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Special Article

Regulation and Function of Mast Cell Proteases in Inflammation

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INTRODUCTION

Mast cells (MC), which reside in connective tissue matrices and epithelial surfaces, are effector cells that initiate inflammatory responses. Activated MC release a variety of proinflammatory mediators, including cytokines, chemokines, histamine, prostaglandins, leukotrienes, and serglycin proteoglycans. However, on a weight and molar basis, proteases that are enzymatically active at neutral pH are the major protein constituents exocytosed from activated MC. Tryptases, chymases, and carboxypeptidase A (MC-CPA) represent the three major families of proteases stored in the secretory granules of MC. Whereas only a few granule proteases have been identified in human (1–10), dog (11, 12), and gerbil (13, 14) MC, mouse (15–26) and rat (27–34) MC express various combinations of MC-CPA and at least 10 distinct serine proteases. MC are heterogeneous in tissues, and the specific panel of proteases a particular mouse MC expresses is a consequence of the regulatory factors to which this cell is exposed in its current and previous tissue microenvironments. Because MC express so many different granule proteases, the challenge ahead is to identify the biologic substrates of each enzyme. This review focuses on some of the recent data relating to the regulated expression of MC proteases, as well as some of the technological advances in protease expression that are yielding insight into protease function.

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REGULATION OF MC PROTEASES

When multipotential stem cells from BALB/c mouse bone marrow are cultured for 2 to 3 weeks in the presence of interleukin-3 (IL-3), an immature population of MC is obtained (35) that expresses mouse MC protease (mMCP) 5 (22), mMCP-6 (18), mMCP-7 (23), and mMC-CPA (16). These *in vitro*-differentiated MC do not express mMCP-1, mMCP-2, or mMCP-4, but they can be induced to express different types of granule proteases if they are subsequently exposed to various combinations of IL-4, IL-9, IL-10, c-*kit* ligand, and glucocorticoids (36–40). MC use multiple mechanisms to control reversibly the types and quantities of proteases stored in their secretory granules. Other control mechanisms regulate the retention and enzymatic activity of these proteases in tissues after their exocytosis from the MC. The fact that MC proteases are regulated at more levels in the body than other mammalian proteins indicates the importance of these enzymes in immune responses.

Chromatin Structure. The genes that encode the varied mouse chymases and tryptases are clustered on chromosomes 14 and 17, respectively (26, 41, 42). The chromosome 14 complex also contains the genes that encode cathepsin G (43) and numerous granzymes (44–46). No crossover point has been noted in the chromosome 14 complex (41, 45), and the finding that the mMCP-1 and mMCP-9 genes are separated only by ~7 kb of flanking DNA (26) suggests that the low recombination frequency

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of the complex is a consequence of the proximity of its genes. Each of the five ~3-kb trypsinogen genes in the human β T cell receptor locus is separated from its adjacent gene by ~7 kb (47). Thus, the 7-kb spacing appears to be a general feature for the chromosomal organization of serine protease genes.

The tight clustering of the MC serine protease genes probably affords the first level of regulation. CpG islands are believed to control nucleosome positioning, and the methylation of the CpG islands in the 5′ and 3′ flanking regions of genes generally influences their transcription (48). While the methylation state of the chromosome 14 complex of serine protease genes has not been studied, the state of methylation of the serglycin gene in MC and other hematopoietic cells is strongly correlated with its level of expression (49). If the serine protease genes are closely packed on chromosome 14 in a highly methylated state, this probably ensures their silencing in any nonhematopoietic cell that expresses abnormally high levels of *trans*-acting factors which induce their transcription.

Transcriptional Regulation. MC express at least three members of the GATA family of transcription factors (50). Although MC can be developed by culturing progenitors from GATA-1-null mice (51), they cannot be developed from GATA-2-null mice (52). With the use of transient transfection/site-directed mutagenesis approaches, it has been found that the conserved GATA-motif residing 42 base pairs (bp) upstream of the transcription-initiation site of the MC-CPA gene probably controls the expression of this gene in MC. Subsequent *in vivo* and *in vitro* promoter studies have revealed that the transcription of the mMCP-5 gene and its dog, baboon, and human homologues also are regulated by the GATA family of transcription factors (53–55). GATA-2 is present in those mouse bone marrow-derived MC (mBMMC) (50) that give rise to multiple populations of MC when adoptively transferred into MC-deficient W/W^v mice (56). *In situ* hybridization studies revealed that cutaneous MC express GATA-2, but not GATA-1, as they proliferate and begin to differentiate in the skin (57). However, because cutaneous MC decrease their expression of GATA-2 as they undergo their final stages of granule maturation, protease expression in MC must be controlled by additional transcription factors. The 571-bp sequence that resides just upstream of the baboon chymase gene is sufficient to induce the cell-specific expression of a reporter gene in transgenic mice (53). This *in vivo* study indicates that the most critical *cis*-acting elements reside relatively close to the transcription-initiation site of the baboon chymase gene, but transient-transfection analysis with DNA constructs containing portions of the 5′ flanking region of the rat MC protease (RMCP) 2 gene suggests that more distant nucleotide sequences contribute to the cell-specific transcription of rat chymase genes (58).

The serglycin transcript is abundantly expressed in MC but not fibroblasts, even if multiple copies of the entire gene are inserted into the genome of the fibroblast (59). The subsequent finding that fibroblasts express *trans*-acting factors that dominantly prevent transcription of the serglycin gene in nonhematopoietic cells (60) highlights the importance of negative transcriptional regulation of those genes that are intended to be expressed in MC but not in fibroblasts. Although this aspect of protease expression has not been investigated in detail, a *cis*-acting element residing ~3.5 kb upstream of the mMCP-5 gene suppresses transcription of a reporter gene in transiently transfected cells (55).

The GATA family of transcription factors binds to (A/T)GATA(A/G) motifs in the promoter and enhancer elements of genes. Nearly every gene in the body has multiple GATA-binding sites in its nucleotide sequence. Yet GATA-1, GATA-2, and/or GATA-3 selectively induce the transcription of the mMCP-5 and mMC-CPA genes in MC. Studies on the basic helix–loop–helix (bHLH) family of transcription factors have provided insight into the process by which a generic transcription factor like GATA-2 induces a multipotential stem cell to differentiate into a MC (61). The bHLH transcription factors recognize E-boxes in promoters with the consensus sequence of CANNTG, where N can be any nucleotide. These transcription factors fall into two classes and are active only when they dimerize. The A subclass of bHLH transcription factors is ubiquitous but specific differentiation pathways are allowed to proceed when A-type bHLH factors bind to the more restricted B-type bHLH factors. *Mi/mi* mice are MC deficient (62) due to a genetic defect in the bHLH *mi*-transcription factor (MITF) (63). MITF recognizes the CANNTG motif and probably directly controls the transcription of the mMCP-6 gene (64) but not the mMCP-5 gene (65) in cutaneous MC.

Id proteins are related to the bHLH family of transcription factors. They cannot bind to DNA, but they can interact tightly with the A subclass of bHLH transcription factors. Because Id proteins control the steady-state levels of class A/B heterotypic complexes and class A homotypic complexes in cells, Id proteins can act as dominant negative or positive regulators of differentiation pathways. In addition to the events that modify the activity of the transcription factors in MC (e.g., phosphorylation of MITF), the role of the four known Id

proteins in the regulated transcription of protease genes in MC must be considered.

Posttranscriptional Regulation. The cutaneous MC of the C57BL/6 mouse do not contain mMCP-7 mRNA or protein (66), and nucleotide sequence analysis revealed that the mMCP-7 gene in this mouse strain has a point mutation at its exon 2/intron 2 splice site (67). This mutation introduces a premature stop codon in the alternatively spliced transcript, which, in turn, causes the expressed protein to consist of only 18 amino acids. Nuclear run-on and reverse transcriptase–polymerase chain reaction experiments revealed that the mMCP-7 gene is transcribed at a high rate in the C57BL/6 mouse MC. However, the level of the defective mMCP-7 transcript in C57BL/6 mouse MC is below detection by RNA blot analysis because it is rapidly catabolized. Thus, MC contain a posttranscriptional mechanism for quickly eliminating transcripts that encode nonfunctional proteases.

MC express a second posttranscriptional mechanism for regulating the steady-state levels of protease transcripts (68). As assessed by RNA blot analysis, BALB/c mBMMC developed with IL-3 contain high levels of the mMCP-5 transcript but extremely low to undetectable levels of the transcripts that encode the homologous chymases mMCP-1, mMCP-2, and mMCP-4. Treatment of these MC with IL-10 results in a rapid rise in the steady-state levels of the mMCP-1 and mMCP-2 transcripts but essentially no change in the steady-state levels of the mMCP-4 or mMCP-5 transcripts (37, 38). Subsequent nuclear run-on and pulse-chase experiments revealed that all four chymase genes are transcribed at a high rate in IL-3-developed mBMMC (68). IL-10 does not influence the rate of transcription of the mMCP-2 gene in mBMMC. Rather, this cytokine induces the expression of an undefined *trans*-acting factor that prevents the mMCP-2 transcript from being rapidly degraded. Glucocorticoids prevent the IL-10-mediated induction of mMCP-1 and mMCP-2 expression by controlling this posttranscriptional pathway (40).

The *cis*-acting motifs in the mMCP-1, mMCP-2, and mMCP-4 transcripts that regulate their stability have not been defined experimentally, but repetitive sequences in the 3′ untranslated regions (UTR) of transcripts often regulate their rate of catabolism. The most thoroughly investigated destabilization motif is the AUUUA motif in the 3′-UTR of numerous cytokine transcripts (69). The lack of a repetitive AUUUA motif in the 3′-UTR of any MC protease transcript indicates that a novel posttranscriptional mechanism must dominantly control the expression of mMCP-1, mMCP-2, and mMCP-4. The presence of variable numbers of the UGNCCCC motif in different positions in the 3′-UTR of the mMCP-1, mMCP-2, mMCP-4, and mMCP-9 transcripts raises the possibility that this repetitive sequence influences transcript stability in MC. Whereas nonsense-mediated decay appears to be a ubiquitous mechanism by which multiple cell types rid themselves of defective transcripts, the cytokine-regulated posttranscriptional pathway appears to be a specialized mechanism by which MC rapidly dispose of functional transcripts that are unwanted.

Translational and Posttranslational Regulation. The steady-state levels of mRNA and protein are not always correlated in MC. For example, peritoneal MC have >100-fold more mMC-CPA protein in their secretory granules than IL-3-developed mBMMC, yet the latter contain >100-fold more mMC-CPA mRNA than the former (16, 70). Thus, translational and/or posttranslational mechanisms play critical roles in granule maturation. The nucleotide sequences immediately upstream of the translation-initiation sites in the transcripts that encode mMCP-6 (18), RMCP-6 (33, 34), dog tryptase (11), human α-tryptase (4), and human β-tryptase (5) are homologous. However, they are very different from the corresponding sequences in the transcripts that encode mMCP-7 (23), RMCP-7 (34), and gerbil MC tryptase (13). The 5′-UTR of the mMCP-7 transcript is also considerably longer than that in the mMCP-6 transcript due to a failure of the former to splice out the nucleotide sequence corresponding to the first intron of the ancestral tryptase gene (23). Although these findings suggested that the mMCP-7 transcript might not bind to the ribosome for its translation, the abnormal transcript is readily translated because heart and muscle MC in the BALB/c mouse contain large amounts of immunoreactive mMCP-7 protein (71). The accumulated data suggest that the 5′-UTR is only marginally important in the regulation of tryptase expression.

Neutral proteases are the major constituents of MC secretory granules. Nevertheless, these organelles contain small amounts of β-hexosaminidase and other lysosomal enzymes (72). Because many mMCPs contain Asn-linked oligosaccharides (66, 73) which are essential for the lysosomal targeting of β-hexosaminidase by mannose 6-phosphate/insulin-like growth factor II receptors (74), it was thought that newly synthesized mMCPs and proteoglycans are targeted to secretory granules by the mannose-6 phosphate-dependent pathway. The first demonstration that this was not the case occurred with the discovery that β-D-xyloside-linked glycosaminoglycan chains lacking mannose-6-phosphate are readily targeted to the secretory granules of cultured rat peritoneal MC (75). Confirmation that MC use a distinct

mechanism to sort serglycin proteoglycans and neutral proteases to the granule compartment occurred with the recognition that mMCP-4 does not have in its primary amino acid sequence an Asn–X–Thr/Ser attachment site for mannose-rich oligosaccharides (20), even though this mMCP readily accumulates in the secretory granules of peritoneal MC (17).

All MC granule proteases are stored in the secretory granule ionically bound to the negatively charged glycosaminoglycans of serglycin proteoglycans (17, 70, 76, 77). Newly synthesized proteases are targeted to and then activated in these organelles by a serglycin proteoglycan-dependent pathway. MC are <25 μm in size. Because the amount of room in the cytoplasm of a MC limits the number of secretory granules it can retain, MC have devised posttranslational control mechanisms that ensure that only biologically active proteins are targeted to the granules in defined molar ratios. Even though some mMCPs have an overall net negative charge at neutral pH, all granule proteases have a positively charged, proteoglycan-binding face on the surface of the folded protease that resides away from the catalytic site (78). This region interacts with short sequences in the glycosaminoglycan chains of serglycin proteoglycans (79). Data suggest that granule proteases and serglycin proteoglycans form macromolecular complexes in the *trans* region of the Golgi just before they are targeted to the secretory granule. The fact that the positively charged face on the surface of each mMCP is made up of noncontiguous residues in the primary sequence of the protease ensures that only properly folded, enzymatically active protease is targeted to the granule.

All MC granule proteases are initially translated as inactive zymogens containing a hydrophobic signal peptide followed by a propeptide that consists of 2 to 94 amino acids. Based on numerous biosynthetic studies of homologous proteins, the ~20-mer signal peptide is presumed to be removed before the translated mMCP leaves the endoplasmic reticulum. Like the other serine proteases whose genes reside at the chromosome 14 complex, the propeptides of the MC chymases consist of either Glu–Glu or Gly–Glu. MC proteases are stored in the granules in their mature, enzymatically active forms. Because premature activation of a mMCP could cause serious damage to the cell, MC activate their proteases only after they are safely sequestered in the secretory granule. Dipeptidyl peptidase I (DPPI) is a minor constituent of secretory granules, but inhibitors of this thiol enzyme block chymase activation (80). Although these observations imply that DPPI is the processing enzyme that activates chymase zymogens, DPPI is not a highly selective aminopeptidase in terms of its substrate preference. For example, its activity in cells is routinely assayed with hydrophobic substrates such as Gly–Phe–βNA (81) rather than acidic substrates such as Glu–Glu–βNA. Thus, it was not clear at first why the negatively charged propeptides of the chromosome 14 family of serine proteases are so conserved. Subsequent studies revealed that serglycin proteoglycans probably play an essential role in the DPPI-dependent activation process (82). It now appears that the negatively charged propeptide initially binds to the positively charged, proteoglycan-binding face of the chymase to ensure that the zymogen remains catalytically inactive during its translocation from the endoplasmic reticulum to the *trans* region of the Golgi. Serglycin proteoglycans are the most negatively charged molecules in the body. Thus, when the inactive zymogen comes in contact with a serglycin proteoglycan as the macromolecular complex is being transported in small vesicles to the larger-sized storage granule, the negatively charged glycosaminoglycan successfully competes for the positively charged face on the protease. This process causes the liberated propeptide to be susceptible to DPPI as soon as the complex gets deposited in the granule. Immediately after the two-residue propeptide is removed by DPPI, the processed serine protease undergoes a subtle but critical structural change, with the newly formed N-terminal Ile residue triggering the conformational change that activates the serine protease. In addition, the protease is no longer susceptible to further processing by DPPI.

mMCP-5 and mMC-CPA are packaged in the secretory granules of peritoneal MC at an ~1:1 molar ratio. The finding that the two proteases remain bound to heparin-containing serglycin proteoglycans outside of the MC (16, 17, 70, 77) suggests that they coordinately degrade substrates in the extracellular matrix, such as low-density lipoprotein (83). Analysis of mMCP-5-null transgenic mice have given insight into how MC ensure that the chymase and exopeptidase are packaged in granules in equimolar amounts. With a homologous recombination approach, transgenic mice have been generated that possess a disrupted mMCP-5 gene (84). The disruption of this chymase gene does not alter the granulation of the mMCP-1^+/mMCP-2^+ MC that increase in number in the jejunal epithelium of helminth-infected mice. In contrast, the cutaneous MC from mMCP-5-null mice contain almost no mMC-CPA protein in their granules even though they have high levels of mMC-CPA mRNA. Unlike the homologous chymases mMCP-1 and mMCP-2, mMCP-5 has a second, more pronounced positively charged face opposite its putative proteoglycan-binding domain (78). Both mouse (16) and human (6) MC-CPA have a 94-residue propeptide that

resembles the propeptide in its pancreatic homologue (85). Based on the crystallographic structure of the pancreatic enzyme (86), this propeptide covers the entire face of the exopeptidase where the active site resides. The 94-residue propeptide of the pancreatic carboxypeptidase has a second function, namely, to control the formation of a 1:1:1 multimeric complex of the exopeptidase with pancreatic pro-chymotrypsin C and pro-proteinase E (87). The observation that the propeptide of mMC-CPA is negatively charged (88) raises the possibility that it interacts with the more positively charged face on the surface of mMCP-5 to form a binary ionic complex before the two proteases come in contact with serglycin proteoglycan. Once in the granule, the propeptides of each are removed, resulting in enzymatically active mMCP-5 and mMC-CPA packaged side by side on the glycosaminoglycan chain of the proteoglycan. Although this is the first example of how the expression of one granule protease in the MC dramatically affects another, the importance of multimeric protease complexes involving a carboxypeptidase has been documented in Cpe^{fat} mice (89) and humans with a galactosialidosis lysosomal disorder (90).

The posttranslational mechanisms that control the granule targeting and activation of the MC tryptases have not been conclusively determined because they appear to be even more complex than that of the chymases and MC-CPA. MC tryptases have a 10-residue propeptide. Based on *in vitro* studies with insect cell-derived recombinant human α- and β-tryptases, it has been proposed that the tryptases themselves somehow remove the first eight residues of the propeptide immediately after they interact with serglycin proteoglycans; they then can be processed further by the DPPI pathway (91). The finding that a monoclonal antibody influences the substrate specificity of recombinant human MC tryptase β suggests the existence of an unknown cofactor inside the MC that controls the posttranslational activation of MC tryptases (92). Unexplained in this proposed processing pathway is how enzymatically active human α-tryptase can be generated from transiently transfected COS cells (93), how human β-tryptase can spontaneously cleave the Arg^{-3}–Val^{-2} bond in its propeptide yet remain an inactive zymogen against all other substrates (91), and how MC tryptases can form homotypic tetramers (71, 94, 95) if they must bind to heparin-containing proteoglycans before their propeptides are removed.

Although all MC store serglycin proteoglycans in their secretory granules, these proteoglycans can have very different types of glycosaminoglycans covalently attached to them (96–100). MC must express an enormous number of glycosyltransferases, deacetylases, epimerases, and sulfotransferases to enable them to synthesize so many different types of glycosaminoglycans onto the serglycin peptide core (101). The reason that serglycin proteoglycans have various types of glycosaminoglycans remains to be determined, but each protease probably prefers to interact with a specific type of glycosaminoglycan. IL-3-developed mBMMC contain high levels of mMCP-5 (22) and mMC-CPA (16) mRNA but relatively low protein levels of both proteases unless they are cocultured with fibroblasts (70). Because maturing MC switch their biosynthesis of chondroitin sulfate E-containing proteoglycans (97) to heparin-containing proteoglycans (102), rather than increase their level of protease transcripts. Heparin is probably synthesized in $mMCP\text{-}5^{+}$/$mMC\text{-}CPA^{+}$ MC to interact selectively with these two proteases. In support of this hypothesis, tryptase resides in a protease/proteoglycan macromolecular complex in human cutaneous MC different from that of MC-CPA and chymase (103).

Inactivation of Exocytosed MC Proteases. The neutral proteases of the MC are stored in secretory granules in the mature forms, but the low pH of the granule helps to prevent their autolysis (104–106). After their exocytosis into a pH 7.0 environment, most MC proteases remain in the macromolecular complex because their proteoglycan-binding domains are Lys and Arg rich. The large size of the exocytosed protease/proteoglycan macromolecular complexes physically hinders the diffusion of the proteases in tissues and, in some cases, restricts their substrate specificities (107, 108). In most cases, the exocytosed proteases are retained in inflammatory sites for >1 hr. The tryptase mMCP-7 is an exception because its proteoglycan-binding domain is rich in His (24, 109). In the acidic granule, mMCP-7 interacts ionically with serglycin proteoglycan via its positively charged His residues. However, the proteoglycan-binding face on the surface of mMCP-7 tryptase loses much of its overall positive charge when it is exocytosed into a neutral pH environment. This allows the rapid dissociation of the tryptase from the macromolecular complex.

Numerous adjacent cell types in connective tissue matrices can endocytose and inactivate protease/proteoglycan macromolecular complexes exocytosed from activated MC (110–114). Although the major way MC proteases are cleared from inflammatory sites appears to be through this ubiquitous endocytic pathway, plasma-localized inhibitors have been identified that can inactivate some MC proteases *in vitro*. In the case of the chymases, these inhibitors include α_1-antichymotrypsin inhibitor, α_1-proteinase inhibitor, secretory leukocyte proteinase inhibitor, lactoferrin, and α_2-macroglobulin (115–120). The role of serglycin proteoglycans in chy-

mase catabolism is controversial. Heparin has been reported to prevent the inactivation of RMCP-1 by the multitude of inhibitors in plasma (120) but has been reported to enhance the inactivation of its human homologue by secretory leukocyte proteinase inhibitor (119). MC tryptases purified from human lung are somewhat resistant to inactivation by plasma-localized protease inhibitors (93, 121, 122). However, at least one rat MC tryptase readily binds the inter-α-trypsin inhibitor (123, 124) and α_1-macroglobulin (125) families of protease inhibitors. Recombinant mMCP-6 (108) is quite susceptible to plasma-localized protease inhibitors, but recombinant mMCP-7 is not (126). mMCP-7 possesses a restricted substrate specificity (126). Thus, it probably escapes inactivation because it fails to cleave the bait regions in the various protease inhibitors.

CURRENT APPROACHES FOR DETERMINING THE PHYSIOLOGIC FUNCTION OF A MC PROTEASE

Different molecular, immunologic, and biochemical approaches have been used in *in vitro* systems in an attempt to determine the physiologic function of each purified MC granule protease. Although hundreds of proteins and biologically active peptides can be cleaved by MC chymases *in vitro*, angiotensin I (127) and procollagenase (128–130) are the two candidate substrates that have been most thoroughly studied. The presence of increased numbers of activated MC in a number of fibrotic conditions (131) has led to the proposal that MC-derived granule mediators enhance fibrosis rather than inhibit it. mMCP-5-null transgenic mice have relatively normal connective tissues (Stevens, unpublished observation). Thus, the significance of the *in vitro* observations that MC chymases can activate those collagenases that cause extensive destruction of connective tissues remains to be determined. MC tryptases induce cultured epithelial cells (132) and fibroblasts to proliferate (133–135); and kininogens, vasoactive intestinal peptide, β-lipotropin, adrenocorticotropic hormone, calcitonin gene-related peptide, pro-opiomelanocortin, pro-atrial natriuretic factor, pro-metalloproteinase-3, pro-urokinase, complement protein C3, fibrinogen, fibronectin, protease-activated receptor 1 (thrombin receptor), and protease-activated receptor 2 are some of the many substrates cleaved *in vitro* by purified human MC tryptase (93, 136–146). Schwartz and co-workers have cloned two human MC tryptase cDNAs (4, 5) but have concluded that human α-tryptase is not converted into an active enzyme due to an Arg $\rightarrow$ Gln conversion at residue -3 in its propeptide (91). Because human β-tryptase cannot activate multiple cell types and cleave all of the above candidate substrates *in vivo* and because pancreatic trypsin also degrades most of the above mammalian proteins *in vitro*, the physiologic substrates of the human MC β-tryptase remain to be elucidated. To overcome the limitations of *in vitro* studies with purified enzymes, investigators are beginning to rely more heavily on *in vivo* systems.

Generation of Transgenic Mice, MC-Deficient Mice, Mastocytosis Mice, and MC Lines. To study the physiologic and pathologic roles of a MC protease, transgenic mice have been developed with one or more disrupted MC protease gene (84), and mouse strains have been identified that exhibit strain-dependent expression of specific granule proteases (66, 67, 147) or the inhibitors that inactivate them (148). Animal models that are MC deficient (149) or contain too many MC (150, 151) permit the functional assessment of MC proteases. The V3 mastocytosis mouse (151), for example, was used in the determination that fibrinogen is a physiologic substrate of mMCP-7 (71, 126). Because no organ in the body contains a high density of MC, MC lines from the rat (152), dog (150), and mouse (151, 153) have been invaluable for the identification, cloning, and expression of certain MC proteases currently being used in *in vivo* studies.

Generation of Recombinant Proteases and Deduction of the Physiologic Functions of mMCP-6 and mMCP-7. The ability to obtain large amounts of recombinant MC proteases with the insect cell-expression technology has made it possible to study *in vivo* function. Previous functional studies of MC proteases required purification of the enzyme from tissues or cultured MC. However, because MC are not a major cell type in any body organ in any species, it is not possible to isolate enough MC protease from any tissue source for in-depth study. Moreover, because neutral proteases tend to undergo autolysis as they are purified and concentrated, they tend to lose and/or change their activity during the isolation process. Finally, all mouse MC have more than one neutral protease in their granules. Because many of these proteases are so similar biochemically and physicochemically, they generally cannot be purified to homogeneity. The danger of using nonrecombinant material was recognized more than a decade ago in the cytokine field. Because enzymes are catalytic, the presence of a trace protease contaminant in an analyzed preparation can have a profound effect on the interpretation of data generated from a substrate-specificity study. Thus, to ensure that a MC protease is not contaminated with another MC protease, recombinant MC protease should be used in studies of function.

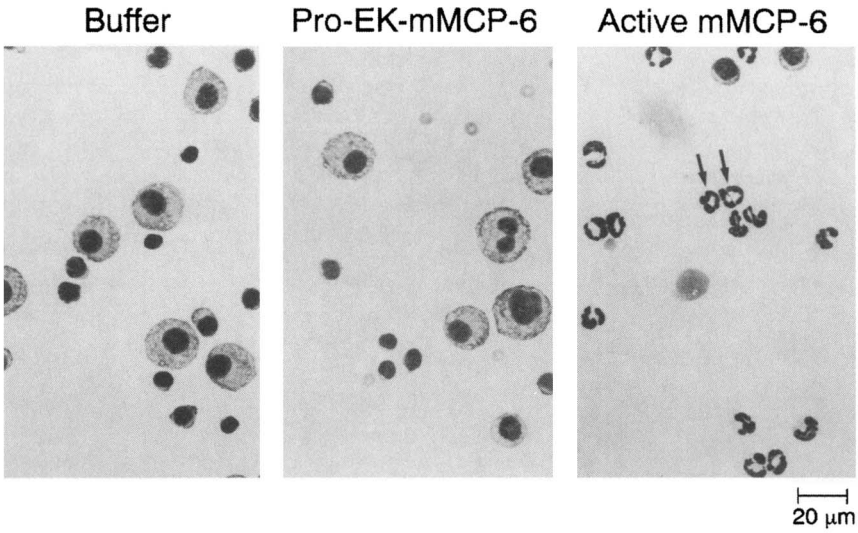

Fig. 1. mMCP-6-induced, neutrophil-specific peritonitis. The peritoneal cavities of separate BALB/c mice were injected with buffer alone (left), buffer containing enzymatically inactive pro-EK-mMCP-6 (middle), or buffer containing enzymatically active mMCP-6 (right). Thirty-six hours later, slides containing cytocentrifugation preparations of the cells in the peritoneal exudates of the treated mice were incubated with Diff-Quik stain. Arrows indicate two of the neutrophils that have extravasated into the peritoneal cavity of the mMCP-6-treated animal.

Of the different expression systems, the baculovirus/insect cell expression system has proven to be the most useful for the production of large amounts of properly folded mammalian proteins. In 1993, Urata and co-workers (154) infected insect cells with baculovirus constructs that encode the mature form of human MC chymase. Although recombinant chymase was obtained, the expressed protein was not properly processed by the insect cell and was not enzymatically active. This observation indicated that its natural propeptide probably plays an essential role in the proper folding of the chymase in the insect cell. Although mature chymase was not obtained with this direct approach, the natural zymogen form of human MC chymase was obtained, which could be purified and activated *ex vivo* by DPPI. Using this recombinant chymase, Husain and co-workers (82, 127, 154) confirmed the findings of their previous *in vivo* and *in vitro* studies that the chymase converts angiotensin I into the hypertensive factor angiotensin II.

In 1995 and 1996, pro-mMCP-7 (109) and human MC pro-tryptase β (91, 155) were expressed in insect cells. Both recombinant tryptases were secreted into the conditioned medium as properly folded zymogens, but neither was enzymatically active. MC tryptases have 10-residue propeptides. Thus, DPPI cannot activate the tryptases in the same manner as it does the chymases. Using a bioengineering approach, an alternate way of obtaining enzymatically active serine protease was therefore developed. In most instances the natural propeptide of the serine protease appears to be essential for the intracellular transport and/or folding of the initially translated zymogen. All mature serine proteases have an N-terminal Ile because, when the propeptide is enzymatically removed, the newly created N-terminal residue must bury itself in the activation groove before the precursor is converted into an active enzyme. Enterokinase (EK) is a highly specific enzyme that cleaves the Lys–Ile bond in its Asp–Asp–Asp–Lys–Ile recognition motif (156). Thus, a baculovirus construct was prepared which encoded a pseudozymogen form of mMCP-7 containing the 5-residue EK-susceptible site in between the 10-residue natural propeptide and the mature enzyme. Because EK is a relatively stable enzyme active at pH 5.0, it was anticipated that a secreted recombinant pseudozymogen could be purified and then activated with EK under conditions where the generated tryptase would have little enzymatic activity until the pH was raised to 7.0.

In V3 mastocytosis mice undergoing systemic anaphylaxis, exocytosed mMCP-7 rapidly makes its way into the blood, where it circulates for >1 hr (71). The discovery that this plasma form of mMCP-7 is enzymatically active even though 10% or more of the proteins in plasma are protease inhibitors raised the possibility that one of its physiologic substrates resides in blood. Relative to plasma from mice that are sensitized with IgE but

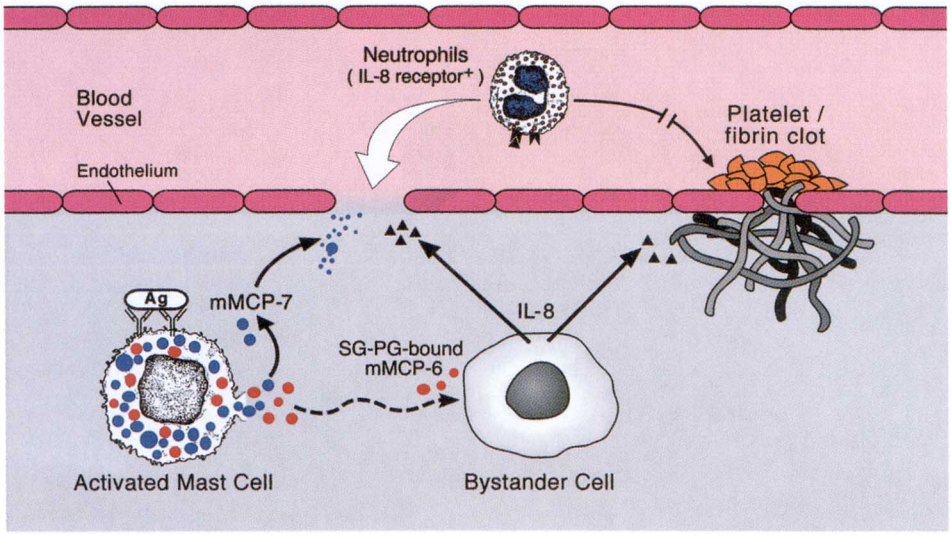

Fig. 2. Roles of mMCP-6 and mMCP-7 in a MC-mediated inflammatory reaction in the skin. Within minutes after a MC that resides deep in the epidermis is immunologically activated, exocytosed mMCP-7 diffuses away from the MC toward the blood/endothelial barrier. There is a marked influx of fibrinogen and other plasma proteins into the inflammatory site during the edema reaction caused by MC-derived histamine and other vasopermeability factors. If the N terminus of the α chain of fibrinogen is cleaved at the appropriate site by thrombin during this inflammatory reaction, cross-linked fibrinogen will accumulate at the blood/endothelial cell barrier. Platelets have an integrin receptor that recognizes fibrin. Thus, the deposited fibrin will bring about the accumulation of aggregated platelets at the site, which, in turn, will physically prevent the extravasation of peripheral blood leukocytes into the site to inactivate the noxious foreign agent. In the proposed mechanism, a gradient of mMCP-7 rapidly inactivates most of the fibrinogen that initially enters the site. Although 10% or more of the proteins in plasma are protease inhibitors, mMCP-7 is not inactivated by the plasma protease inhibitors that also diffuse into the inflamed site because it is essential to prevent the formation of the fibrin/platelet clot. Thus, mMCP-7 is an immunoprivileged protease. In contrast, mMCP-6 is preferentially retained in the inflammatory site bound to serglycin proteoglycan (SG-PG) so that it can act directly on an undefined receptor on bystander cells to induce them to produce large amounts of IL-8. This chemokine, in turn, induces neutrophils to extravasate into the inflamed tissue.

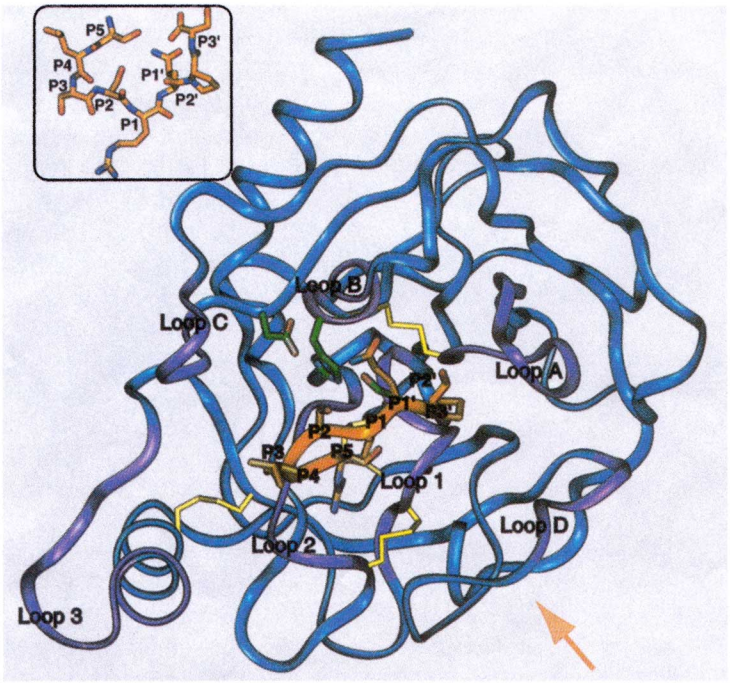

Fig. 3. Comparative protein structure modeling of the complex between mMCP-7 and its preferred peptide substrate. The backbone of the mMCP-7 model, built by MODELLER (164), is represented by a blue and purple ribbon. The seven loops (purple) that form the substrate-binding pocket of mMCP-7 are labeled according to Perona and Craik (165) for the serine protease family. The active-site residues (His, Asp, and Ser) and disulfide bonds are green and yellow, respectively. The peptide substrate (Ser–Leu–Ser–Ser–Arg–Gln–Ser–Pro) is shown as a ribbon with explicit side chains (orange). The substrate P5 to P3′ residues are numbered at their C_α atoms. The bound peptide resembles the letter "U." The inset shows the peptide substrate viewed sideways from that in the enzyme pocket. The oxygen and nitrogen atoms in the substrate are shown in red and blue, respectively. The plots were prepared by Insight II.

not challenged with antigen, plasma from IgE/antigen-treated V3 mastocytosis mice contained large amounts of four peptides derived from fibrinogen (126). These findings suggested that fibrinogen is the physiologic substrate of mMCP-7. However, with this *in vivo* model system alone, it was not possible to deduce whether the tryptase exerts its effect directly or indirectly. Thus, the derivation of recombinant mMCP-7 was instrumental in showing that this tryptase selectively degrades the α chain of fibrinogen before it can be converted into cross-linked fibrin.

The primary amino acid sequences of mMCP-6 and mMCP-7 are 71% identical (17, 18, 23, 24), but *in vivo* and *in vitro* studies carried out with the two recombinant mouse tryptases revealed that they have very different physiologic functions. Because the MC that reside in the peritoneal cavity of BALB/c mice contain mMCP-6 but not mMCP-7 (147), it was anticipated that mMCP-6 exerts its function in the peritoneal cavity. When recombinant mMCP-6 was injected into the peritoneal cavity of BALB/c (Fig. 1), MC-deficient WCB6F$_1$-*Sl/Sl^d*, C5-deficient, or mMCP-5-null mice, the number of neutrophils in this tissue site increased >50-fold (108). In contrast, neither pro-mMCP-6 nor mature mMCP-7 induced the neutrophil peritonitis. Unlike most forms of acute inflammation, the mMCP-6-mediated peritonitis was relatively long-lasting and neutrophil-specific. mMCP-6 did not induce cultured human endothelial cells to express tumor necrosis factor-α, RANTES, IL-1-α, or IL-6, but it did induce endothelial cells to express large amounts of IL-8. A pronounced neutrophil peritonitis also occurs when a purified human lung tryptase is injected into BALB/c mice (157). Although the mechanism at the molecular level by which mMCP-6 and the human lung tryptase induce neutrophil accumulation in tissues remains to be determined, the finding that both tryptases induce cultured human endothelial cells to selectively release large amounts of IL-8 (108, 132) suggests that they regulate the steady-state levels of neutrophil-specific chemokines *in vivo* during MC-mediated inflammatory events. It appears that mMCP-6 and mMCP-7 evolved to work in concert to control inflammatory responses in skin, heart, and other organs that contain MC which express both tryptases (Fig. 2).

Because mMCP-6 and mMCP-7 play important but distinct roles in inflammation, it is necessary to understand the substrate specificity of each protease for the design of mMCP-specific protease inhibitors. All three chains of fibrinogen contain a large number of Lys and Arg residues, yet mMCP-7 preferentially cleaves a site in this plasma protein to liberate the globular C-terminal portion of the α chain. Thus, the mMCP-7 has a substrate specificity that is considerably more restricted than that of trypsin. Phage-display peptide libraries have been used primarily to map the antigenic epitopes in proteins and to identify antibodies that are relatively specific for the protein of interest. The genome of the filamentous bacteriophage consists of the 11 genes designated gI to gXI. Although the protein (pIII) encoded by gIII is chymotrypsin, thermolysin, and subtilisin susceptible (158), phage-display peptide libraries can give insight into the substrate specificities of proteases that have restricted activities (159–162). With this approach, it was discovered that mMCP-6 (108) and mMCP-7 (126) preferentially cleave Lys/Arg-rich peptides and Ser-rich peptides, respectively. Comparative protein modeling based on the crystallographic structure of trypsin (163) indicates that MC tryptases have more restricted substrate specificities than pancreatic trypsin, due, in part, to residue insertions in three loops that form part of the substrate-binding cleft of each tryptase (24, 108, 126). It is predicted that these insertions in mMCP-7 cause the substrate-binding cleft to be deeper than that in trypsin. The three-dimensional model of mMCP-7 (Fig. 3) shows that the favored peptide, Ser–Leu–Ser–Ser–Arg–Gln–Ser–Pro, isolated from the screening of the phage-display peptide library can fit well in the active-site cleft.

CONCLUDING REMARKS

Over the last decade, many MC proteases have been identified, purified, and cloned from different species. The recent discovery of mMCP-9 and its selective expression in uterine MC raises the possibility that not all mouse MC proteases have been identified. Whereas the abundance of homologous proteases in mouse MC suggests that some of them are functionally redundant, the recent *in vivo* and *in vitro* studies carried out on recombinant mMCP-6 and mMCP-7 document that two homologous MC proteases can have very different functions. Molecular approaches should permit the function of each MC protease to be deduced. Transgenic mice that cannot express a particular MC protease or its inhibitor will be needed in these functional studies. Strain-dependent differences in the amino acid sequence of mMCP-5 (the homologue of human MC chymase) have already been found. As shown for granzyme B, a change in a single amino acid in the substrate-binding cleft of a serine protease can dramatically alter its substrate specificity. Once all MC proteases have been identified in humans, their allelic variants will be sought and then the functional consequences of the differences in primary amino acid sequences will be determined experimentally. Finally, it is anticipated that data from modeling, com-

binatorial, and expression approaches will lead to the development of new therapeutic approaches for treating the protease component of MC-mediated inflammatory reactions.

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