrophilic inflammation of the lung airways and parenchyma. Traditionally, neutrophil elastase is placed at the top of the list of potential offending enzymes in the protease/antiprotease imbalance hypothesis of emphysema pathogenesis [105]. Recent evidence from smoking mice does in fact suggest that elastase contributes to experimental emphysema, for neutrophil-depleted or α_1 -antitrypsin-supplemented mice are protected from acute lung matrix breakdown [106], and *NE*-null mice are partially protected from emphysema [107]. Furthermore, long-term pharmacological inhibition of elastase decreases emphysema severity in smoking guinea pigs [108]. However, genetic deletion of at least one other peptidase (MMP12, otherwise known as macrophage metalloelastase, which is not a GASPID) also is protective-possibly more so [109]. One potential explanation for protection by either peptidase is that each cross-inactivates a major inhibitor of the other [107], i.e., TIMP-1 for MMP12 and

α_1 -antitrypsin for elastase. Another explanation is that neutrophil recruitment to lungs of smokers depends on smoke-stimulated production of MMP12 by macrophages.

Factor D/Adipsin

In *Df*^{-/-} mice, the alternative pathway of complement activation is defective, as reflected by impaired hydrolysis of factor B and slower opsonization of the common respiratory pathogen *Streptococcus pneumoniae*. On the other hand, there are no defects in development or body weight, despite the initial characterization of factor D as the fat cell differentiation marker, adipsin [110].

Cathepsin G

Ctsg-null mice were reported to be free of overt defects in neutrophil structure or microbial killing [111]. More recent data suggest that cathepsin G promotes adhesion-dependent cytoskeletal reorganization and cell spreading in neutrophils [112]. In mice, some defects associated with lack of cathepsin G may be more manifest when combined with deficiency of neutrophil elastase, as in *Ctsg* (-/-) x *NE* (-/-) double knockout animals [34,112]. In humans, the only known deficiency state is Papillon-Lefevre syndrome, which also affects other GASPIs, as noted above. Thus, the extent to which cathepsin G contributes to defense against to human lung immune defense is not yet clear. However, the observation that some cases of early-onset emphysema occur in patients with inherited deficiency of α_1 -antichymotrypsin, as noted above, fuels speculation that overactive cathepsin G can destroy lung parenchyma over time.

Chymases and Fellow-Travelers

Efforts to use mice to predict the importance of mast cell chymase in human biology and disease have been slowed by the multiplicity of chymase-like genes (compared to just one in humans) and by the more recent discovery that the mouse enzyme most similar in primary structure to human chymase has acquired a mutation that strikingly alters its substrate specificity from primarily chymotryptic to elastolytic [20,21]. Nonetheless, several mast cell protease-null mice have been generated and demonstrate that this group of enzymes has important roles to play in rodents. The clearest role for an individual chymase-like enzyme has been established for mast cell protease 1. *Mcp1*-null mice have impaired ability to expel certain intestinal parasites [113]. On the other hand, mice deficient in mast cell protease 4 (MCP4) manifest reduced activation of pro-gelatinase B/MMP9 (as predicted by *in vitro* studies [114-116]), increased deposition of retention of tissue fibronectin, and thickened ears [117], suggesting a role for MCP4 in processing of extracellular matrix. Decreased processing of fibronectin was also noted by mast cells from sulfotransferase *NDST2*-deficient mice, which have decreased mast cell chymase activity because of a lack of sulfated heparins needed for MCP4 packaging in secretory granules [118]. Even though MCP4 is not the chymase most structurally and phylogenetically related to human chymase [75,119], it is sufficiently similar in activity profile and tissue expression that it may play an equivalent role. Mice deficient in elastolytic MCP5, which is similar to human chymase in structure and distribution but dissimilar in substrate preferences, resist skeletal muscle ischemia-reperfusion injury [120]. Chymases also are implicated indirectly in promoting neoplastic progression in a model of skin cancer [116]. Mice with globally depressed chymase activity, such as *Ctsc*-null mice discussed above in connection with GAS PID activation and Papillon-Lefevre syndrome, have host defense defects, which have yet to be linked to severely reduced activity of chymase-like enzymes [33]. They also exhibit the interesting and unanticipated phenotype of protection from death from septic peritonitis, which may due to reduced levels of mast cell tryptase, which therefore fails to inactivate IL-6, which is required for the protective effect [121].

Mast Cell Tryptases

As of this writing, no genetically engineered mouse with selective mast cell tryptase deficiency has been reported. However, it has been observed that the C57BL/6 strain naturally lacks MCP7 [122], which is one of two soluble mast cell tryptases in mice. Because tryptases are linked to bronchoconstriction and hyperresponsiveness in asthma [123-127], the hyporesponsive airway phenotype of this strain [128] may relate to partial tryptase deficiency. In other experiments, recombinant human β 1-tryptase instilled into lungs of mast cell-deficient *Kit^W/Kit^{W-v}* mice [129] caused pulmonary neutrophilia and provided partial protection from *Klebsiella pneumoniae*. This is not the case with instilled α -tryptase. These findings hint that active β -tryptases are important in host defense against bacterial lung infection (as are mast cells in mice [130,131]), although the species mismatch between instilled human enzyme and the murine host prevents free extrapolation of results to either species. Given the ongoing effort to develop β -tryptase-targeted inhibitor drugs for treatment of asthma and other inflammatory diseases, the potential involvement of tryptases in anti-bacterial defense is an issue that could influence the safety of long-term tryptase inhibition. Soluble human γ -tryptase, on the other hand, caused mouse airways to become hypersensitive to contractile stimuli [47]. Whether this is a property of the native, membrane-anchored form remains to be determined.

Granzymes

Granzymes A and K are tryptic serine peptidase whose genes form their own mini-cluster. Granzyme A-deficient *Gzma*^{-/-} mice retain cell-mediated killing capacity [132] but have a subtle late defect in cytotoxicity. Granzyme K probably accounts for residual tryptic granzyme activity in cytotoxic T cells from *Gzma*-null mice [88]. A double knockout of granzymes A and B produces more profoundly defective killing phenotypes [133]. Mouse B-type granzyme genes, like the many chymase-related genes in the same chromosomal neighborhood, are numerous-and most have no counterpart in humans. Most granzymes in mice appear to be most abundant in cytolytic CD8⁺ T lymphocytes and natural killer cells, although some are not fully characterized in this regard. Studies in granzyme B-deficient *Gzmb*^{-/-} mice suggest that cytotoxic lymphocytes require granzyme B for early induction of apoptosis in target cells [134] but eventually can kill them. Studies in single and double knockout *Gzma*^(-/-) x *Gzmb*^(-/-) mice show that these granzymes protect against a variety of virus infections [135-137], including early cytomegalovirus infection [138]. Studies with recently generated granzyme M-null (*Gzmm*^{-/-}) mice also support a role for this less extensively studied GASPID in control of murine CMV infection, but not a role in natural killer cell-mediated cytotoxicity [139].

Cautionary Tales

Not all mouse knockout experiments involving GASPIDs are “clean”, which is to say that the ensuing phenotype reveals something about the role of that GASPID and only that GASPID. For example, the tendency of GASPID genes to cluster means that manipulation of the genome in the vicinity of one GASPID gene may affect expression of nearby GASPID genes. This has happened in experiments designed to eliminate expression of the granzyme B gene [140, 141], which is in the middle of a large locus of numerous granzymes and chymase-like genes in mice [1,6] as well as in humans [17]. Some members of these clusters may be subject to “locus control”, with some sharing of regulatory elements in the genome, thereby increasing the likelihood that modifications in the region will alter expression of more than one gene. Another caveat concerns potential effects of removing one GASPID on other GASPIDs packaged within the same granule, and on non-GASPID proteins and biomolecules. For example, mast cells from *Mcpt5*-null and *Ctsc*-null mice have markedly reduced and elevated levels of granule-associated carboxypeptidase A [143,144], respectively. Investigators also need to be cognizant of background-specific variations of GASPID expression, such as the

inability of C57BL/6 mice to express the MCP7 tryptase [122] and the markedly increased expression of γ /transmembrane-tryptase in C57BL/6 mice relative to BALB/c [38].

CONCLUSIONS

The rapidly evolving GASPID family of peptidases is phylogenetically related but functionally diverse. Multiple lines of evidence in mouse models, including genetically modified animals, suggest important roles for GASPIDs-individually and in various combinations--especially in host defense against infections and in tissue destruction in non-infectious autoimmune and inflammatory syndromes. In humans, we know the most about the chromosome 19p13.3 GASPIDs, especially neutrophil elastase and proteinase 3, which are linked to emphysema and Wegener's autoimmune vasculitis, respectively. However, based on *in vitro* data involving human enzymes and cells, and upon extrapolations from animal models, it seems likely that other GASPIDs will be found to play importance roles in human defense and disease, and that some GASPIDs will be appropriate targets for pharmaceutical inhibition.

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Fig. 1.

Structural/evolutionary relationships among GASPIDs. This “best” tree was prepared by neighbor-joining from a multiple sequence alignment of GASPID catalytic domain primary sequence. The length of lines is proportional to sequence differences between any given pair of peptidases and also reflect evolutionary distance. Orthologous pairs of human and mouse sequences are compared when expressed. Note that some GASPIDs are expressed in humans but not mice (such as azurocidin and granzyme H) and many more are expressed in mice but not humans (chymase- and granzyme B-related peptidases). This tree also reveals that the tryptase/mastin group of GASPIDs are more distantly related than the other GASPIDs. In this analysis, γ -tryptase serves as an outgroup.

Fig. 2.

General scheme of GASPID maturation, secretion and inactivation. GASPIDs are initially synthesized as prepro-proteins with a signal and activation peptide. The signal peptide is removed during translation and translocation into the endoplasmic reticulum, leaving an inactive pro-GASPID with residual propeptide, as shown. Subsequent propeptide removal (typically by dipeptidylpeptidase I/cathepsin C) generates "activated", mature GASPID, which is stored in a granule. Most GASPIDs have limited activity in this milieu because of low granule pH and tight packing in a matrix of uncleavable glycosaminoglycan. In response to signals appropriate for the immune cell of origin, the mature GASPID is secreted. Outside of the cell, the GASPID can remain associated with the outer surface of the plasma membrane, which sometimes affords protection from inhibition, or is secreted in a soluble, "free" form, which is more vulnerable to inactivation by extracellular anti-peptidases. Binding to a circulating inhibitor is usually irreversible, eventuating in clearance and destruction of the enzyme-inhibitor complex.

Fig. 3. Patterns of immune cell expression among GASPIDs. Transcripts arising from human GASPID genes in four clusters are dispersed in immune cells in idiosyncratic patterns.

Table 1

Human GASPID Genes, Proteins and Enzyme Specificity

Chromosome Cluster	Gene	Protein	Activity	P1 Preference ^a
5q11.2	<i>GZMA</i>	Granzyme A	Tryptic	Arg, Lys
	<i>GZMK</i>	Granzyme K	Tryptic	Arg, Lys
14q11.2	<i>CMA1</i>	Chymase	Chymotryptic	Phe, Tyr, Trp, Leu
	<i>CTSG</i>	Cathepsin G	Chymotryptic, tryptic, met-ase	Phe, Tyr, Met, Arg, Lys
	<i>GZMH</i>	Granzyme H	Chymotryptic	Phe, Tyr
	<i>GZMB</i>	Granzyme B	"Asp-ase"	Asp, Glu
	<i>TPSG</i>	γ -Tryptase	Tryptic	Arg, Lys
16p13.3	<i>TPSB2</i>	β II & β III Tryptase	Tryptic	Arg, Lys
	<i>TPSAB1</i>	β I Tryptase	Tryptic	Arg, Lys
	"	α -Tryptase	Nearly inactive	Arg, Lys
	<i>TPSD</i>	δ -Tryptase	Nearly inactive	?
	<i>NE/ELA2</i>	Neutrophil elastase	Elastolytic	Ala, Val
19p13.3	<i>PRTN3</i>	Proteinase 3	Elastolytic	Ala, Val
	<i>DF</i>	Factor D	Tryptic	Arg
	<i>GZMM</i>	Granzyme M	"Met-ase"	Leu, Ile, Met
	<i>AZU1</i>	Azuricidin	Inactive	-

^aP1 is the substrate amino acid on the amino-terminal side of the site of hydrolysis.

Table 2

Human Versus Mouse GASPIDs

Human Chromosome	Human Gene/Protein	Mouse Gene/Protein
5q11.2	<i>GZMA</i> /Granzyme A	<i>Gzma</i> /Granzyme A
	<i>GZMK</i> /Granzyme K	<i>Gzmk</i> /Granzyme K
14q11.2	<i>CMA1</i> /Chymase	<i>Cma1</i> (<i>Mcpt5</i>)/mast cell protease 5
		<i>Mcpt1</i> /mast cell protease 1
		<i>Mcpt2</i> /mast cell protease 2
		<i>Mcpt4</i> /mast cell protease 4
		<i>Mcpt9</i> /mast cell protease 9
		<i>Mcpt10</i> /mast cell protease 10
		<i>McptL</i> /mast cell protease-like
	<i>CTSG</i> /Cathepsin G	<i>Ctsg</i> /Cathepsin G
	<i>GZMH</i> /Granzyme H	No mouse equivalent
	<i>GZMB</i> /Granzyme B	<i>Gzmb</i> /Granzyme B
		<i>Gzmc</i> /Granzyme C
		<i>Gzmd</i> /Granzyme D
		<i>Gzme</i> /Granzyme E
		<i>Gzmf</i> /Granzyme F
		<i>Gzmg</i> /Granzyme G
		<i>Gzmn</i> /Granzyme N
		<i>Gzmo</i> /Granzyme O
16p13.3	<i>TPSG/γ</i> -Tryptase	<i>Tpsg/γ</i> (transmembrane)-Tryptase
	<i>TPSB2/βII- & βIII</i> -Tryptase	<i>Mcpt6</i> /Mast cell protease 6
	<i>TPSAB/βI- & α</i> -Tryptase	No direct mouse equivalent
	<i>TPSD/δ</i> -Tryptase	<i>Mcpt7</i> /Mast cell protease 7
19p13.3	<i>NE/ELA2</i> /Neutrophil elastase	<i>NE</i> /Neutrophil elastase
	<i>PRTN3</i> /Proteinase 3	<i>Prtn3</i> /Proteinase 3
	<i>DF</i> /Factor D	<i>DF</i> /Factor D
	<i>GZMM</i> /Granzyme M	<i>Gzmm</i> /Granzyme M
	<i>AZU1</i> /Azuricidin	No mouse equivalent

Table 3

Native Inhibitors of Human GASPIDs

Chromosome Cluster	Protein Name	Native Inhibitor(s)
5q11.2	Granzyme A	Antithrombin III
	Granzyme K	Bikunin
14q11.2	Mast cell chymase	α_1 -Antichymotrypsin, α_2 -macroglobulin, others
	Cathepsin G	α_1 -Antichymotrypsin, α_2 -macroglobulin, others
	Granzyme H	None known
	Granzyme B	Proteinase inhibitor 9
16p13.3	γ -Tryptase (soluble form)	α_1 -Antitrypsin, secretory leukoelastase inhibitor
	β II- & β III-Tryptase	?Lactoferrin
	β I- & α -Tryptase	?Lactoferrin
	δ -Tryptase	None known (or needed)
19p13.3	Neutrophil elastase	α_1 -Antitrypsin, α_2 -macroglobulin, secretory leukoelastase inhibitor, elafin, others
	Proteinase 3	α_1 -Antitrypsin, elafin
	Factor D	None known (or needed)
	Granzyme M	Serpin SCI-CI
	Azuricidin	None known (or needed)

Table 4

Crystallized GASPIDs

GASPID	References	Special Features
Rat mast cell protease II + inhibitor	[79,80]	Deep, extended substrate binding pocket
Human chymase + inhibitor	[81,82]	High density of surface charge explains high affinity for proteoglycan
Human pro-chymase	[28]	Changes in active site conformation explain inactivity of pro-enzyme
Human cathepsin G + inhibitor	[86]	Active site mutations explain unusually broad specificity
Human β -tryptase + inhibitor	[54]	Toroidal, self-compartmentalizing tetramer; structure explains heparin stabilization
Human α -tryptase	[19]	Tetramer, with contorted, self-blocked active site
Human neutrophil elastase + inhibitor	[85]	Asymmetry of charge distribution explains proteoglycan binding
Rat Granzyme B + inhibitor	[87]	Structure explains unusual Asp specificity
Human Granzyme B	[142]	Structure explains unusual Asp specificity
Human granzyme A + inhibitor	[56,57]	Dimer extends & protects active site
Human pro-granzyme K	[88]	Rigid, non-productive active site
Human Factor D	[52,53]	Auto-blocked active site

Table 5

GASPID-Defective Mice

Genotype	GASPID Deficiency	Lung-Relevant Phenotype
C57Bl/6 mice	Tryptase MCP7	Airway hyporesponsiveness; resistance to allergen sensitization
<i>Kit^W/Kit^{W-v}</i> or <i>Kit^{W-sh}/Kit^{W-sh}</i>	Mast cell GASPIs	Lack of mast cells; decreased allergic inflammation; increased mortality from sepsis; enhanced bacterial bronchitis and pneumonia;
Ctsc $-/-$	Activated chymases Activated cathepsin G Activated granzymes A & B Activated tryptase MCP6	Decreased mortality from septic peritonitis; enhanced pneumonia
Gzma $-/-$	Granzyme A	Defective cell killing ^a
NE $-/-$	Neutrophil elastase	Protection from emphysema ^a ; defective bacterial killing ^a
Gzmm $-/-$	Met-ase/Granzyme M	Defective control of CMV infection
Gzmb $-/-$	Granzyme B	Defective cell killing ^a
Ctsg $-/-$	Cathepsin G	Defective neutrophil function ^a
Mcpt1 $-/-$	Chymase MCP1	Delayed parasite expulsion; delayed mast cell recruitment & structure
Mcpt4 $-/-$	Chymase MCP4	Decreased activation of MMP gelatinases
Mcpt5 $-/-$	Chymase/elastase MCP5 (Carboxypeptidase A3)	Resistance to ischemia-reperfusion injury

^aMany of these phenotypes are more dramatic when combined with other GASPID defects, as in Gzma ($-/-$) x Gzmb ($-/-$) or NE ($-/-$) x Ctsg ($-/-$) double-knockout strains (see text).