Mechanism by Which Heparin Proteoglycan Modulates Mast Cell Chymase Activity†

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ABSTRACT: Chymases are highly basic chymotrypsin-like serine proteases expressed exclusively by mast cells. Large amounts of chymases complexed with heparin proteoglycan (PG) are released in vivo during mast cell activation. The tight binding of chymase to heparin PG results in increased activity of the protease toward certain substrates, e.g., thrombin and MeO-Suc-Arg-Pro-Tyr-pNA (S-2586). In this study, the mechanism by which heparin PG modulates chymase activity was investigated, using thrombin and various chromogenic peptide substrates as model substrates. Incubation of thrombin with oligonucleotides that block the heparin-binding site of thrombin abolished the stimulatory effect of heparin PG on thrombin inactivation. Further, thrombin mutants with defects in their heparin-binding regions were less efficiently inactivated by chymase−heparin PG than wild type thrombin. These findings suggest a model for chymase stimulation where heparin PG may promote the chymase-catalyzed cleavage of heparin-binding substrates by simultaneously binding to both chymase and substrate. Experiments in which various chromogenic peptide substrates were utilized showed that heparin PG enhanced the activity of chymase toward positively charged peptide substrates such as S-2586, whereas the cleavage of uncharged substrates was not affected by the presence of heparin PG. On the basis of the latter findings, an alternative stimulation mechanism is discussed where heparin PG may stimulate chymase activity by blocking positively charged regions in chymase, thereby reducing the level of electrostatic repulsion between chymase and positively charged substrates.

Mast cells (MCs)[1] are recognized as key effector cells in immediate hypersensitivity reactions. When MCs are activated, they respond by releasing the contents of their secretory granules to the extracellular milieu. The MC granules contain a broad spectrum of inflammatory mediators, e.g., histamine, cytokines, proteases, and heparin/chondroitin sulfate proteoglycans (PGs), that together produce the clinical symptoms associated with allergic inflammation (*1*, *2*). The MC proteases comprise a group of serine proteases with substrate cleavage specificities similar to that of either pancreatic trypsin (tryptases) or chymotrypsin (chymases), as well as a carboxypeptidase A (*3*). Heparin is a highly sulfated polysaccharide belonging to the glycosaminoglycan family (*4*), expressed exclusively by MCs. In the PG form, several heparin chains are covalently attached to a small protein core denoted serglycin (*5*).

The specific pattern for protease and proteoglycan expression varies between different species and between different MC subtypes within each species. In rats, for example, MCs of the connective tissue subtype express two different chymases, rat mast cell protease 1 (RMCP-1) and RMCP-5, whereas MCs of the mucosal type express a different chymase, RMCP-2 (*6*). Further, whereas connective tissue type MCs predominantly attach heparin chains to their serglycin core proteins, the major glycosaminoglycan associated with serglycin in mucosal MCs is chondroitin sulfate. The MC proteases are generally stored as tight macromolecular complexes with PGs and are released in such complexes during MC degranulation (*7*). Whereas the tryptases after degranulation slowly dissociate from the PG, thereby irreversibly losing activity (*8*), the chymases remain associated with the PGs (*7*, *9*).

This study was undertaken to investigate the functional consequences of the association between RMCP-1, the major chymase expressed by rat connective tissue type MCs, and heparin PG. Previous studies have shown that heparin PG enhances chymase activities toward certain substrates, e.g., thrombin (*9*), the chromogenic peptide substrate S-2586 (*10*), procollagenase (*11*), and anaphylatoxin C3a (*12*), whereas the cleavage of the serpins α_1-antichymotrypsin and α_1-antitrypsin is blocked by heparin PG (*13*). However, the mechanism by which heparin PG influences chymase activity has previously not been elucidated.

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[1] Abbreviations: MC, mast cell; PBS, phosphate-buffered saline; PG, proteoglycan; RMCP, rat mast cell protease.

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Table 1: Structure and Designation of Oligonucleotides Used for Blocking the Heparin-Binding Exosite of Thrombin

		Kd for thrombin
G15D:	5'-GGTTGGTGTGGTTGG-3'	~2.7 nM
30-8[15]:	5'-GGTAGGGTCGGATGG-3'	>1000 nM
60-18[29]:	5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3'	~0.5 nM
30-8[30]:	5'-GTGAATAGGTAGGGTCGGATGGGCTACGGT-3'	>1000 nM

In this report, the mechanism by which heparin PG stimulates MC chymase was investigated. To this end, thrombin and various chromogenic peptides were used as model substrates. The results that were obtained indicate that heparin PG may stimulate chymase-catalyzed thrombin degradation by binding simultaneously to both chymase and thrombin, forming a ternary complex. Further, evidence showing that heparin PG preferentially stimulated the hydrolysis of peptide substrates carrying positive charge was obtained. The possibility that the effect of heparin may involve blocking of electropositive regions at the surface of the chymase, thereby reducing the level of electrostatic repulsion between chymase and positively charged substrates, is discussed. The results reported here thus describe two possible mechanisms by which heparin may modulate MC chymase activity.

MATERIALS AND METHODS

RMCP-1 (EC 3.4.21.39) and heparin PG were purified from rat peritoneal MCs by a combination of anion exchange chromatography on DEAE-Sephacel and HPLC on a Superdex 75 column as described previously (*14*). No contaminating components were observed after these components were first analyzed by SDS−PAGE and then silver-stained. The chromogenic peptide substrates S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA), S-2590 (MeO-Suc-Ala-Pro-Tyr-pNA), and S-2238 (H-D-Phe-Pip-Arg-pNA) were obtained from Chromogenix (Mölndal, Sweden). Suc-Ala-Lys-Pro-Phe-pNA was purchased from Bachem (Bubendorf, Switzerland). The latter substrate was treated with methanol in HCl, yielding its methylated form, MeO-Suc-Ala-Lys-Pro-Phe-pNA. Additional chromogenic peptide substrates with various structures (see Table 2) were purchased from Åke Engström, BMC (Uppsala, Sweden). Bovine α-thrombin was kindly provided by I. Björk (Department of Veterinary Medical Chemistry, Uppsala University, Uppsala, Sweden). Oligonucleotides with the sequences listed in Table 1 were obtained from DNA Technology A/S (Aarhus, Denmark). Pig mucosal heparin ("commercial heparin") was a gift from U. Lindahl (Department of Medical and Physiological Chemistry, Uppsala University). Even-numbered heparin oligosaccharides, obtained by partial depolymerization of the polysaccharide with nitrous acid (pH 1.5, cleavage at N-sulfated GlcN units; see ref *15*), were gifts from D. Spillmann Department of Medical and Physiological Chemistry, Uppsala University). Thrombin mutants were prepared and purified to homogeneity as described previously (*16*).

Enzymatic Assays. RMCP-1 activities were measured in 96-well microtiter plates. RMCP-1 (20−50 ng) was diluted with phosphate-buffered saline (PBS)/0.1% Triton X-100 to a final volume of 200 μL. RMCP-1 activity was measured either in the absence or in the presence of rat heparin PG. In standard incubations, a 1.25/1 ratio (by mass) of RMCP-1/heparin PG was used (e.g., 25 ng of RMCP-1/20 ng of heparin PG). This protein/PG ratio was chosen to provide optimal conditions for potentiation of RMCP-1 activity. RMCP-1 activity was measured after addition of 20 μL of solutions of various chromogenic peptide substrates, and the absorbance at 405 nm was monitored with a Titertek Multiscan spectrophotometer (Flow Laboratories). When thrombin was used as a substrate for purified RMCP-1, 10 ng of thrombin was added to wells containing RMCP-1 with or without heparin, followed by an additional period of incubation. Next, 20 μL of a solution of the chromogenic thrombin substrate S-2238 (2.5 mM in H_2O) was added, and finally, residual thrombin activity was monitored.

Peritoneal cells from Sprague-Dawley rats (females, ~15 weeks old) were collected by peritoneal washing with 0.025 M Tris-HCl (pH 7.6) containing 0.12 M NaCl and 0.01 M EDTA. MCs (connective tissue type) that were approximately 90% pure, as judged by staining with toluidine blue, were prepared by density gradient centrifugation on metrizamide as described by Sterk and Ishizaka (*17*). In experiments where the thrombin-inactivating activities of intact MCs were determined, 2×10^4 purified MCs were incubated in 400 μL of a 1:1 mixture of Dulbecco's Modified Eagle's Medium and PBS, supplemented with 0.4 mg/mL bovine serum albumin. Incubations were conducted in a humidified atmosphere of 5% CO_2 in air at 37 °C. Thrombin (0.5 μg) was added, and samples (20 μL) from the media were collected at 5, 20, 50, 90, and 130 min. The samples were mixed with 200 μL of PBS and were analyzed for residual thrombin activities after addition of 20 μL of a solution of S-2238 (2.5 mM in H_2O).

The $K_{m,app}$ and k_{cat} values of RMCP-1 for the various chromogenic peptide substrates were determined after incubation of the enzyme with increasing concentrations of substrate, followed by the monitoring of protease activity. The data that were obtained were used for calculations of kinetic parameters after nonlinear regression analysis.

Results are expressed as the means of duplicate determinations. Duplicate determinations were generally within ±5% of the mean.

RESULTS

Effect of Heparin on RMCP-1-Catalyzed Inactivation of Thrombin. Thrombin was previously shown to be a substrate for RMCP-1. Incubation of thrombin with RMCP-1 results in the rapid degradation of thrombin accompanied by the loss of enzyme activity (*14*). The Phe1G−Gly1F, Trp148−Thr149, and Tyr117−Ile118 bonds in thrombin (numbering of amino acid residues according to topological equivalencies with chymotrypsinogen) were previously identified as major targets for MC chymase (*18*). This degradation and inactivation process has been shown to be greatly enhanced by heparin PG (*10*). The importance of polysaccharide size for the stimulation of RMCP-1-catalyzed thrombin inactivation was investigated (Figure 1A). The strongest stimulation of thrombin inactivation was produced by intact heparin PG at a concentration of 100 ng/mL, with maximal ~16-fold enhancement. Pig mucosal heparin also stimulated thrombin inactivation, although to a lesser extent (maximal stimulation of ~6-fold). In addition, heparin oligosaccharides of sizes

Table 2: Kinetic Constants for the Interaction of RMCP-1 with Various Chromogenic Peptide Substrates in the Absence or Presence of Heparin PG[a]

(P4) P3 P2 P1	$K_{m,app}$ (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \times s^{-1}$)
MeO-Suc-Arg-Pro-Tyr-pNA (S-2586)	2.1 ± 0.2	5.0 ± 0.3	2.4
+heparin PG	0.22 ± 0.02	3.1 ± 0.1	14
MeO-Suc-Ala-Pro-Tyr-pNA (S-2590)	0.32 ± 0.01	3.0 ± 0.04	9.4
+heparin PG	0.31 ± 0.03	2.7 ± 0.1	8.7
Ac-Arg-Pro-Phe-pNA	1.0 ± 0.1	1.2 ± 0.08	1.2
+heparin PG	0.33 ± 0.01	1.5 ± 0.03	4.4
Ac-Ala-Pro-Phe-pNA	0.73 ± 0.04	1.4 ± 0.04	2.0
+heparin PG	0.64 ± 0.04	1.3 ± 0.05	2.0
Ac-Arg-Arg-Pro-Phe-pNA	0.79 ± 0.2	0.094 ± 0.02	0.12
+heparin PG	0.086 ± 0.006	0.22 ± 0.005	2.6
Ac-Ala-Arg-Pro-Phe-pNA	1.8 ± 0.5	2.1 ± 0.4	1.1
+heparin PG	0.42 ± 0.03	1.6 ± 0.05	3.9

[a] For experimental details, see Materials and Methods.

down to tetradecasaccharides showed some stimulatory effect on thrombin inactivation (maximal enhancement of ~3-fold by the "22-24" oligosaccharide). Interestingly, the smallest oligosaccharides that were able to stimulate RMCP-1-catalyzed thrombin inactivation (tetradecasaccharides) corresponded to the minimal oligosaccharide size required for binding to RMCP-1 (*19*). These data thus indicate a strong dependence on polysaccharide size for stimulation of RMCP-1-catalyzed thrombin inactivation. Optimal stimulation of thrombin inactivation was observed at 100 ng/mL PG. At higher PG concentrations, the stimulatory effect decreased dramatically (Figure 1A). Similarly, the stimulation by pig mucosal heparin showed a distinct optimum at 100 ng/mL saccharide, and a decreased effect at higher concentrations. In contrast, the chymase stimulation produced by the various heparin oligosaccharides appeared to level off at higher concentrations.

Thrombin contains two large electropositive patches, denoted anion-binding exosites I and II, respectively. Previous work has shown that heparin binds to anion exosite II, whereas anion-binding exosite I is involved in interaction with fibrinogen (*16*). Since thrombin is a heparin-binding protein, we speculated that heparin PG may stimulate thrombin degradation by binding simultaneously to both RMCP-1 and thrombin, thereby facilitating contact between the two molecules. To test this hypothesis, we investigated whether blocking of the heparin-binding domain of thrombin had any effect on the rate of thrombin inactivation by RMCP-1−heparin PG. For this purpose, we used oligonucleotides that were recently reported to bind with high affinity to the heparin-binding site of thrombin. Padmanabhan et al. (*20*) reported that a 15-mer oligonucleotide denoted G15D (Table 1) bound to the fibrinogen recognition exosite as well as to the heparin binding exosite of thrombin. The affinity of G15D for thrombin has been reported to be ~2.7 nM (*21*). Later, Tasset et al. (*22*) reported a 29-nucleotide sequence denoted 60-18[29] (Table 1) that binds to the heparin-binding exosite of thrombin with a K_d of ~0.5 nM. The specific binding of these oligonucleotides to thrombin involves the formation of intramolecular G-quadruplex structures (*22*). The effect of these oligonucleotides on the rate of inactivation of thrombin by RMCP-1−heparin PG was studied (Figure 2A). As controls, two oligonucleotides with structures and lengths similar to those of the ones described above, denoted 30-8[30] and 30-8[15], respectively (Table 1), were tested. The latter oligonucleotides exhibit a very low affinity for thrombin, with K_d values of >1000 nM (*22*).

In the presence of the oligonucleotide 60-18[29], the rate of thrombin inactivation by RMCP-1−heparin PG was markedly reduced, with 50% inhibition achieved at ~20 nM 60-18[29]. At ~400 nM 60-18[29], the stimulatory effect of heparin PG on thrombin inactivation was almost completely abolished. The oligonucleotide G15D also caused a substantial reduction in the rate of thrombin inactivation.

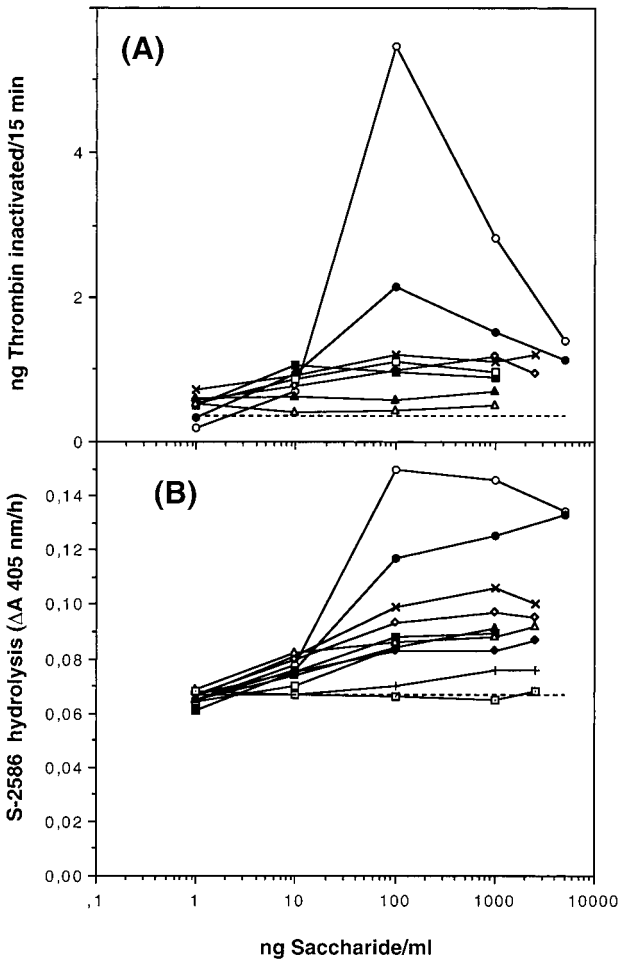

FIGURE 1: Stimulation of RMCP-1 activity by heparin. RMCP-1 (125 ng/mL) was incubated with either heparin PG (○), pig mucosal heparin (●), or heparin oligosaccharides of various sizes: 4-saccharides (⊡), 6-saccharides (+), 8-saccharides (◆), 10-saccharides (△), 12-saccharides (▲), 14-saccharides (□), 16-saccharides (■), 18-saccharides (◇), and 22−24-saccharides (×). The dashed lines (- - -) represent the baseline activities in the absence of saccharide. (A) After 15 min, 10 ng of bovine α-thrombin was added, and after an additional 15 min, 20 μL of a solution of the chromogenic thrombin substrate S-2238 (2.5 mM) was added, followed by the determination of residual thrombin activity. (B) After 15 min, 20 μL of a solution of S-2586 (1.8 mM) was added and RMCP-1 activity was measured.

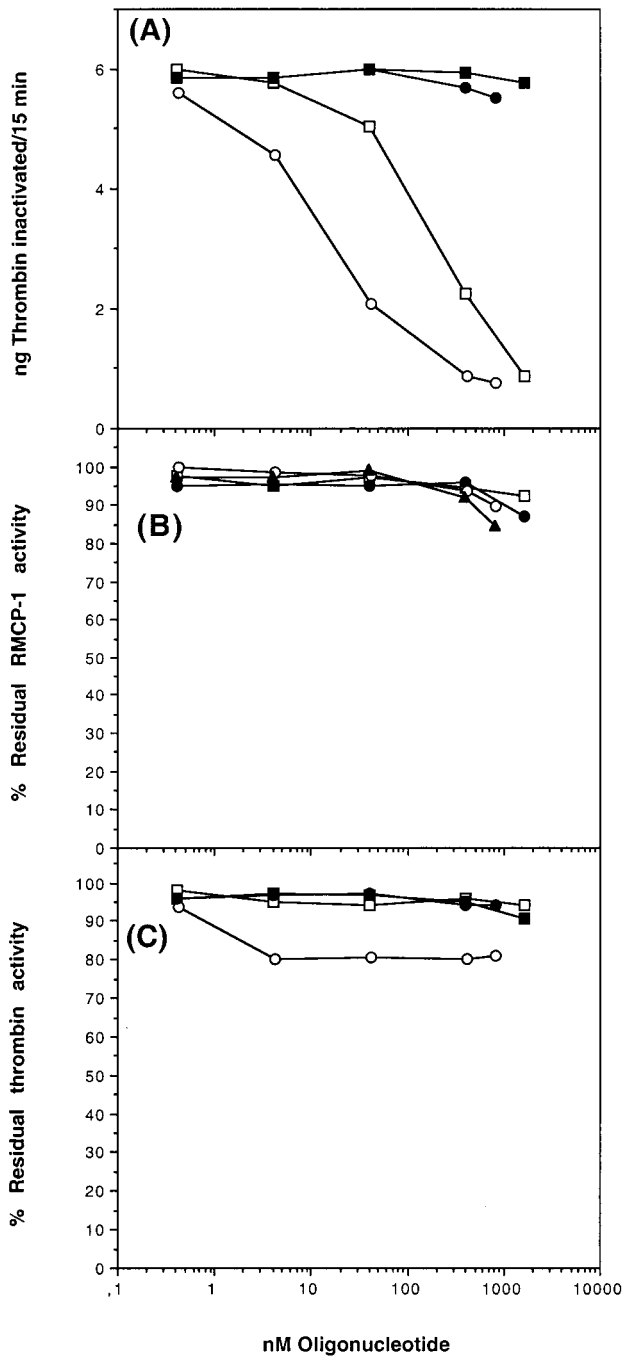

FIGURE 2: Inactivation of thrombin by RMCP-1−heparin PG in the presence of oligonucleotides that recognize the heparin-binding region of thrombin. (A) RMCP-1 (125 ng/mL) was incubated together with heparin PG (100 ng/mL) and either G15D (□), 30-8[15] (■), 60-18[29] (○), or 30-8[30] (●) at the indicated concentrations. After 15 min, bovine α-thrombin (10 ng) was added followed by incubation for 15 min. Subsequently, 20 μL of a solution of S-2238 (2.5 mM) was added and residual thrombin activity was monitored. (B) Direct effect of oligonucleotides on RMCP-1 activity. RMCP-1 (125 ng/mL) was incubated with 100 ng of heparin PG/mL and either G15D (□), 30-8[15] (■), 60-18[29] (○), or 30-8[30] (●) at the indicated concentrations. After 15 min, 20 μL of a solution (1.8 mM) of S-2586 was added followed by monitoring of RMCP-1 activity. (C) Direct effect of oligonucleotides on thrombin activity. Bovine α-thrombin (10 ng) was incubated with either G15D (□), 30-8[15] (■), 60-18[29], (○) or 30-8[30] (●) at the indicated concentrations. After 15 min, 20 μL of a solution of S-2238 (2.5 mM) was added and residual thrombin activity was monitored.

However, higher concentrations of G15D (∼250 nM) than 60-18[29] were required to achieve 50% inhibition, in agreement with the fact that G15D has a lower affinity than 60-18[29] for thrombin. Neither of the control oligonucleotides 30-8[15] or 30-8[30] caused any significant inhibition of the thrombin inactivation process, at concentrations of up to 1.6 μM (Figure 2A).

Control experiments were performed to determine that the inhibitory effect of the oligonucleotides was not due to direct effects on the catalytic activity of either thrombin or RMCP-1. Neither of the oligonucleotides at concentrations of up to ∼400 nM caused any change in chymase activity, as measured by S-2586, in the presence of heparin PG. However, at high concentrations (≥0.8−1.6 μM), all of the tested oligonucleotides produced slight reductions (7−16%) in RMCP-1−heparin PG activity (Figure 2B). The various oligonucleotides were tested for their direct effects on the catalytic activity of thrombin. Neither G15D, 30-8[15], nor

30-8[30] caused any significant changes in thrombin activity as assessed with the chromogenic substrate S-2238. In contrast, 60-18[29] at concentrations of ≥4 nM caused a moderate (~20%) loss of thrombin activity (Figure 2C). These experiments thus show that the inhibitory effects of the various oligonucleotides on thrombin inactivation by RMCP-1−heparin PG are not caused by direct effects on the catalytic activity of either RMCP-1 or thrombin.

The importance of the heparin-binding region of thrombin for the stimulatory effect of heparin PG on thrombin inactivation was studied further utilizing various recombinant thrombin mutants. Previous work showed that K248E and R89E mutations in the anion-binding exosite II resulted in a drastically reduced level of heparin-dependent inhibition by antithrombin, whereas a R68E mutation in anion-binding exosite I did not affect its heparin-dependent inhibition (*16*). Neither of these mutations significantly altered the cleavage rate for S-2238. The inactivation of these thrombin mutants as well as wild type recombinant thrombin by RMCP-1 in the presence of heparin PG was monitored (Figure 3A). The results clearly showed that thrombins carrying mutations in their heparin-binding region, R89E and K248E, were inactivated significantly more slowly than wild type thrombin. The R68E mutation in the anion-binding exosite I did not reduce the ability of RMCP-1−heparin PG to inactivate this thrombin variant. In fact, thrombin R68E was inactivated at a higher rate than wild type thrombin. The rates of inactivation of wild type thrombin and the various thrombin mutants were measured at different heparin PG concentrations (Figure 3B). The inactivation of both wild type thrombin and R68E thrombin was significantly enhanced by heparin PG, with the optimal effect of heparin PG obtained at ~100 ng of PG/mL. Again (see above), at higher PG concentrations, the stimulatory effect was decreased. The inactivation of the R89E and K248E thrombin mutants by RMCP-1 was also stimulated to some extent by heparin PG. However, in contrast to the wild type and R68E thrombins, no decrease in the level of stimulation was observed at the higher PG concentrations.

The inactivation of thrombin by native chymase−heparin PG complexes was studied. For this purpose, wild type thrombin or thrombin mutants were added to intact peritoneal MCs, followed by monitoring of thrombin inactivation. Wild type thrombin was rapidly inactivated by purified rat peritoneal MCs (~15 μg of thrombin inactivated per hour per 1×10^6 cells; Figure 4). The thrombin mutant with a defect in fibrinogen binding, R68E, was inactivated at a rate similar to that of wild type thrombin. In contrast, thrombin mutant R89E, with a defective heparin binding region, was inactivated only very slowly by the MCs (Figure 4). The inactivation of thrombin by intact MCs was strongly inhibited by the heparin antagonist protamine, further supporting the importance of heparin PG in the thrombin inactivation process (not shown).

Effect of Heparin PG on RMCP-1-Catalyzed Cleavage of Chromogenic Substrates. Heparin PG binds strongly to RMCP-1 (*19*) and enhances the activity of the chymase toward the chromogenic substrate S-2586 (*13*), although the underlying mechanism for this enhancement has not been clarified. To determine the effect of polysaccharide size on chymase activation, RMCP-1 activity toward S-2586 was assessed after incubation with either heparin PG (M_r ~

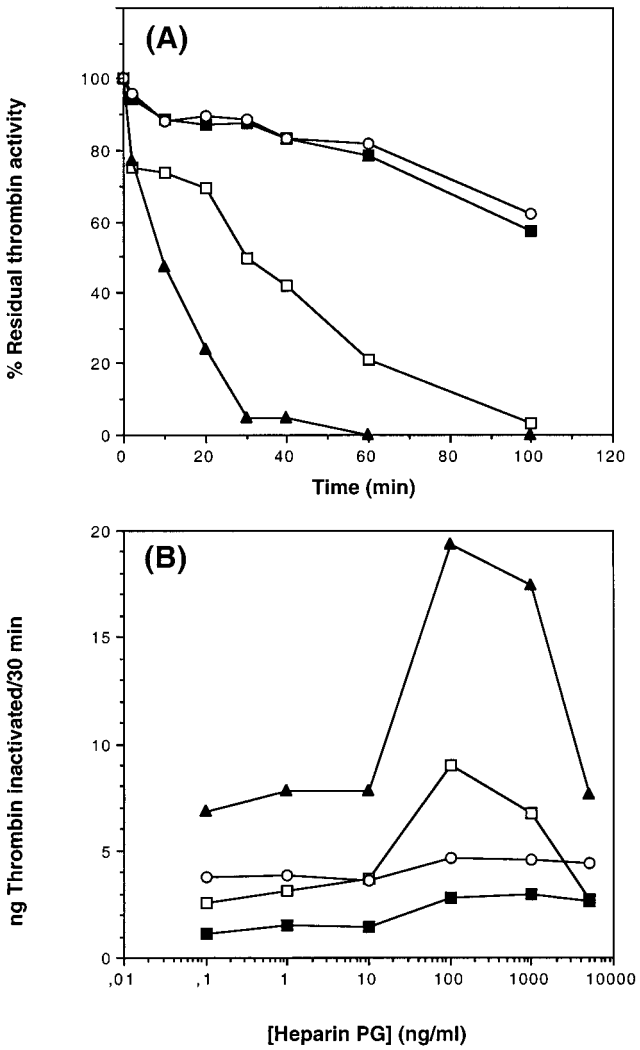

FIGURE 3: Inactivation of wild type thrombin and thrombin mutants by RMCP-1−heparin PG. (A) RMCP-1 (125 ng/mL) was incubated with heparin PG (100 ng/mL). After 15 min, 10 ng of either wild type human recombinant thrombin (□) or recombinant thrombins with the mutations R68E (▲), R89E (■), and K248E (○) was added. After various periods of time, 20 μL of a solution (2.5 mM) of the thrombin substrate S-2238 was added and residual thrombin activity was measured. (B) Dose response for the stimulation of the RMCP-1-catalyzed inactivation of wild type (□) or mutant thrombins R68E (▲), R89E (■), and K248E (○) by heparin PG. RMCP-1 (125 ng/mL) was incubated for 15 min with heparin PG at the indicated concentrations. Ten nanograms of wild type or mutant thrombins was added. After 30 min, residual thrombin activity was monitored after addition of 20 μL (2.5 mM) of a solution of S-2238.

100 000 for the polysaccharide chains), pig mucosal heparin ("commercial heparin", M_r ~ 15 000), or heparin oligosaccharides of various sizes (Figure 1B). Heparin PG produced the most profound stimulatory effect on RMCP-1 activity, with a maximal ~2.5-fold stimulation. Also, pig mucosal heparin and, to a lesser extent, heparin oligosaccharides of sizes down to octasaccharides exhibited some stimulatory effect on RMCP-1 activity toward S-2586. The stimulation of RMCP-1 toward S-2586 thus exhibited a strong dependence on the size of the polysaccharide. Optimal stimulation occurred at ~100 ng/mL proteoglycan. At higher heparin PG concentrations, no further stimulation of RMCP-1 was observed.

S-2586 contains a positively charged amino acid residue (Arg, Table 2). The possibility that this positive charge is

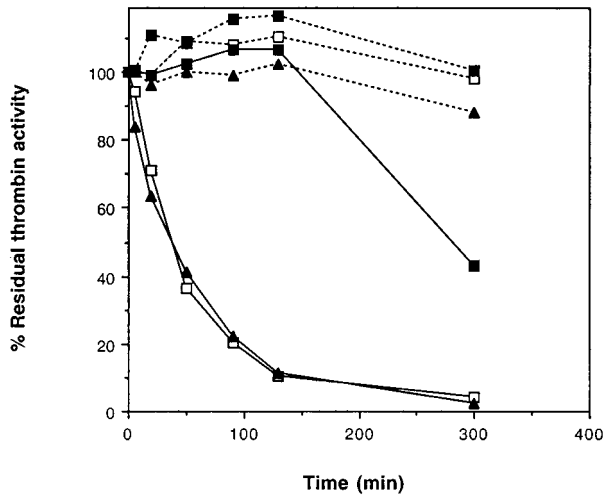

FIGURE 4: Inactivation of recombinant thrombins by intact peritoneal mast cells. Recombinant wild type thrombin (□), R68E thrombin (▲), or R89E thrombin (■) (0.5 μg) was added to 2×10^4 intact purified rat peritoneal MCs in 400 μL of cell culture medium (solid lines). As a control, the various recombinant thrombins were added to 400 μL of cell culture medium in the absence of cells (dashed lines). After various periods of time, samples were taken from the supernatants and were analyzed for residual thrombin activities as described in Materials and Methods.

important for the stimulatory effect of heparin was tested. To this end, we compared the effect of heparin PG on the rate of RMCP-1-catalyzed cleavage of S-2586 and an uncharged substrate, S-2590. S-2590 has the same structure as S-2586 except that an Arg residue is substituted for an Ala (Table 2). Heparin PG caused a ~10-fold reduction of the $K_{m,app}$ of RMCP-1 for S-2586 but only a minor effect on the k_{cat} value for this substrate. In contrast, heparin PG did not influence either the $K_{m,app}$ or k_{cat} of RMCP-1 for S-2590. These results thus indicate that heparin preferentially stimulates the cleavage of positively charged substrates.

To confirm and extend these observations, several new chromogenic substrates were synthesized. A substrate with the structure Ac-Arg-Pro-Phe-pNA, i.e., a Tyr-Phe substitution at the P1 position as compared with S-2586 (see Table 2), was hydrolyzed with a $K_{m,app}$ value lower than that of S-2586 in the absence of heparin PG. In the presence of heparin PG, the $K_{m,app}$ value was decreased significantly (3-fold), although the stimulatory effect of heparin PG on substrate hydrolysis was not as strong as for S-2586. Again, very little effect of heparin PG on the k_{cat} value was seen. When the Arg residue of the latter substrate was exchanged for an Ala (Ac-Ala-Pro-Phe-pNA), no effect of heparin PG on either the $K_{m,app}$ or k_{cat} values was observed, in agreement with the suggested critical importance of positive charge in obtaining stimulation of substrate cleavage by heparin PG. The substrate Ac-Ala-Arg-Pro-Phe-pNA, i.e., extension with a P4 Ala residue, was cleaved by RMCP-1 with or without heparin PG with kinetics similar to those of Ac-Arg-Pro-Phe-pNA, indicating that the extra Ala in position P4 did not affect the kinetics of substrate hydrolysis. In contrast, the corresponding substrate with an Arg at the P4 position (Ac-Arg-Arg-Pro-Phe-pNA) was a very poor substrate for RMCP-1, as reflected by a low k_{cat} as compared with that of Ac-Ala-Arg-Pro-Phe-pNA. However, heparin PG stimulated the hydrolysis of this substrate to a larger extent (~9-fold decrease in the $K_{m,app}$ value) than the corresponding substrate with an Ala at the P4 position (Table 2).

The cleavage of MeO-Suc-Ala-Lys-Pro-Phe-pNA was stimulated by heparin PG to approximately the same extent as the cleavage of Ac-Ala-Arg-Pro-Phe-pNA, indicating that Lys can be substituted for Arg, with the maintained stimulatory effect of heparin PG on substrate hydrolysis (not shown). Interestingly, the cleavage of the nonmethylated form of this substrate, Suc-Ala-Lys-Pro-Phe-pNA, was not stimulated by heparin PG (not shown). Since the nonmethylated succinyl group carries a negative charge, the net charge of Suc-Ala-Lys-Pro-Phe-pNA is zero. The absence of a stimulatory effect of heparin PG on the cleavage of the latter substrate is thus in agreement with the notion that heparin PG preferentially stimulates the cleavage of positively charged peptides.

The possibility that the effect of heparin PG on the hydrolysis of positively charged peptide substrates involves interaction of these substrates with heparin was tested. However, neither S-2586 nor Ac-Arg-Arg-Pro-Phe-pNA remained bound to heparin−Sepharose at 0.15 M NaCl, an indication of a low affinity for heparin. S-2586 was completely eluted from heparin−Sepharose at 0.05 M NaCl, whereas Ac-Arg-Arg-Pro-Phe-pNA was eluted at 0.1 M NaCl (not shown).

A substrate with a negatively charged amino acid residue (Glu) was also tested (Ac-Glu-Pro-Phe-pNA). The rate of hydrolysis of this substrate was compared with those for the other available chromogenic substrates at a substrate concentration of 80 μM.[2] At this substrate concentration, Ac-Glu-Pro-Phe-pNA was cleaved at a higher rate by free RMCP-1 than any of the other tested substrates, e.g., ~20 times faster than Ac-Arg-Pro-Phe-pNA or Ac-Ala-Arg-Pro-Phe-pNA and ~200 times faster than Ac-Arg-Arg-Pro-Phe-pNA. In the presence of heparin PG, the cleavage rate for this substrate was decreased ~50% (not shown).

The rate of hydrolysis of the various chromogenic substrates by pancreatic chymotrypsin was assessed. Heparin PG did not influence the activity of pancreatic chymotrypsin toward any of the chromogenic substrates that were investigated (not shown).

DISCUSSION

MCs synthesize and store very large amounts of proteases. It has been calculated that various proteases constitute up to ~25% of the total protein content in connective tissue type MCs (*7*). Moreover, we have recently shown that up to 4% of the mRNA in rat peritoneal MCs encodes RMCP-1 (*6*). The high abundance of these enzymes suggests an important function in inflammatory conditions involving MC degranulation. Indeed, recent data implicate potent proinflammatory properties of both tryptases (*23*) and chymases (*24*). However, despite the identification of several substrates for various MC proteases in vitro (*9*, *11*, *25*−*32*), the physiological substrates for these proteases remain to be identified.

MC chymase is stored in a tight complex with heparin PG and remains associated with the PG after MC degranu-

[2] Due to the limited available quantities of this substrate, accurate kinetic measurements were not possible.

lation. It is therefore relevant to regard the chymase−heparin PG complex, rather than free chymases, as the physiological form of these enzymes. The interaction of MC chymase with heparin PG appears to have several functional consequences. First, the chymase−heparin PG complexes tend to remain associated with the MC surface after degranulation, thus possibly preventing the chymases from diffusing away from the site of inflammation (*7*, *14*). It has also been proposed that the interaction with heparin PG is necessary to achieve the proteolytic removal of the N-terminal activation peptide that is present in chymases (*33*). Further, we and others have shown that heparin PG modulates the activity of mature MC chymase in different ways. In a previous study (*34*), it was observed that heparin PG altered the substrate specificity of chymase in the cleavage of insulin; in the absence of heparin, a Tyr−Leu bond was cleaved, whereas the adjacent Leu−Tyr bond was the preferred target in the presence of heparin. Heparin PG has been demonstrated to accelerate the chymase-catalyzed degradation and inactivation of thrombin (*10*, *18*), to stimulate the cleavage of the chromogenic peptide substrate S-2586 (*13*), to enhance the degradation of procollagenase (*11*) and anaphylatoxin C3a (*12*), and to promote the interaction of chymase with secretory leukocyte proteinase inhibitor (*35*).The importance of the interaction with heparin PG is further supported by our findings that various heparin antagonists, by displacing chymase from heparin PG, interfere with protease function (*10*, *36*). Further, we showed previously that when RMCP-1 is bound to heparin PG it is largely resistant to inhibition by a variety of plasma protease inhibitors, including the serpins α_1-antichymotrypsin and α_1-protease inhibitor (*13*). Since serpins function by acting as substrates for their target proteases, it is apparent that, for these substrates, heparin PG decreases the rate of substrate cleavage.

From the above findings, it is apparent that the association of chymase with heparin PG favors the interaction with certain substrates, whereas the cleavage of other substrates is prevented. However, the mechanism by which heparin PG modulates chymase activity has not been clear. One possible mechanism for chymase stimulation would be that heparin PG, when bound to chymase, interacts simultaneously with chymase substrates, thus facilitating contact between chymase and substrate. Such a model implies that heparin PG should preferentially accelerate the cleavage of heparin-binding chymase substrates, whereas the cleavage of heparin nonbinding substrates is not favored (Figure 5, mode 1). Another possibility would be that heparin PG, by binding to positively charged regions in the chymase, reduces its overall positive charge. This may facilitate the contact between chymase and substrates that are positively charged but are lacking a functional heparin binding site (Figure 5, mode 2).

In this paper, we have examined the stimulatory effect of heparin PG on chymase activity, utilizing two different model substrates: thrombin and S-2586. In the case of thrombin, much of the obtained data suggests a stimulation mechanism involving interaction of both chymase and thrombin with heparin PG (Figure 5, mode 1). First, if such a mechanism is operating, it should be expected that the heparin PG concentration is critical; i.e., the heparin PG concentration should be appropriate for allowing binding of chymase and substrate to the same proteoglycan molecule (Figure 5, mode 1). A superoptimal proteoglycan concentration should reduce

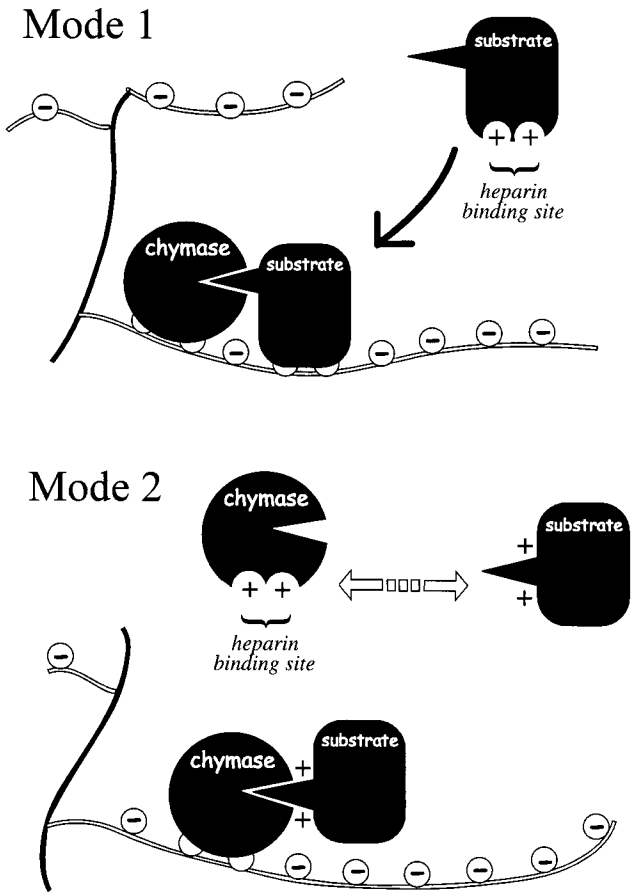

FIGURE 5: Proposed mechanisms for the stimulation of mast cell chymase by heparin PG. In mode 1, stimulation occurs by a mechanism where heparin PG binds simultaneously to both chymase and heparin-binding substrates. In mode 2, heparin PG accelerates the interaction between chymase and substrates that are positively charged but lack a heparin-binding site. This mechanism involves blocking of electropositive regions in the chymase, thereby reducing the level of electrostatic repulsion between such regions and positively charged substrates.

the likelihood that chymase and substrate interact with the same proteoglycan molecule, and would thus result in a decreased level of stimulation of substrate cleavage. The data depicted in Figure 1A clearly demonstrate that superoptimal heparin PG concentrations result in a decreased level of stimulation of chymase-catalyzed thrombin inactivation, in agreement with such a mechanism. Second, blocking of the heparin-binding region of thrombin with specific oligonucleotides virtually abolished the stimulatory effect of heparin PG on thrombin inactivation, further supporting such a mechanistic model (Figure 2). Third, thrombin mutants with defects in their heparin-binding regions were less susceptible to inactivation by chymase−heparin PG than either wild type thrombin or thrombin with a mutation in the fibrinogen-binding region. Taken together, the data presented here strongly indicate that heparin PG stimulates RMCP-1-catalyzed thrombin inactivation by the mechanism depicted as mode 1 in Figure 5. According to this model, heparin PG thus acts by capturing heparin-binding substrates from the solution. In this way, the probability that PG-associated chymase will encounter its substrate is increased compared to that for the situation when the substrate is free in solution. Interestingly, it has recently been proposed that this type of mechanism may be a general mechanism by which pro-

teoglycans facilitate contact between different proteins (*37*). A notable example is the interaction of thrombin with the protease inhibitor antithrombin, which is accelerated by heparin via a mechanism analogous to the one described previously (*38*). Other examples include the interaction between certain growth factors and their cell surface receptors, which may be facilitated by cell surface proteoglycans in a similar fashion (*39*). Proteoglycans may thus be envisaged as "catalysts of molecular encounter" (*37*), and in many cases, it is possible that this constitutes, at least partly, the biological function of the polysaccharide part of various proteoglycans.

The mechanism for the modulation of S-2586 hydrolysis is more uncertain. Although heparin PG stimulates the chymase-catalyzed hydrolysis of S-2586, the results that are obtained indicate that the mechanism may differ, at least partly, from the mechanism for the stimulation of thrombin degradation. First, S-2586 has a low affinity for heparin, arguing against a mode 1 type of mechanism. However, the lack of binding of these substrates to heparin−Sepharose at physiological ion strength does not completely rule out involvement of the mode 1 mechanism. Even weak binding may be sufficient for allowing interaction of the peptide substrates with heparin for short times, thus making the mode 1 type mechanism possible also for these substrates. It is apparent from Figure 1 that the dose−response curves for chymase stimulation by heparin PG differ significantly for S-2586 and thrombin. The course of stimulation of thrombin inactivation exhibits a distinct optimum, with reduced levels of stimulation at higher proteoglycan concentrations. This kind of dose−response curve is, as discussed above, compatible with a stimulation mechanism where heparin binds simultaneously to both chymase and substrate. In contrast, the course of stimulation of S-2586 cleavage reaches a maximum at the same PG concentration as that required for optimal stimulation of thrombin degradation, but exhibits very little decrease at the higher proteoglycan concentrations. Possibly, this may further point to an alternative stimulation mechanism. One possibility, although at present tentative, is a stimulation mechanism depicted as mode 2 in Figure 5, i.e., a mechanism where the effect of heparin involves blocking of positively charged regions in the chymase. RMCP-1 is a highly basic protein with a net charge of +19 at neutral pH (*6*), and it is thus possible that shielding of positively charged regions in RMCP-1 by heparin PG may reduce the level of electrostatic repulsion between the chymase and substrates that are positively charged. In this mode of stimulation, only the chymase but not its substrate is bound to the proteoglycan; i.e., no ternary complex between the substrate, chymase, and proteoglycan is formed. Therefore, proteoglycan concentrations increased above that required for maximal stimulation are not expected to reduce the stimulatory effect.

According to the mode 2 type of stimulation, heparin PG should stimulate the cleavage of positively charged substrates whereas the cleavage of uncharged substrates is expected to be unaffected. Indeed, the results obtained with the various chromogenic peptide substrates (Table 2) indicated that the cleavage of positively charged substrates was stimulated by heparin PG, whereas the cleavage rate for uncharged peptide substrates was unaffected by heparin PG. The presence of a negative charge in the substrate appeared to result in a high rate of hydrolysis by free RMCP-1. Moreover, the extent of cleavage of the negatively charged substrate was decreased by heparin PG. These results may thus further support the suggested mechanism for stimulation; the presence of negative charge may result in a higher affinity of this substrate for the free (positively charged) chymase than of the positively charged or uncharged peptide substrates. When chymase is bound to heparin PG, the resulting net loss of exposed positive charge may lead to a lower affinity for negatively charged substrates and thus decreased rates of substrate cleavage. However, we cannot at present completely exclude the possibility that properties of the amino acid side chains other than their actual electrostatic charges are important in determining whether their rates of chymase-catalyzed hydrolysis are modulated by heparin PG.

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