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Packaging of Proteases and Proteoglycans in the Granules of Mast Cells and Other Hematopoietic Cells

A CLUSTER OF HISTIDINES ON MOUSE MAST CELL PROTEASE 7 REGULATES ITS BINDING TO HEPARIN SERGLYCIN PROTEOGLYCANS*

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Mouse mast cell protease 7 (mMCP-7) is a tryptase stored in the secretory granules of mast cells. At the granule pH of 5.5, mMCP-7 is fully active and is bound to heparin-containing serglycin proteoglycans. To understand the interaction of mMCP-7 with heparin inside and outside the mast cell, this tryptase was first studied by comparative protein modeling. The "pro" form of mMCP-7 was then expressed in insect cells and studied by site-directed mutagenesis. Although mMCP-7 lacks known linear sequences of amino acids that interact with heparin, the three-dimensional model of mMCP-7 revealed an area on the surface of the folded protein away from the substrate-binding site that exhibits a strong positive electrostatic potential at the acidic pH of the granule. In agreement with this calculation, recombinant pro-mMCP-7 bound to a heparin-affinity column at pH 5.5 and readily dissociated from the column at pH > 6.5. Site-directed mutagenesis confirmed the prediction that the conversion of His residues 8, 68, and 70 in the positively charged region into Glu prevents the binding of pro-mMCP-7 to heparin. Because the binding requires positively charged His residues, native mMCP-7 is able to dissociate from the protease/proteoglycan macromolecular complex when the complex is exocytosed from bone marrow-derived mast cells into a neutral pH environment. Many hematopoietic effector cells store positively charged proteins in granules that contain serglycin proteoglycans. The heparin/mMCP-7 interaction, which depends on the tertiary structure of the tryptase, may be representative of a general control mechanism by which hematopoietic cells maximize storage of properly folded, enzymatically active proteins in their granules.

As much as 30% of the total protein of a mature mouse mast cell consists of 26- to 36-kDa proteases that are enzymatically active at neutral pH (1–5). A cDNA that encodes mouse mast cell carboxypeptidase A (mMC-CPA)[1] (4) has been cloned, as have the cDNAs and genes that encode six of the mast cell's seven granule serine proteases, designated mouse mast cell protease (mMCP) 1 to mMCP-7 (3–13). Based on the deduced amino acid sequences of their cDNAs, all mast cell proteases are initially translated as zymogens that possess a 15- to 19-residue hydrophobic signal peptide and a 2- to 94-residue "pro" activation peptide. NH_2-terminal amino acid analyses of the SDS-PAGE-resolved proteins in isolated granule preparations have revealed that these proteases are preferentially stored intracellularly in their mature forms (3–5). The function(s) of the propeptides remain to be elucidated. Although they may be important for proper folding and/or intracellular targeting of the translated mast cell serine proteases, most likely the propeptides simply prevent the proteases from exhibiting enzymatic activity until they are safely sequestered in the granule. The specific substrates of the mMCPs also remain to be determined, but mMCP-6 and mMCP-7 have been classified as tryptases. The other serine proteases have been classified as chymases.

The mature mast cells that reside in the ear and skin of the BALB/c mouse and the immature mast cells that are derived by culturing BALB/c mouse bone marrow cells for 2–3 weeks in medium containing interleukin 3 (mBMMC) express mMCP-7, in addition to mMC-CPA, mMCP-5, and mMCP-6. BALB/c mBMMC activated at neutral pH through their FcεRI receptors (14) exocytose their proteases (2). When the resulting supernatants are chromatographed on a Sepharose CL-2B column, most mMC-CPA (1) and serine protease (15) activities filter in the column's excluded volume as a $>10^7$ Da complex with heparin-containing serglycin proteoglycans. Rat (16–20) and human (21, 22) mast cell proteases also strongly bind heparin-containing serglycin proteoglycans. In the case of rat serosal mast cells, 1 M NaCl or 2 M KCl is required to separate the components of the macromolecular complex. Thus, even in a neutral pH environment, much of the macromolecular complex exocytosed from an activated mast cell remains intact outside the cell.

Johnson and Barton (13) noted that mast cell tryptases have a higher content of His than pancreatic trypsin and the other proteases found in the mast cell granule. While the specific amino acid residues that interact with heparin-containing ser-

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[1] The abbreviations used are: mMC-CPA, mouse mast cell carboxypeptidase A; mBMMC, mouse bone marrow-derived mast cells; mMCP, mouse mast cell protease; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

glycin proteoglycans were not predicted, comparative modeling of a human skin mast cell tryptase suggested that many of the His residues were on the surface of the folded human protease, as were Arg and Lys residues. Thus, it was proposed that the binding of this mast cell tryptase to heparin-containing serglycin proteoglycans inside the granule might depend on His residues as well as on Arg and Lys residues. Against this hypothesis was the observation that human lung mast cell tryptase bound heparin strongly at neutral pH and that a NaCl concentration >0.8 M was required to dissociate the complex *ex vivo* (21). Moreover, even though BALB/c mBMMC express two tryptases (6, 11), ~80% of the total serine protease activity in the pH 7.2 supernatants of FcεRI-activated cells resides in the macromolecular complex (15). Nevertheless, the observation that mMCP-6 and mMCP-7 are proteins rich in His raised the possibility that some proteases might dissociate from the protease-proteoglycan macromolecular complex after the granules are exocytosed from an activated mouse mast cell into a neutral pH environment.

To understand how mMCP-7 is packaged in granules and whether or not this tryptase dissociates from heparin-containing serglycin proteoglycans outside the mast cell, a comparative modeling study of mMCP-7 was combined with electrostatic calculations to predict which residues participate in heparin binding. To test the model, the pro form of mMCP-7 was expressed in insect cells. Using site-directed mutagenesis, we confirmed the prediction that His residues 8, 68, and 70 in the properly folded protein are essential for its ionic interaction with heparin inside and outside the mast cell. We have also shown that mMCP-7 dissociates from the macromolecular complex exocytosed at neutral pH from FcεRI-activated mBMMC.

MATERIALS AND METHODS

Comparative Modeling of mMCP-7—A detailed comparative modeling study of mMCP-7 was combined with electrostatic calculations to obtain specific predictions about which residues participate in heparin binding. A three-dimensional model of mMCP-7 was built by MODELLER-11,[2] a program that implements comparative modeling by satisfaction of spatial restraints (23–26). The multiple alignment of mMCP-7 with serine proteases of known three-dimensional structures indicated that the crystallographic structure of bovine pancreatic trypsin (Bookhaven Protein Data Bank code 2PTN) (27) was a suitable template structure for comparative modeling of mMCP-7. Because of the relatively high similarity (39% identity) between the amino acid sequences of pancreatic trypsin and mMCP-7, the alignment was prepared by hand, ensuring that the gaps were located in the exposed loops outside of helices and strands. The sequence alignment between pancreatic trypsin and mMCP-7 was used with program MODELLER-11 to produce a mMCP-7 model containing all main chain and side chain heavy atoms without further manual intervention. First, MODELLER-11 was used to derive many distance and dihedral angle restraints on the mMCP-7 sequence from its alignment with the template trypsin structure. Then, the spatial restraints and CHARMM-22[3] energy terms (28) enforcing proper stereochemistry were combined into an objective function. The variable target function procedure, which employs methods of conjugate gradients and molecular dynamics with simulated annealing, was used to obtain three-dimensional models by optimizing the objective function. Ten slightly different three-dimensional models of mMCP-7 were calculated by varying the initial structure. The root mean square difference for superposition of the main chain atoms of these models was generally less than 0.2 Å. The structure with the lowest value of the objective function was selected as the representative model. The model of mMCP-7 passes all the stereochemistry checks implemented in the program PROCHECK-3 (29). The three-dimensional models of the $His^{8}\rightarrow Glu^{8}$, $His^{68}\rightarrow Glu^{68}$, $His^{70}\rightarrow Glu^{70}$, $His^{80}\rightarrow Glu^{80}$, and $His^{187}\rightarrow Glu^{187}$ mutants of mMCP-7 were then calculated in a similar manner. The 10-residue activation peptide of pro-mMCP-7 (11) was not considered in any of the three-dimensional models because of its low sequence similarity to the structurally defined propeptides of trypsinogen and chymotrypsinogen.

Electrostatic Potential of mMCP-7 and Five His→Glu Mutants—The electrostatic potentials of mMCP-7 and its five His→Glu mutants were calculated with the UHBD-3 program (30), using standard charges from the CHARMM-22 force field (28). Two calculations were performed in which each His was assigned either a positive or neutral charge, corresponding to the pH below and above the pK_a 6.5 of this amino acid, respectively.

Expression of Normal and Mutated pro-mMCP-7 in Insect Cells—To obtain recombinant pro-mMCP-7 in insect cells, a cDNA that encodes prepro-mMCP-7 (11) was inserted in the correct orientation into the multiple cloning site of pVL1393 (PharMingen) downstream of the promoter of the polyhedrin gene (31). The expression construct was designed to encode prepro-mMCP-7 rather than pro-mMCP-7 because a hydrophobic signal peptide is generally needed for expressed foreign proteins to traverse the endoplasmic reticulum of the insect cell. Mature mMCP-7 was not expressed in insect cells because of potential autolysis of the recombinant mature protease and because other mast cell granule proteases initially bind to serglycin proteoglycans as inactive zymogens (32, 33). Plasmid DNA (~2 μg), purified by CsCl density gradient centrifugation (34), was mixed with 0.5 μg of linearized BaculoGold™ DNA (PharMingen) and calcium phosphate in a total volume of 1 ml. After a 5-min incubation at room temperature, the resulting DNA solution was added to a 6-cm Falcon tissue culture dish containing 1 ml of Grace's insect medium and 3×10^{6} adherent *Spodoptera frugiperda 9* (Sf9) (Invitrogen) cells that were in their log phase of growth. After a 4-h incubation at 27 °C, the infection medium was replaced with 3 ml of TNM-FH insect medium (Invitrogen) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (Sigma). The infected insect cells were cultured for 7 days at 27 °C. After the amplification of a single virus clone, $\geq 3 \times 10^{7}$ recombinant virus particles were added to each 10-cm tissue culture dish containing 6×10^{6} Sf9 cells or *Trichoplusia ni* High Five™ cells (Invitrogen) in their log phase of growth. Three to 4 days later, the collected insect cells and supernatants were separated from one another by a 5-min room temperature centrifugation at ~300 × *g*. In those experiments in which recombinant pro-mMCP-7 had to be purified from the conditioned medium, infected High Five cells were cultured in the presence of Insect Xpress (Whittaker) protein-free medium.

His^{8}, His^{68}, His^{70}, His^{80}, and His^{187} in pro-mMCP-7 were each individually changed to Glu residues with a three-step, site-directed mutagenesis approach. To make the $His^{8}\rightarrow Glu^{8}$ mutated form of pro-mMCP-7, the first polymerase chain reaction (PCR) was carried out using a synthetic oligonucleotide (5′-TTCCGGATTATTCATACCGTCCCACCA-3′) that corresponds to a 5′-region in pVL1393 and a synthetic oligonucleotide (5′-GGACAGGAGGCAGAGGGGAACAAGTGG-3′) that spans the desired mutation site in the mMCP-7 cDNA. A second PCR was carried out using a synthetic oligonucleotide (5′-CCTTTCCATCCAACGACAAGC-3′) that corresponds to a 3′-region in pVL1393 and a synthetic oligonucleotide (5′-CCACTTGTTCCCCTCTGCCTCCTGTCC-3′) that also spans the desired mutation site in the mMCP-7 cDNA but in the opposite direction. The two PCR-generated products were purified and mixed in equal proportions, and then a third PCR was performed with the pVL1393-specific oligonucleotides. The final PCR product was digested with *Eco*RI and inserted into *Eco*RI-digested pVL1393, and the DNA sequence of the mutated construct was confirmed in both directions. Synthetic oligonucleotides 5′-CAGTACCTCTATTACGAGGACCACCTGATGACT-3′ and 5′-AGTCATCAGGTGGTCCTCGTAATAGAGGTACTG-3′, 5′-CTCTATTACCATGACGAGCTGATGACTGTGAGC-3′ and 5′-GCTCACAGTCATCAGCTCGTCATGGTAATAGAG-3′, 5′-AGCCAGATCATCACAGAGCCCGACTTCTACATC-3′ and 5′-GATGTAGAAGTCGGGTCTTGTGATGATCTGGCT-3′, and 5′-GCTGGGAATGAAGGAGAAGACTCCTGCCAG-3′ and 5′-CTGGCAGGAGTCTTCTCCTTCATTCCCAGC-3′ were used to generate the $His^{68}\rightarrow Glu^{68}$, $His^{70}\rightarrow Glu^{70}$, $His^{80}\rightarrow Glu^{80}$, and $His^{187}\rightarrow Glu^{187}$ mutants of pro-mMCP-7, respectively.

SDS-PAGE, Immunoblotting, and NH_2-terminal Amino Acid and Carbohydrate Analysis of Recombinant pro-mMCP-7—A SDS-PAGE buffer (20% glycerol, 4% SDS, 5% 2-mercaptoethanol, 0.004% bromphenol blue, and 125 mM Tris-HCl, pH 6.8) was added (1:1 v/v) to each culture supernatant, and 2 ml of another SDS-PAGE buffer (0.5% SDS,

[2] MODELLER is available by anonymous FTP from tammy.harvard.edu:pub/modeler and also as part of QUANTA (MSI, Burlington, MA; E-mail, jcollins@msi.com).

[3] A. D. MacKerell, Jr., D. Bashford, M. Bellott, R. L. Dunbrack, Jr., M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, D. Joseph, L. Kuchnir, K. Kuczera, F. T. K. Lau, C. Mattos, S. Michnick, T. Ngo, D. T. Nguyen, B. Prodhom, B. Roux, M. Schlenkrich, J. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorkiewicz-Kuczera, and M. Karplus, manuscript in preparation.

100 μg/ml phenylmethylsulfonyl fluoride, 50 mM 2-mercaptoethanol, and 20 mM sodium phosphate, pH 7.6) was added to each pellet of ~10^7 baculovirus-infected insect cells. The samples were sonicated, boiled, and loaded onto 12 or 15% Tris/Tricine gels (35). After a 16-h electrophoresis (anode buffer = 0.2 M Tris-HCl, pH 8.9; cathode buffer = 0.1 M Tris-HCl, 0.1% SDS, 0.1 M Tricine, pH 8.25) at 20 mA under reducing conditions, the gels were stained with Coomassie Blue or were electroblotted onto Immobilon membranes in a transfer buffer consisting of 10% methanol and 10 mM CAPS, pH 11.3. The resulting protein blots were then analyzed for their reactivity with a mMCP-7-specific peptide antibody (36). For NH_2-terminal amino acid analysis of recombinant mMCP-7, proteins resolved from replicate samples by SDS-PAGE were transferred to Immobilon and briefly stained with 0.5% Ponceau S (Sigma) (5). The immunoreactive protein bands were excised, placed in the reaction cartridge of an Applied Biosystems model 475A gas-phase protein sequencer, and subjected to automated Edman degradation by the Biopolymer Facility at Brigham and Women's Hospital. Phenylthiohydantoin-derivatives, obtained after each cycle, were resolved by high performance liquid chromatography and were identified by manual inspection of the resulting chromatograms.

To determine whether any of the recombinant pro-mMCP-7 produced by infected High Five cells was *N*- or *O*-glycosylated, SDS-PAGE buffer was added to each medium and cell pellet. Samples were briefly sonicated and then boiled for 5 min. A 50-μl portion of 7.5% Nonidet P-40 was added to each 100-μl sample, followed by the addition of 2 μl (~0.5 milliunits) of *Flavobacterium meningosepticum N*-glycanase (Genzyme) with or without 2 μl (~2 milliunits) of *Diplococcus pneumoniae O*-glycanase (Genzyme). After the samples were incubated overnight at 37 °C, the resulting digests were subjected to SDS-PAGE/immunoblot analysis to assess for an *N*- or *O*-glycanase-induced change in the size of recombinant pro-mMCP-7.

Binding of Recombinant pro-mMCP-7 to Heparin-Sepharose CL-6B—The pH of each 3-day culture supernatant of insect cells induced to express normal or mutated pro-mMCP-7 was adjusted to 5.0. This supernatant was applied to a 1-ml precolumn of Sepharose CL-6B (Pharmacia) and then to a 1–3-ml column of heparin-Sepharose CL-6B (Pharmacia). After each heparin-Sepharose CL-6B column was washed with pH 5.0 phosphate-buffered saline (150 mM NaCl and 10 mM sodium phosphate), the bound proteins were eluted with a step gradient in which the pH of the buffer was adjusted sequentially to 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. Alternatively, bound proteins were eluted from the affinity column with a continuous linear salt gradient. In this procedure, the heparin-Sepharose CL-6B column was washed with 1 column volume of pH 5.0–5.2 buffer (0.1% Triton X-100, 100 mM NaCl, and 50 mM sodium acetate), and subjected to a 50-ml gradient in which the NaCl concentration in this buffer varied from 100 to 500 mM.

FcεRI-dependent Activation of mBMMC, and Sepharose CL-2B Chromatography of Exocytosed Serine Proteases—mBMMC (~10^8), obtained as described previously (37), were sensitized with 50 μg of mouse monoclonal anti-2,4,6-trinitrophenol IgE for 1 h at 37 °C, washed once in modified Tyrode's buffer, resuspended in 1 ml of the same buffer, and then challenged for 10 min at 37 °C with 100 ng of trinitrophenol conjugated to bovine serum albumin. The activated mBMMC were concentrated by centrifugation at ~150 × *g* for 10 min at 4 °C, and the supernatants were removed by aspiration. The cell pellets were resuspended in modified Tyrode's buffer and assessed for their exclusion of Trypan blue. Samples of the supernatants and cell pellets were analyzed for their histamine content with a radioimmunoassay kit (AMAC, Westbrook, ME) (38) to evaluate the extent of exocytosis of this preformed mediator. The remaining supernatants (0.2 ml) were applied at 4 °C to an 80 × 0.8-cm column of Sepharose CL-2B (Pharmacia) that had been equilibrated with 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.2 (15). Samples (0.2 ml) of each 1-ml column fraction were applied to replicate Immobilon-P PVDF membranes (Millipore) with a Bio-Dot SF Blotting Apparatus (Bio-Rad). The membranes were incubated sequentially for 1 h in Tris-HCl-buffered saline containing 5% nonfat milk, for 17 h in a 1/500 dilution (~1.5 μg/ml) of either affinity-purified anti-mMCP-5 Ig (39) or affinity-purified anti-mMCP-7 Ig (36) in TBST buffer (Tris-HCl-buffered saline containing 0.1% Tween 20), for 30 min in TBST buffer, and then for 1 h in a 1/1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) in TBST buffer. After five additional washes, the blots were developed with a chemiluminescence kit (Amersham Corp.). Bound antibodies were visualized by autoradiography.

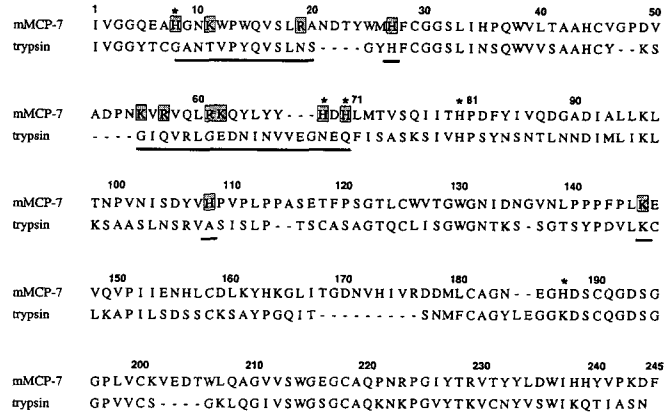

FIG. 1. **Amino acid alignment of mMCP-7 and bovine pancreatic trypsin.** The *numbers* in the top line refer to the residues in mMCP-7. The *five horizontal lines* under the alignment indicate the sequences that comprise the positively charged, heparin-binding region in mMCP-7. The Arg, Lys, and His residues in this region are *highlighted*. The *stars* indicate the five His residues that were individually mutated to Glu.

RESULTS

Comparative Modeling of mMCP-7—Trypsin and mMCP-7 have 223 and 245 residues, respectively, and the comparison of their sequences revealed 7 insertions and 2 deletions in mMCP-7 (Fig. 1). The largest insertion, at position 168, consists of 9 residues. The approximate positions of the regions comprising the heparin-binding domain described below are likely to be modeled correctly for the following three reasons. First, although insertions are difficult to model, none are in the putative heparin-binding domain. Second, all gaps could be easily inserted on the surface of the fold, outside the strands and helices. Third, the alignment of equivalent segments was straightforward due to relatively high similarity between the two sequences. The three-dimensional model of mMCP-7 (Fig. 2*A*) suggests that this mast cell tryptase has a trypsin-like fold consisting of two domains with the active site located in the cleft at the interface between the domains. The backbone structure of the mMCP-7 model is virtually indistinguishable from that of pancreatic trypsin. The probable error in the model of mMCP-7 is ~1Å for most exposed backbone atoms. Approximately 70, 70, 50, and 50% of the side chains are expected to have dihedral angles χ_1, χ_2, χ_3, and χ_4, respectively, in the correct minima. Although there are considerable uncertainties in the positions of the positive charges at the end of the long Lys and Arg side chains on the surface of mMCP-7, these uncertainties do not significantly influence the global electrostatic potential around mMCP-7.

Electrostatic Potential of mMCP-7 and Identification of a Putative Heparin-binding Region—Based on its three-dimensional model (Fig. 2*A*), mMCP-7 has a His-rich region (Fig. 2*B*) on its surface that contains considerably more positive than negative charges at acidic pH (Fig. 3*A*). This positively charged region is located far from the active site. It is a convex strip approximately 20 Å long and 10 Å wide, and therefore could interact with ~5 monosaccharides within heparin glycosaminoglycan. The chain segments comprising this region are not contiguous. The region is defined by residues His^{27}, His^{108}, Lys^{146}, and the two long segments spanning residues 8–20 and residues 55–70 (Figs. 1–3). Each long segment consists of an irregular structure and a β-strand. The β-strands from the two long segments are anti-parallel and are hydrogen bonded to each other. In total, there are 5 His, 4 Lys, 3 Arg, and 1 Asp residues in this region, resulting in a net charge of +11. Of the 5 His residues (residues 8, 27, 68, 70, and 108 in the mature

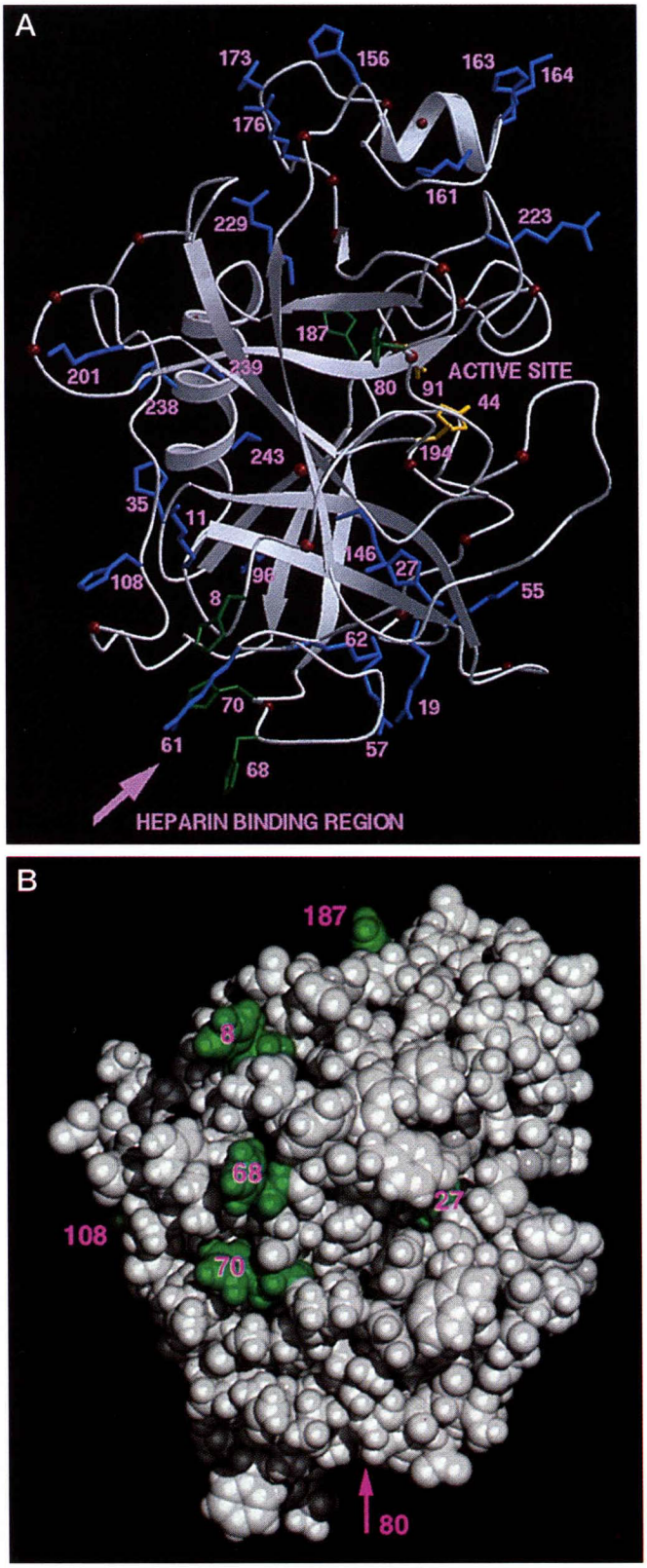

FIG. 2. **Three-dimensional model of mMCP-7 at pH 5.5.** In the ribbon diagram (*A*), the positively charged side chains (Lys, Arg, and His), the C_α atoms of the negatively charged residues (Asp, Glu), the active site side chains (His^{44}, Asp^{91}, and Ser^{194}), and the mutated His side chains are shown in blue, red, yellow, and green, respectively. The figure was prepared by programs MOLSCRIPT (40) and RASTER3D (41). The His, Arg, Lys, and active site residues are numbered in pink. The space-filling model of the heparin-binding region (*B*) has been obtained by rotating 90 degrees the model depicted in *panel A*. His residues 8, 27, 68, 70, 80, 108, and 187 are shown in green. The rest of the residues are shown in white.

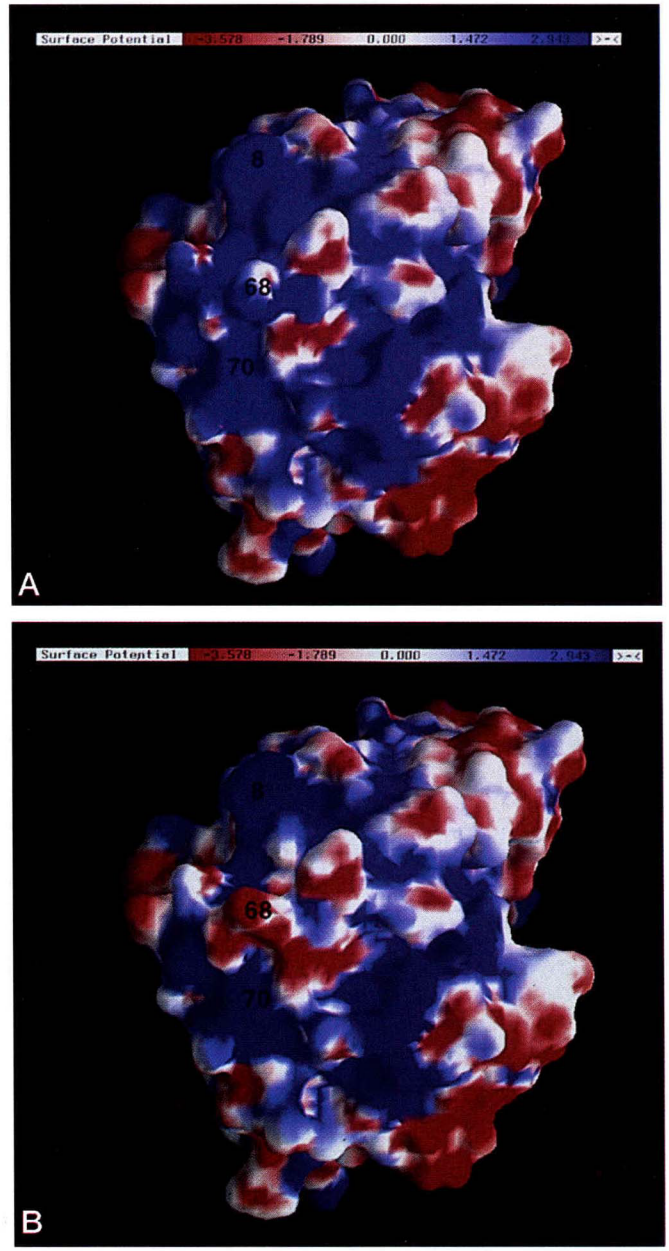

FIG. 3. **Electrostatic potential at the positively charged region of normal and altered mMCP-7.** Non-mutated mMCP-7 (*A*) and the $His^{68} \rightarrow Glu^{68}$ mutant of mMCP-7 (*B*) at the acidic pH of the granule. The molecular surfaces of the models are colored by the electrostatic potential, as shown by the color bar on each panel (in units of kT; 1 kT unit = 0.58 kcal/electron mol). The figures were prepared by program GRASP (42), using the relative dielectric constants of 2 and 78 for protein and solvent, respectively, and the salt concentration of 150 mM. The three His residues in the positively charged region are numbered in black. The orientation of the tryptase is the same as in Fig. 2*B*.

protease), His^{68} is located in the center of the region; His^{27} and His^{108} are on the periphery and are less exposed.

To determine whether the positively charged region in mMCP-7 is likely to bind to the heparin chain of serglycin proteoglycan, it is necessary to calculate the overall electrostatic potential, which is a sum of the contributions from positive and negative charges from all parts of the molecule. A plot of the electrostatic potential around mMCP-7 with positively charged His residues is shown in Fig. 3*A*. There is only one region of pronounced positive electrostatic potential and the electrostatic potential at the molecular surface of this region is diminished significantly at neutral pH when the positive

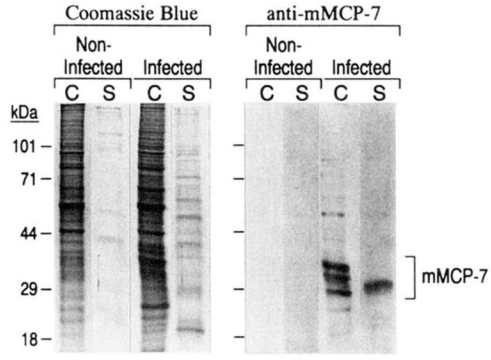

FIG. 4. **Expression of pro-mMCP-7 in High Five cells.** High Five cells were either non-infected or were infected with the pVL1393 construct that encodes prepro-mMCP-7. Three days later, samples of the cell pellets (*C*) and culture supernatants (*S*) were applied to individual wells of two polyacrylamide gels. After SDS-PAGE, the gel on the left was stained with Coomassie Blue. On the right, the protein blot from the duplicate gel was stained with anti-mMCP-7 Ig. Molecular mass markers in kDa are indicated on the *left*.

charges on the His residues are eliminated (data not shown). Moreover, the positive electrostatic potential of this region is also greatly diminished if His^{8}, His^{68} (Fig. 3*B*), or His^{70} is mutated to Glu. In contrast, His^{80} and His^{187} do not reside in the positively charged region, and their conversion to Glu does not influence the electrostatic potential of the region (data not shown).

Expression of Normal and Mutated pro-mMCP-7 in Insect Cells—As assessed by SDS-PAGE/immunoblot analysis, High Five cells infected with a pVL1393-derived construct encoding prepro-mMCP-7 released substantial amounts of soluble 28- and 29-kDa proteins into the 3-day culture medium that reacted strongly with affinity purified anti-mMCP-7 Ig (Fig. 4). As assessed by Coomassie Blue staining, these expressed proteins represented as much as 10% of the total protein produced by the infected cells. Neither immunoreactive protein was produced by control non-infected High Five cells. NH_2-terminal amino acid analysis of the 28- and 29-kDa proteins in the supernatant revealed in each instance a single prominent sequence of Ala-Pro-Gly-Pro-Ala-Met-Thr-Arg-Glu-Gly-Ile-Val. This 12-mer sequence is identical to the NH_2-terminal sequence of pro-mMCP-7 deduced from its cDNA. Besides the 28- and 29-kDa immunoreactive proteins found in the culture medium, additional ~32 and ~27-kDa immunoreactive proteins occasionally were detected inside the infected insect cells (Fig. 4). All immunoreactive cell-associated proteins possessed an amino terminus identical to that of recombinant pro-mMCP-7 recovered from the culture medium. *N*-Glycanase converted the 29-kDa immunoreactive protein in the culture medium to a 28-kDa protein, whereas the combination of *N*-glycanase and *O*-glycanase converted the 32-kDa cell-associated immunoreactive protein and the 28-kDa medium-associated immunoreactive protein to 27-kDa proteins (data not shown). Infected Sf9 cells also produced pro-mMCP-7, but the amount released into the culture medium when these cells were cultured in protein-free medium was less than that of High Five cells.

Binding of Recombinant pro-mMCP-7 and Its His→Glu Mutants to Heparin-Sepharose CL-6B—Recombinant pro-mMCP-7 bound to heparin-Sepharose CL-6B, but only when the pH was <6.5 (Fig. 5) and the NaCl concentration was <240 mM (Fig. 6). These findings suggested that the binding of pro-mMCP-7 to heparin is probably His-dependent. In protein modeling and electrostatic potential analysis, His^{8}, His^{68}, and His^{70} were speculated to be critical for the binding of pro-mMCP-7 to heparin but not His^{80} or His^{187}. Thus, five derivatives of pro-mMCP-7 were expressed in insect cells in which in each instance one of these specific His residues was point mutated to Glu. At pH 5.0–5.2, both the $His^{80}\rightarrow Glu^{80}$ mutant and the $His^{187}\rightarrow Glu^{187}$ mutant bound to the heparin-affinity column (Fig. 7) and were eluted when the NaCl concentration reached 240 mM (data not shown), analogous to non-mutated pro-mMCP-7. In contrast, neither the $His^{8}\rightarrow Glu^{8}$ mutant, the $His^{68}\rightarrow Glu^{68}$ mutant, nor the $His^{70}\rightarrow Glu^{70}$ mutant of pro-mMCP-7 bound to the heparin-affinity column (Fig. 7).

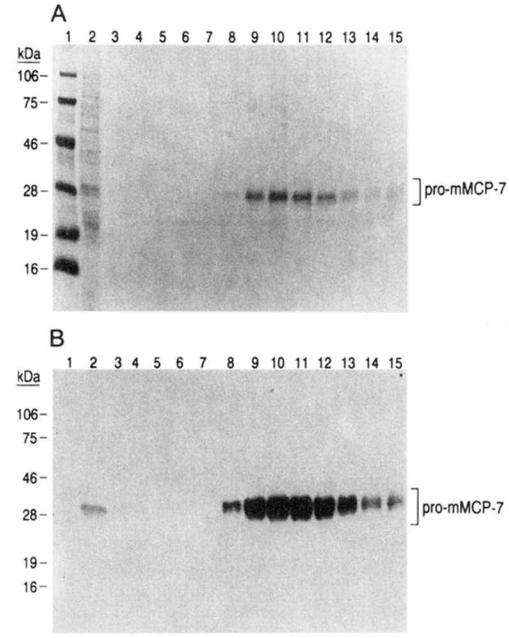

FIG. 5. **Effect of pH on the binding of recombinant pro-mMCP-7 to heparin-Sepharose CL-6B.** In this experiment, the culture supernatant from infected High Five cells was adjusted to pH 5.0 and applied to the heparin affinity column. The column was sequentially washed with phosphate-buffered saline adjusted to pH 5.0, 4.5, 5.0, 5.5, 6.0 (*lanes 3–5*), 6.5 (*lanes 6–8*), and 7.0 (*lanes 9–15*). The gel depicted in *panel A* was stained with Coomassie Blue. The SDS-PAGE/immunoblot depicted in *panel B* was prepared from a second gel and was stained with anti-mMCP-7 Ig. No ~28-kDa immunoreactive protein eluted when the affinity column was washed with pH 4.5, 5.0, or 5.5 buffer (data not shown). *Lane 2* contains a sample of the culture supernatant that was initially applied to the column. Molecular mass markers in kDa are indicated on the *left* and in *lane 1* of *panel A*.

Gel Filtration Chromatography of Supernatants from FcεRI-Activated mBMMC—A 24 ± 17% (mean ± S.D., $n = 4$) release of histamine was obtained from BALB/c mBMMC sensitized with IgE and activated with antigen. When the supernatants from these activated mast cells were filtered on a Sepharose CL-2B column equilibrated in pH 7.2 buffer and the individual fractions analyzed for their mMCP-5 and mMCP-7 contents, most of the immunoreactive mMCP-5 filtered in the column's excluded volume in fractions 13 and 14 as if its molecular mass were $>10^{7}$ Da (Fig. 8*A*). In contrast, most of the immunoreactive mMCP-7 exocytosed from these activated mast cells filtered in the column's included volume in fractions 31 to 34 (Fig. 8*B*).

DISCUSSION

At the granule pH of 5.5 (43), mMC-CPA and all mMCPs are positively charged proteins. Depending on their cytokine microenvironment, mouse mast cells will synthesize either chondroitin sulfate E or heparin onto the serglycin proteoglycan peptide core (44). Because chondroitin sulfate E and heparin are two of the most negatively charged molecules in the mouse, it has been assumed that mMC-CPA and most, if not all, mMCPs are ionically bound to serglycin proteoglycans within the granule. To investigate the interaction of mMCP-7 with heparin-containing serglycin proteoglycans inside and outside

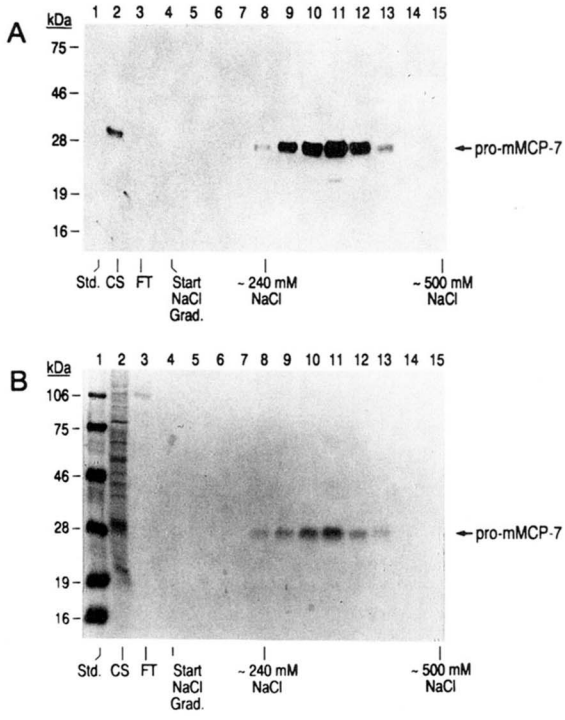

FIG. 6. **Effect of NaCl concentration on the binding of recombinant pro-mMCP-7 to heparin-Sepharose CL-6B.** Cultured supernatant from infected High Five cells was adjusted to pH 5.2 and applied to the heparin affinity column. The column was washed with pH 5.2 buffer before the start of the salt gradient. The proteins recovered in the column fractions were separated by SDS-PAGE and transferred to Immobilon, and the resulting protein blot depicted in *panel A* was stained with anti-mMCP-7 Ig. *Lanes 1, 2,* and *3* consist of molecular weight standards (*Std.*), a sample of the culture supernatant (*CS*) before application to the column, and the unbound proteins in the fall-through fraction (*FT*), respectively. Fractions 4–15 represent the initial gradient fractions. pro-mMCP-7 begins to elute at a NaCl concentration of ~240 mM. The duplicate SDS-PAGE gel depicted in *panel B* was stained with Coomassie Blue. Molecular mass markers in kDa are indicated on the *left* and in *lane 1* of *panel B*.

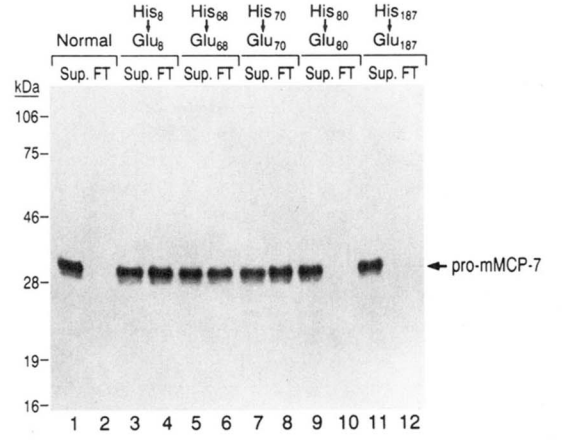

FIG. 7. **Binding of normal pro-mMCP-7 and five His→Glu mutants of pro-mMCP-7 to heparin-Sepharose-6B.** Samples of the cultured supernatants from High Five cells induced to express normal non-mutated pro-mMCP-7 (*lanes 1* and *2*), or the $His^{8}\rightarrow Glu^{8}$ (*lanes 3* and *4*), $His^{68}\rightarrow Glu^{68}$ (*lanes 5* and *6*), $His^{70}\rightarrow Glu^{70}$ (*lanes 7* and *8*), $His^{80}\rightarrow Glu^{80}$ (*lanes 9* and *10*), or $His^{187}\rightarrow Glu^{187}$ (*lanes 11* and *12*) mutants of pro-mMCP-7 were adjusted to pH 5.0 and applied to replicate columns of heparin-Sepharose CL-6B. The starting supernatants (*Sup.*) (*lanes 1, 3, 5, 7, 9,* and *11*) and the non-bound proteins in the fall-through fractions (*FT*) (*lanes 2, 4, 6, 8, 10,* and *12*) were analyzed for the presence of immunoreactive mMCP-7. Molecular mass markers in kDa are shown on the *left*.

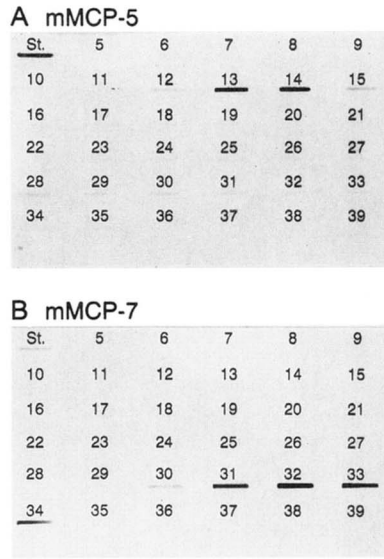

FIG. 8. **Sepharose CL-2B chromatography at neutral pH of immunoreactive mMCP-5 and mMCP-7 released from FcεRI-activated mBMMC.** Supernatants from FcεRI-activated mBMMC were chromatographed on a Sepharose CL-2B column, and samples of fractions 5–39 were assessed for their presence of immunoreactive mMCP-5 (*A*) and mMCP-7 (*B*). Fractions 13 and 14 and fractions 34–39 represent the column's excluded and total volumes, respectively. A sample of starting supernatant (*St.*) before gel filtration chromatography was also assessed for the presence of mMCP-5 and mMCP-7. Similar findings were obtained in three other experiments.

the mast cell, this tryptase was modeled, expressed in insect cells, and studied by site-directed mutagenesis.

Cathepsin G is a chymase serine protease that, like mMCP-7 in the mouse (45), is found in the secretory granules of human skin mast cells (46). Based on biosynthetic radiolabeling techniques, it takes ~90 min for newly translated cathepsin G to be converted to active protease in U937 cells (47) and in transfected rat basophil leukemia mast cells (48). If it is assumed that its signal peptide is enzymatically removed in the endoplasmic reticulum, then the prolonged time it takes to generate mature cathepsin G suggests that the final post-translational maturation of this and other granule proteases in mast cells occurs after the zymogens are targeted to granules. In agreement with this conclusion, Dikov and co-workers (32) found small but significant amounts of pro-mMC-CPA in the secretory granules of cultured mouse mast cells. Certain mast cell, myeloid, and lymphoid granule serine proteases are activated by the granule-localized thiol protease, dipeptidyl peptidase I (49, 50). Moreover, recombinant human mast cell pro-chymase can be efficiently activated by dipeptidyl peptidase I only after the zymogen binds to heparin (33). Heparin biosynthesis is completed relatively late during the post-translational modification of serglycin proteoglycan peptide core in the *trans*-region of the Golgi (51). These and other studies suggest that the chymase and mMC-CPA zymogens bind to serglycin proteoglycans during or shortly after exit from the Golgi and are slowly converted to active, mature enzymes in the granule.

Because mMC-CPA and the mast cell chymases are first packaged in granules as zymogens, it was hypothesized that the mast cell tryptase, mMCP-7, also is first packaged in granules as a zymogen. Thus, mMCP-7 was expressed in its pro form rather than its "mature" form. In preliminary experiments, both COS-1 cells and rat basophil leukemia mast cells were transfected with a pSRα expression construct containing a full-length mMCP-7 cDNA. However, because the amount of recombinant protease obtained in these transfected mammalian cells was insufficient for conducting extensive biochemical studies, we turned to insect cell expression systems. As shown in Fig. 4, High Five cells produced and secreted large amounts

of soluble 27- to 32-kDa proteins that were recognized by anti-mMCP-7 Ig. NH_2-terminal amino acid analysis indicated that all immunoreactive proteins secreted into the culture medium and all immunoreactive cell-associated proteins were pro-mMCP-7. In BALB/c mBMMC, mMCP-7 exhibits considerable size heterogeneity due to its variable Asn-linked carbohydrate content (36). *N*- and *O*-glycanase analyses revealed that recombinant pro-mMCP-7 was heterogeneous in its size primarily because of variable glycosylation. Insect cell-derived pro-mMCP-7 bound to the heparin-affinity column at pH <6.5 (Fig. 5) and NaCl concentration <0.24 M (Fig. 6). Since the pH of the granule in the mast cell has been estimated to be ~5.5 (43), these data support the hypothesis that pro-mMCP-7 binds to serglycin proteoglycans inside the granule.

All mouse mast cell proteases eventually are stored for long periods of time in the secretory granule in their mature, enzymatically active states (1–5). How the mast cell minimizes autolysis of its granule proteases and prevents degradation of other granular constituents has not been determined. Although these proteases are optimally active at neutral pH, many of them possess significant enzymatic activity at pH 5.5 (18, 52). Skin mast cells were first discovered because of the affinity of their heparin-rich (53) secretory granules for cationic dyes (54). All hematopoietic cells that store biologically active proteins in their secretory granules then were found to contain serglycin proteoglycans in this intracellular compartment. It has been presumed that, by binding to serglycin proteoglycans, the proteases are sterically prevented from degrading each other or any other large-sized protein that may be in the granule. Mast cell-derived heparin binds to numerous proteins *in vitro* that have a consensus amino acid sequence of X-B-B-X-B-X, or X-B-B-B-X-X-B-X, where X and B are non-charged and positively charged amino acids, respectively (55). Another heparin-binding motif is the sequence, Trp-Ser-X-Trp (56). However, none of these amino acid sequences are present in mMCP-7 (Fig. 1) or in any other known mouse mast cell granule protease. It has been suggested that His residues could play an important role in heparin binding to mast cell tryptases (13). There are 14 His residues in mMCP-7, but these His residues appear to be randomly distributed throughout the amino acid sequence of the translated protein (11, 13). A comparison of the mMCP-7 amino acid sequence with that of pancreatic trypsin (Fig. 1) does not indicate which His residues in mMCP-7 control its ionic binding to heparin.

A previous protein modeling study predicted that the chymases mMCP-4 and mMCP-5 have two regions with net positive charges ranging from +6 to +10 (23). The regions are located at the opposite ends of the molecule and are away from the substrate-binding cleft. It also was predicted that the chymases mMCP-1 and mMCP-2 have one of these regions. We now show that mMCP-7 has a positively charged region at approximately the same location as the four chymases (Figs. 2 and 3*A*). The mast cell chymases have 0 to 1 His residue in their positively charged regions, whereas mMCP-7 has 5 His residues. Thus, the potential in the positively charged region in mMCP-7 is diminished substantially when the positive charge on its His residues is eliminated at neutral pH. Moreover, the positive electrostatic potential of this region is greatly diminished if His^8, His^{68} (Fig. 3*B*), or His^{70} is mutated to Glu. Because these three His residues are predicted to be important for heparin binding, they were studied by site-directed mutagenesis. In contrast, His^{80} and His^{187} do not reside in the positively charged region, and their conversion to Glu does not influence the electrostatic potential of the region. Because these two His residues were predicted not to be important for heparin binding, they were used as controls in the site-directed mutagenesis study. Site-directed mutagenesis confirmed that a mutation of any of the three His residues in the identified positively charged region into Glu abolishes the binding of recombinant pro-mMCP-7 to the heparin-affinity column at pH 5.0–5.5 (Fig. 7). The substitution of the two His residues outside the positively charged region does not affect this binding. It is possible that the mutations caused a global change in the three-dimensional structure of pro-mMCP-7. However, generally the mutation of a single charged amino acid that resides on the surface of a protein will not cause a global change in its three-dimensional structure (57, 58). Moreover, because similar findings were obtained if His^8, His^{68}, or His^{70} were mutated (Fig. 7), it is highly unlikely that each of these mutations would cause the same global change in the three-dimensional structure of the protein. Thus, the functional differences among the mutants are most likely due to the charge differences between the mutated side chains.

The modeling study and experiments with recombinant pro-mMCP-7 indicate that the tertiary structure of mMCP-7 is critical for enabling the mast cell to store large amounts of the enzymatically active protease in its granules. A binding site consisting of several segments that are brought together in the folded protein is highly advantageous because it prevents the mast cell from storing denatured protease in its granules. Inasmuch as mast cells, monocytes/macrophages, natural killer cells, cytotoxic lymphocytes, basophils, eosinophils, neutrophils, and platelets/megakaryocytes all contain positively charged proteins in their granules along with negatively charged serglycin proteoglycans, the manner by which mMCP-7 is packaged in the granules of mast cells may represent a general control mechanism used by hematopoietic effector cells to package biologically active proteins in high concentration in a small number of secretory granules.

The mast cells in the ear and skin of the BALB/c mouse and the interleukin-3-dependent mBMMC developed *in vitro* from this strain all express mMCP-5, mMCP-6, mMCP-7, and mMC-CPA. When mBMMC are activated through their FcεRI receptors, they exocytose nearly all of their granule mMC-CPA and ~80% of their granule serine proteases as a $>10^7$ Da macromolecular complex with heparin-containing serglycin proteoglycans (1, 15). Three-dimensional modeling of four mouse mast cell chymases predicted that their heparin-binding domains are comprised predominantly of Lys and Arg residues rather than His residues. These findings explain why the mast cell chymases fail to dissociate from serglycin proteoglycan outside the mast cell at neutral pH. The three-dimensional modeling and heparin-interaction studies with recombinant pro-mMCP-7 predicted that mMCP-7 would differ from other proteases in that it would dissociate from the macromolecular complex exocytosed from mast cells. Thus, the status of mMCP-7 released from immunologically activated mBMMC was re-examined. We now report that native mMCP-7, but not mMCP-5, dissociates at neutral pH from the endogenous macromolecular complex exocytosed from activated mBMMC (Fig. 8).

Based on its amino acid sequence (10) and three-dimensional model (23), mMCP-5 is likely to be a chymase with specificity for aromatic and/or aliphatic amino acids. In contrast, mMCP-7 is likely to be a tryptase with specificity for basic residues at the P1 position (11, 13). Since mMC-CPA (1) and rat MC-CPA (18) are exopeptidases that prefer carboxyl-terminal aromatic and aliphatic amino acids, the continued physical association of mMCP-5 (Fig. 8*A*) and mMC-CPA (1) in the macromolecular complex outside the mast cell may be a mechanism by which these two neutral proteases coordinate degradation of common protein substrates. In addition, because of its large physical size, the macromolecular complex may represent a way to

retain enzymatically active mMCP-5 and mMC-CPA around the mast cell in inflamed tissue sites. mMCP-7 is likely to be quite different from mMCP-5 in its substrate specificity. Thus, there would be no need for mMCP-7 to remain physically associated with mMC-CPA outside the mast cell if these proteases degrade distinct proteins. Because mMCP-7 dissociates from the protease-proteoglycan complex outside FcεRI-activated mBMMC (Fig. 8*B*), it is possible that this tryptase is not retained for long periods of time in the inflamed sites in the ear and skin where mast cells containing mMCP-7 reside. It was recently discovered that the liver and spleen mast cells in the V3 mastocytosis mouse express every known mast cell protease, but only mMCP-7 is found in high concentrations in the peripheral blood 20 min after the V3 mastocytosis mouse is systemically activated with IgE and antigen.[4] Most studies carried out to deduce the functions of the mast cell's tryptases have focused on their potential biologic effects in the tissue site immediately adjacent to the activated mast cell. If mMCP-7 is able to retain its enzymatic activity for a substantial length of time after its dissociation from the macromolecular complex, this tryptase could degrade proteins at sites distant from the activated mast cell. Thus, the possible systemic effects of mMCP-7 must be considered in future functional studies of this tryptase.

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