Lactoferrin regulates the activity of heparin proteoglycan-bound mast cell chymase: characterization of the binding of heparin to lactoferrin

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Rat mast cell protease 1 (RMCP-1) is a secretory granule serine protease (chymase) that is recovered *in vivo* in a macromolecular complex with heparin proteoglycan (PG). We have previously shown that heparin activates RMCP-1 and that RMCP-1, when bound to heparin PG, is largely resistant to inhibition by a variety of macromolecular protease inhibitors. In the search for alternative mechanisms in the regulation of RMCP-1 activity, we hypothesized that heparin antagonists, by interfering with the RMCP-1/heparin PG interaction, might influence the activity of heparin-bound mast cell chymase. In the present study, lactoferrin (LF), a heparin-binding protein, was assessed for RMCP-1 inhibiting activity. LF proved to decrease the activity of heparin PG-associated RMCP-1, although a portion of the

enzyme activity was resistant to regulation. The mechanism of regulation was shown to involve the displacement of RMCP-1 from heparin PG, and LF caused an approx. 6-fold increase in the apparent K_m of the RMCP-1–heparin PG complex for the chromogenic substrate S-2586. The interaction of LF with heparin was characterized. Pig mucosal heparin and endogenous heparin PG were equally effective in binding LF, whereas heparan sulphate bound with lower affinity. None of dermatan sulphate, chondroitin sulphate or hyaluronan were effective in binding LF. Further, the 6-*O*-, 2-*O*- and *N*-sulphate groups in heparin were of approximately equal importance for binding. Octasaccharides were the smallest heparin oligosaccharides showing significant binding to LF.

INTRODUCTION

Mast cells (MCs) are of crucial importance in different inflammatory conditions, including immediate hypersensitivity [1–4]. When MCs are activated they respond by releasing various inflammatory mediators from their secretory granule, such as histamine, cytokines, heparin proteoglycan (PG), carboxypeptidase and serine proteases [5,6]. The MC serine proteases constitute a family of closely related enzymes with substrate specificities similar to those of either trypsin (tryptases) or chymotrypsin (chymases) [7–11]. Rat mast cell protease 1 (RMCP-1) is a chymase specifically expressed by connective tissue-type MCs. It is stored in a macromolecular complex with heparin PG and remains associated with the PG after MC degranulation [12–14].

Heparin is a polysaccharide belonging to the glycosaminoglycan family. It consists of repeating disaccharide units of iduronic/glucuronic acid and glucosamine residues, in which the glucosamine residues are either *N*-sulphated or *N*-acetylated (free amino groups are rare). In addition, the uronic acid residues are sometimes 2-*O*-sulphated and the glucosamine residues can carry 6-*O*- and 3-*O*-sulphate groups [15]. In the PG form, the heparin chains are attached to a small protein core [16]. Owing to the presence of the sulphate and carboxy groups, heparin has a high anionic charge density and is known to interact with a large number of proteins through electrostatic interactions [17–22].

Heparin binds to RMCP-1 with high affinity. This binding is dependent on the anionic charge density of the polysaccharide and requires a minimal size of approx. 14 monosaccharide units [23]. We recently showed that if RMCP-1 is bound to heparin PG it is largely resistant to inhibition by various plasma protease

inhibitors such as α_1 -protease inhibitor, α_1 -antichymotrypsin and α_2 -macroglobulin [24]. Because RMCP-1 bound to heparin PG seems to be inaccessible to inhibition by plasma protease inhibitors, I decided to investigate whether antagonists of heparin were capable of expressing RMCP-1-inhibiting activity, by interfering with the chymase/heparin PG interaction. Heparin antagonists of possible physiological relevance, i.e. those accessible to the chymase–heparin complexes *in vivo*, were of particular interest. Lactoferrin (LF), an iron-binding protein with heparin-binding properties [25], is present in milk, tears and saliva and is a component of the secondary granules of neutrophils. During an inflammatory reaction, neutrophils that have migrated to the extravascular compartment would thus, after cellular activation, release LF that might have the potential to interact with chymase–heparin PG complexes secreted by MCs. The present study demonstrates that LF can regulate MC chymase activity by displacing RMCP-1 from its physiological ligand, heparin PG.

MATERIALS AND METHODS

Rat peritoneal MCs were purified and cultured as described [14]. 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 [14]. No contaminating components were observed after these components were first analysed by SDS/PAGE and then silver-stained. The chromogenic peptide substrates S-2586 (MeO-succinyl-Arg-Pro-Tyr-p-nitroanilide, a chymotrypsin substrate) and S-2238 (H-D-Phe-piperidine-Arg-p-nitroanilide, a thrombin substrate) were obtained from Chromogenix (Mölnå, Sweden).

Sweden). Human LF purified from milk was purchased from Calbiochem (La Jolla, CA, U.S.A.). Bovine α -thrombin was kindly given by I. Björk (Department of Veterinary Medical Chemistry, Uppsala, Sweden). Human α_1 -antichymotrypsin was purchased from Calbiochem. DEAE-cellulose (DEAE-Sephacel), heparin-Sepharose and CNBr-activated Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). LF (2 mg) was coupled to CNBr-activated Sepharose 4B in accordance with the procedure described by the manufacturer. Protein concentrations were determined by the Quantigold method (Diversified Biotech, Boston, MA, U.S.A.), in accordance with the procedure described by the manufacturer. Glycosaminoglycan concentrations were determined by the carbazole method for the detection of uronic acid [26].

Polysaccharides

Heparin from pig intestinal mucosa (approx. 2.4 sulphate groups per disaccharide) and [^3H]NAc-heparin were gifts from Ulf Lindahl (Department of Medical and Physiological Chemistry, Uppsala University, Uppsala, Sweden). The specific radioactivity of the [^3H]NAc-heparin was 6×10^5 d.p.m./nmol, assuming a molecular mass of 15 kDa. Even-numbered heparin oligosaccharides, obtained by partial depolymerization of the polysaccharide with nitrous acid (pH 1.5; cleavage at N-sulphated GlcN units) [27] were gifts from Dorothe Spillmann (Department of Medical and Physiological Chemistry, Uppsala University, Uppsala, Sweden). The resulting reducing-end anhydromannose residues were either reduced with NaBH_4 or radiolabelled by reduction with NaB^3H_4 . The specific radioactivities of the radiolabelled oligosaccharides were approx. 10^6 d.p.m./nmol of saccharide; no differences in specific radioactivities were observed for oligosaccharide fractions of different molecular sizes. Dermatan sulphate purified from pig intestinal mucosa, chondroitin sulphate purified from bovine cartilage and heparan sulphate purified from whale lung were gifts from Ulf Lindahl. Samples of selectively 2-O-desulphated (approx. 1.7 sulphate groups per disaccharide), 6-O-desulphated (approx. 1.5 sulphate groups per disaccharide) and N-desulphated heparins (approx. 1.5 sulphate groups per disaccharide) were given by Dorothe Spillmann.

Enzymic assays

RMCP-1 activities were measured in 96-well microtitre plates. RMCP-1 (15–50 ng) was diluted with PBS/0.1 % Triton X-100 to a final volume of 200 μl . Enzyme activity was measured after the addition of 20 μl of a solution of S-2586 (4 mM in water), and the absorbance at 405 nm was monitored with a Titertek Multiscan spectrophotometer (Flow Laboratories). Alternatively, when thrombin was used as substrate for RMCP-1, 10 ng of thrombin was added to wells containing RMCP-1, followed by incubation for an additional 15 min. Next, 20 μl of a solution of S-2238 (2.5 mM in water) was added, and finally residual thrombin activity was monitored. In experiments where the effect of various heparins on RMCP-1 activity was studied, the polysaccharides were added 15 min before the chymase substrates (S-2586 or thrombin). In standard incubations a 2.5:1 ratio (by mass) of RMCP-1 to heparin PG was used (e.g. 25 ng RMCP-1 to 10 ng heparin PG). This protein-to-PG ratio was chosen to provide optimal conditions for the potentiation of RMCP-1 activity (see Figure 1). The effect of LF on chymase activity was determined after the addition of increasing amounts of LF to RMCP-1/heparin mixtures or to free RMCP-1, followed by an additional incubation time of 2 h and monitoring of residual

enzymic activity as above. To study the effect of LF on cell-surface-associated chymase, purified MCs (2×10^4 cells in 0.4 ml of cell culture medium) were preincubated for 10 min with 1, 10 or 100 μg of LF. Subsequently, 0.1 μg of thrombin was added and the rate of thrombin inactivation was monitored. The K_m and k_{cat} values of RMCP-1 for S-2586 were determined after incubation of the enzyme with increasing concentrations of substrate, followed by monitoring of protease activity. The results obtained were used for calculations of kinetic parameters after nonlinear regression analysis. Results are expressed as means of duplicate determinations; duplicate determinations were generally within $\pm 5\%$ of the mean.

DEAE-cellulose precipitation assay

RMCP-1 (50 ng) was incubated in the absence or the presence of 20 ng of heparin PG, and various amounts of LF. Incubations were performed in 1.5 ml Eppendorf tubes in 600 μl of PBS/0.1 % Triton X-100. After 2 h, either (1) 400 μl of a 1:1 mixture of DEAE-Sephacel and PBS/0.1 % Triton X-100 or (2) 400 μl of PBS/0.1 % Triton X-100 was added followed by gentle rotation of the tubes (end over end) for 30 min. The gels were allowed to settle and 200 μl of the resulting supernatants were transferred to 96-well plates, followed by determination of residual RMCP-1 activity by using S-2586 (see above). In some experiments 1 μg of heparin PG had been added to the wells before the supernatants were transferred.

Binding assay

The binding of LF to RMCP-1 was studied with a nitrocellulose filter-disc assay, modified from that described by Pejler et al. [27]. In standard assays, purified LF (5 μg) was incubated in PBS at room temperature together with either [^3H]heparin or [^3H]heparin oligosaccharides in a final volume of 200 μl . In the competition experiments, various unlabelled inhibitors were included in the incubation mixtures. After approx. 1 h the mixtures were passed through nitrocellulose filters (Sartorius; 25 mm diameter; pore size 0.45 μm) that had been prewashed with 5 ml of PBS; they were then washed with 5 ml of the same buffer. The filters were subsequently soaked in 2 ml of PBS/0.1 % Triton X-100 containing 0.5 M NaCl. Finally, radioactivity eluted from the filters was quantified by liquid-scintillation counting.

RESULTS

Activation of RMCP-1 by heparin

We have previously shown that RMCP-1 binds strongly to heparin and that this binding results in potentiation of its enzymic activity [14,23]. To establish optimal conditions for enzyme activation, RMCP-1 activity was measured at different concentrations of heparin. Both S-2586 and a macromolecular substrate, thrombin, were used as substrates. We have previously shown that thrombin is degraded by RMCP-1 in a process that is accompanied by inactivation of the coagulation enzyme [28]. Endogenous MC heparin PG gave a maximal approx. 2.3-fold stimulation of RMCP-1 activity towards S-2586 (Figure 1A). Maximal stimulation occurred at 10 ng of added PG to 25 ng of RMCP-1, and increased amounts of PG did not further affect the level of activity. Pig mucosal heparin ('commercial heparin') also stimulated RMCP-1 activity, although its effect was significantly less than that of the PG. Heparin tetradecasaccharides showed only a small stimulatory effect on RMCP-1 activity. When thrombin was used as substrate, heparin PG produced a maximal approx. 10–20 fold stimulation of RMCP-1 activity

Figure 1 Activation of RMCP-1 by heparin

RMCP-1 (25 ng) was incubated in the presence of heparin PG (●), pig mucosal heparin (○) or heparin tetradesaccharides (■) at the amounts of saccharide indicated. (A) After 15 min, 20 μ l of a solution of S-2586 (4 mM in water) was added, followed by the determination of RMCP-1 activity. (B) After 15 min, 10 ng of thrombin was added and after an additional 15 min, 20 μ l of a solution of S-2238 (2.5 mM in water) was added, followed by the determination of residual thrombin activity.

(Figure 1B). Again, maximal stimulation occurred at 10 ng of added heparin PG. However, at higher concentrations of heparin PG there was a significant decrease in the activity of RMCP-1 towards thrombin.

Regulation of RMCP-1/heparin by LF

LF was assessed for RMCP-1-inhibiting activity, with both S-2586 and thrombin as substrates for the chymase (Figure 2). Increasing amounts of LF were added to RMCP-1 in the presence of either 10 ng of heparin PG or 10 ng of pig mucosal heparin. The results showed that LF decreased RMCP-1 activity both in the presence of heparin PG and in the presence of pig mucosal heparin. In addition, a slight inhibitory effect of LF was observed towards the free chymase. However, complete inhibition of RMCP-1 activity was not achieved with either S-2586 or thrombin as substrate. Approx. 25% of the activity towards S-2586 and approx. 15% of the activity towards thrombin, compared with the activities in the presence of optimal amounts of heparin PG, were resistant to regulation by LF. When increased amounts of heparin PG were present, the regulation of RMCP-1 by LF was partly blocked at lower concentrations of LF. However, at higher concentrations of LF, heparin PG was unable to block the effect of LF (Figure 3).

We have previously shown that RMCP-1 present at the surface of unstimulated peritoneal MCs expresses potent thrombin-inactivating activity [14]. Experiments were performed with purified MCs to study the effect of LF on cell-surface-associated

Figure 2 Inhibition of RMCP-1 by LF

RMCP-1 (25 ng) was incubated in the presence of 10 ng of heparin PG (●) or 10 ng of pig mucosal heparin (○), or without heparin (▲). After 15 min, LF was added at the amounts indicated. (A) After an additional 2 h, 20 μ l of a solution of S-2586 (4 mM in water) was added, followed by the determination of residual RMCP-1 activity; 100% RMCP-1 activity represents the amount of activity in the presence of 10 ng of heparin PG. (B) After 2 h, 10 ng of thrombin was added and after an additional 15 min, 20 μ l of a solution of S-2238 (2.5 mM in water) was added, followed by the determination of residual thrombin activity.

RMCP-1. Intact MCs were preincubated for 10 min with LF at various concentrations. Thrombin (0.1 μ g) was then added and the rate of inactivation of thrombin by cell-surface-associated chymase was monitored. In the absence of LF, thrombin was inactivated at a rate of approx. 0.08 μ g/h. When 10 μ g of LF was added, approx. 50% inhibition of the thrombin inactivation was observed, whereas 100% inhibition of the chymase-mediated thrombin inactivation process was achieved in the presence of 100 μ g of LF.

The process of inhibition of RMCP-1 by LF over time was studied. The regulation process seemed to involve an initial rapid approx. 45% decrease in activity within 1 min, followed by a slow further inhibition of RMCP-1 activity, essentially completed within 2 h (results not shown). Fe^{2+} or Fe^{3+} ions at concentrations up to 100 μ M had no significant effect on the inhibition of RMCP-1 activity by LF (results not shown). The results shown in Figure 2 demonstrate not only that LF regulates heparin PG-bound RMCP-1 but also that it has a slight inhibitory effect on free RMCP-1. To determine whether or not RMCP-1 could interact directly with LF, free RMCP-1 was subjected to affinity chromatography on immobilized LF. The results obtained showed that RMCP-1 did indeed bind to the immobilized LF at physiological ion strength and was eluted from the column at NaCl concentrations between 0.15 and 0.5 M (results not shown).

Figure 3 Effect of heparin PG on the inhibition of RMCP-1 by LF

RMCP-1 (25 ng) was incubated in the presence of 10 (□), 50 (■) or 200 (○) ng of heparin PG. After 15 min, LF was added at the amounts indicated and after 2 h of additional incubation, 20 µl of a solution of S-2586 (4 mM in water) was added, followed by the determination of residual RMCP-1 activity; 100% RMCP-1 activity represents the amount of activity in the presence of 10 ng of heparin PG.

Table 1 Kinetic constants of RMCP-1 for S-2586 in the presence of heparin PG and LF

RMCP-1 (25 ng) was incubated with 10 ng of heparin PG and the amounts of LF indicated. For further experimental details see the Materials and methods section. Results are values $\pm$ S.E. obtained by nonlinear regression analyses of measurements at 7 substrate concentrations.

Addition	K_m (mM)	k_{cat} (s^{-1})	$10^{-3}k_{cat}/K_m$ ($M^{-1} \cdot s^{-1}$)
None	1.6 ± 0.07	8.9 ± 0.2	5.6
Heparin PG	0.30 ± 0.05	7.4 ± 0.4	25
Heparin PG, LF (2.6 µg)	1.4 ± 0.1	6.5 ± 0.3	4.6
Heparin PG, LF (13.3 µg)	1.9 ± 0.1	7.4 ± 0.2	3.9

Kinetic analyses of the interaction of LF with RMCP-1–heparin PG

The K_m and k_{cat} values of RMCP-1 for S-2586 were determined for the free chymase, chymase in the presence of heparin PG, and chymase in the presence of both heparin PG and LF (Table 1). Heparin PG produced an approx. 5-fold decrease in the K_m of RMCP-1 for S-2586, whereas the k_{cat} value was affected only marginally. When LF was added to the chymase–heparin PG mixtures, the apparent K_m values were increased to levels comparable to those of the free chymase, again with only a minor effect on the k_{cat} values. Heparin PG caused an approx. 4.5-fold increase in k_{cat}/K_m compared with free RMCP-1, whereas LF decreased k_{cat}/K_m back to values that were similar to that obtained for the free chymase, or slightly lower.

Displacement of RMCP-1 from heparin PG by LF

The results above are compatible with a mechanism in which LF displaces RMCP-1 from heparin PG. To obtain direct evidence for such a regulating mechanism, an assay involving precipitation of RMCP-1–heparin PG complexes with an anion-exchange resin, DEAE-cellulose, was developed. RMCP-1 was incubated either alone, together with heparin PG, or in the presence of both heparin PG and LF. After 2 h, DEAE-cellulose was added to the

Table 2 Precipitation of RMCP-1–heparin PG with DEAE-cellulose

RMCP-1 (50 ng) was incubated either alone (–PG) or together with 20 ng of heparin PG (+PG) and LF as indicated. After 2 h, either 400 µl of a 1:1 mixture of DEAE-cellulose and PBS/0.1% Triton X-100 (+DEAE), or 400 µl of PBS/0.1% Triton (–DEAE), was added, followed by the determination of residual RMCP-1 activities present in the resulting supernatants.

Incubation conditions	$10^3 \times$ RMCP-1 activity ($\Delta A_{405}/h$)
–PG –DEAE	33
–PG + DEAE	30
+PG –DEAE	79
+PG –DEAE $\rightarrow$ +PG (1 µg)*	80
+PG + DEAE	6.2
+PG + LF (150 ng) + DEAE	21
+PG + LF (500 ng) + DEAE	26
+PG + LF (500 ng) + DEAE $\rightarrow$ +PG (1 µg)*	68
+PG + LF (1 µg) + DEAE	27
+PG + LF (5 µg) + DEAE	28
+PG + LF (25 µg) + DEAE	30

* Heparin PG (1 µg) was added as indicated [$\rightarrow$ +PG (1 µg)] either to RMCP-1 that had been previously incubated with 20 ng of heparin PG (without the DEAE precipitation step), or to supernatants from tubes where RMCP-1 had been first incubated together with 20 ng of heparin PG and 500 ng of LF followed by precipitation with DEAE-cellulose.

incubation mixtures. The gels were allowed to settle in the tubes, and residual chymase activities in the resulting supernatants were measured (Table 2). When RMCP-1 was incubated alone, only marginal reduction of residual chymase activity in the supernatant was observed after the addition of DEAE-cellulose, indicating that free RMCP-1 does not bind to the anion-exchange resin. In contrast, when RMCP-1 was incubated together with heparin PG, most of the chymase activity was precipitated after the addition of DEAE-cellulose. This indicates that heparin PG, as expected, binds to DEAE-cellulose and that the decrease in chymase activity in the supernatants is due to binding of RMCP-1 to the DEAE-cellulose-associated heparin PG. When LF was added to the RMCP-1–heparin PG mixtures, an increased recovery of chymase activity in the supernatants was observed after addition of DEAE-cellulose, indicating that LF had displaced RMCP-1 from heparin PG. The maximum amount of chymase activity that could be recovered in the supernatants after displacement by LF corresponded to a level similar to that of RMCP-1 in the absence of both heparin PG and LF (Table 2). Indeed, if the displaced chymase is present in a free form (devoid of heparin PG) this level of maximal displaceable activity should be expected.

Experiments were performed to confirm that the chymase activity released by LF was due to free RMCP-1. If the displaced activity corresponds to free RMCP-1 it should be possible to stimulate the displaced chymase activity by the addition of heparin PG (see Figure 1). In contrast, if the displaced activity represents RMCP-1–heparin PG complexes, further stimulation with heparin PG should not be possible. Addition of excess amounts of heparin PG to supernatants obtained after displacement by LF resulted in an approx. 2.4-fold stimulation of the RMCP-1 activity, indicating that the chymase activity recovered in the supernatants did indeed correspond to free RMCP-1 (Table 2). (Excess PG was added to block any remaining LF present in the supernatant.) In contrast, RMCP-1 that had been previously incubated with heparin PG in the absence of LF was, as expected (see Figure 1A), not stimulated further by addition of excess amounts of heparin PG (Table 2).

Figure 4 Inhibition of RMCP-1 by α_1 -antichymotrypsin

RMCP-1 (15 ng) was incubated either alone (●, ○) or with 3 ng of heparin PG (■, □). After 5 min, 6 μ g of LF was added either to the RMCP-1–heparin PG mixture (■) or to free RMCP-1 (●). After incubation for a further 2 h, DEAE-cellulose was added (see the Materials and Methods section for further details). Incubations with RMCP-1 and heparin PG (without LF; □) were not subjected to precipitation with DEAE-cellulose. Supernatants (200 μ l), recovered after precipitation with DEAE-cellulose, were transferred to 96-well microtitre wells. Next, α_1 -antichymotrypsin at the amounts indicated was added; after incubation for 10 min, residual chymase activities were determined.

Figure 5 Binding of heparin to LF

LF (5 μ g) was incubated for approx. 1 h with 3 H-labelled pig mucosal heparin at the molar ratios indicated. Radioactivity bound to LF was quantified with a nitrocellulose filter-disc assay (see the Materials and methods section). The line corresponds to the fitted curve generated with the best estimates calculated by nonlinear regression analysis.

Experiments were performed to determine whether the displacement of RMCP-1 from heparin PG by LF resulted in an increased susceptibility of the chymase to inhibition by α_1 -antichymotrypsin (Figure 4). Free RMCP-1 was readily inhibited by α_1 -antichymotrypsin, whereas chymase in complex with heparin PG was largely resistant to inhibition, confirming our previous observations [24]. When LF was added to the heparin PG-associated RMCP-1 followed by precipitation with DEAE-cellulose as above, the displaced chymase showed an inhibition curve similar to that of the free chymase. LF alone did not affect the inhibition process.

Characterization of the binding of heparin to LF

The results described above indicate that most of the RMCP-1-regulating activity of LF is caused by binding to heparin PG, thereby displacing the chymase. Experiments were performed to characterize the interaction between heparin and LF. Initial

Figure 6 Binding of LF to glycosaminoglycans

Purified LF (5 μ g) was incubated with [3 H]NAC-heparin (5000 d.p.m.) and various amounts of unlabelled heparin PG (●), pig mucosal heparin (○), heparan sulphate (▲), chondroitin sulphate (□), dermatan sulphate (■) or hyaluronan (Δ). Radioactivity bound to LF was quantified with a nitrocellulose filter-disc assay (see the Materials and methods section).

experiments showed that the binding of [3 H]heparin (pig mucosal heparin; approx. 15 kDa) to LF was very rapid, maximal binding being achieved within 1 min (results not shown). Titration of LF with [3 H]heparin gave an apparent binding stoichiometry of approx. 0.2, indicating approx. 5 binding sites for LF per heparin chain (Figure 5). Control experiments showed 100% binding of the LF preparation to heparin–Sephacrose at physiological ion strength (results not shown). The binding of [3 H]heparin to LF was too tight to allow accurate calculations of K_d for the interaction at physiological ionic strength. However, an upper limit for K_d at approx. 15 nM was estimated from the data shown in Figure 5. Heparin did not produce any detectable change in the tryptophan fluorescence emission spectrum of LF, measured between 300 and 400 nm, with an excitation wavelength of 280 nm (results not shown).

The specificity of the binding was studied in competition experiments where the binding of [3 H]heparin to LF was determined in the presence of various unlabelled glycosaminoglycans (Figure 6). Unlabelled heparin PG and pig mucosal heparin were approximately equally effective in displacing the radiolabelled heparin from LF, with 50% inhibition achieved at approx. 0.3 μ g of polysaccharide. Heparan sulphate was less effective, with approx. 7 μ g of polysaccharide being required to obtain 50% inhibition. The galactosaminoglycans chondroitin sulphate and dermatan sulphate were both poor competitors for the binding of [3 H]heparin to LF: 50% inhibition by these compounds was not achieved, even when 100 μ g of galactosaminoglycan was added. Hyaluronan actually showed a slight potentiating effect, with an approx. 20% increase in binding of [3 H]heparin to LF at 100 μ g of added polysaccharide.

The contribution by sulphate groups located at various positions in the binding of heparin to LF was examined. Competition experiments were performed where the binding of intact heparin was compared with the binding of selectively 6-O-desulphated, 2-O-desulphated and N-desulphated heparins (Figure 7). All of these modified heparins were able to displace [3 H]heparin from LF, with 50% inhibition at approx. 3 μ g (6-O-desulphated heparin), approx. 1.4 μ g (2-O-desulphated heparin) and approx. 1.3 μ g (N-desulphated heparin) of polysaccharide. However, none of the modified heparins was as effective in the

Figure 7 Binding of LF to structurally modified heparins

LF (5 µg) was incubated with [3 H]NAC-heparin (5000 d.p.m.) and various amounts of unlabelled intact pig mucosal heparin (○), 2-O-desulphated pig mucosal heparin (□), 6-O-desulphated pig mucosal heparin (●) or N-desulphated pig mucosal heparin (■). Radioactivity bound to LF was quantified with a nitrocellulose filter-disc assay (see the Materials and methods section).

Figure 8 Binding of LF to heparin oligosaccharides

LF (1 (○) or 5 (●) µg) was incubated with 3 H-labelled heparin oligosaccharides (20 000 d.p.m.) of the chain lengths indicated. Radioactivity bound to LF was subsequently quantified with a nitrocellulose filter-disc assay (see the Materials and methods section).

displacement of [3 H]heparin as intact heparin (50 % inhibition at approx. 0.3 µg of heparin).

The size of the heparin region required for binding to LF was also examined. 3 H-labelled, even-numbered heparin oligosaccharides prepared by partial nitrous acid deamination of intact heparin (see the Materials and methods section) were assessed for binding to LF in direct binding studies (Figure 8). No binding of tetrasaccharides and very little binding of the 3 H-labelled hexasaccharides to LF was observed. In contrast, 3 H-labelled octasaccharides showed significant binding, and increased binding was observed for larger oligosaccharides up to 10–14 monosaccharide units, where a plateau of bound radioactivity was observed.

DISCUSSION

The biological function(s) of the MC proteases are not known. However, because they are present in the secretory granules and

are released during MC activation it seems reasonable to postulate a role in inflammation. The MC chymases are recovered *in vivo* in macromolecular complexes with heparin PG. The association of the chymases with heparin PG has several functional consequences of potential physiological importance. First, the chymase–heparin PG complexes are poorly soluble and tend to remain associated with the MC surface after exocytosis [13,14], thereby preventing the chymases from diffusing away from the inflammatory site. Secondly, we and others have shown that heparin potentiates the enzymic activity of the proteases [14,29–31] by a mechanism that seems to involve a decrease in the K_m of the chymase for its substrates [24]. Thirdly, we have recently shown that RMCP-1, when bound to heparin PG, is largely resistant to inhibition by various plasma protease inhibitors such as α_1 -protease inhibitor, α_1 -antichymotrypsin and α_2 -macroglobulin [24].

MCs contain very large amounts of chymases stored in their secretory granules in complex with heparin PG [13]. Thus, after MC degranulation, large quantities of protease–heparin PG complexes should be present in the extracellular space, and it seems likely that mechanisms for the regulation of protease activity are important in the control of the inflammatory response. Clearly, because association with heparin PG renders chymases largely resistant to inhibition by conventional protease inhibitors, alternative inhibitory mechanisms might be necessary. The present report shows, to my knowledge for the first time, that LF decreases the activity of MC chymase. The mechanism of regulation is shown to involve displacement of the chymase from heparin PG, its physiological ligand. However, displacement of RMCP-1 from the PG does not account for the entire effect because LF was also shown to interact directly with chymase. Thus the optimal effect might require that LF binds simultaneously to both RMCP-1 and heparin PG, and it is possible that such multivalent binding increases the affinity of the interaction. Indeed, the observed relatively low capacity of excess heparin PG to block the effect of LF (Figure 3) might be due to a limited ability of free PG to compete with heparin PG/chymase complexes for binding to LF.

Although LF is known to express a variety of biological activities, the exact physiological function of the molecule remains to be determined. In addition to its iron-binding properties, LF has been shown to express a variety of activities of potential importance in host defence. LF can be classified as an acute-phase protein and it has been shown that LF has lipopolysaccharide-inactivating [32], bactericidal [33], bacteriostatic [34] and antiviral [35] activities. Moreover, LF deficiency has been demonstrated to be associated with altered granulocyte function [36], and a role for LF in the regulation of cellular proliferation has been implicated [34]. The present report identifies a new activity of LF, one of potential physiological relevance. Because MC degranulation during inflammation is normally associated with neutrophil influx into the tissue, it is clear that LF, released by activated neutrophils, should have the potential for interacting with secreted chymase–heparin PG complexes. Such interactions should result in the release of free chymase molecules accompanied by a decreased activity of the proteases. Because the free chymase molecules are susceptible to inhibition by a variety of conventional protease inhibitors [24], this process should ultimately result in complete inhibition of the proteases. Such a mechanism would indeed represent a plausible pathway for the regulation of MC chymases under inflammatory conditions. Because the exact biological function(s) of the MC chymases are not known, it is difficult to predict the consequences of chymase regulation by LF. However, it seems most likely that chymases have pro-inflammatory activities and the regulation of

the proteases by LF would thus contribute to the generally anti-inflammatory properties of LF.

Before this investigation, the interaction of LF with heparin had been characterized only partly. The heparin-binding site in LF has been located to the N-terminal portion of the molecule and the interaction is thought to be mediated through a GRRRRS sequence in synergy with a RKVR sequence [37,38]. Here I investigated which structural elements of heparin are important for interaction with LF. Free heparin chains (pig mucosal heparin; 'commercial heparin') were as effective in binding to LF as was the endogenous heparin PG, indicating that the protein core of the PG does not influence the interaction. Heparan sulphate, which has the same carbohydrate backbone structure as heparin but has a lower degree of overall sulphation, was less potent in the binding to LF. Neither of the two galactosaminoglycans chondroitin sulphate or dermatan sulphate, although having approximately the same degree of overall sulphation as the heparan sulphate used (approx. 1 sulphate group per disaccharide), was as effective in binding LF as the heparan sulphate. It thus seems that binding to LF is specific for the heparin/heparan sulphate carbohydrate backbone structure. The preference of LF to bind to heparin and heparan sulphate over chondroitin sulphate is also supported by a previous report [39]. Modified heparins produced by the selective removal of either 6-*O*-sulphate, 2-*O*-sulphate or *N*-sulphate groups showed approximately equal affinity for LF, indicating that there is no specific requirement for the presence of sulphate groups in any of these positions. Alternatively, the binding might require a specific sequence containing both (one or more) 6-*O*-sulphate, 2-*O*-sulphate or *N*-sulphate groups. The minimal heparin oligosaccharide showing significant binding to LF was an octasaccharide. The LF-binding site in heparin is thus comparable in size with the binding sites for several other heparin-binding proteins, e.g. antithrombin [17], basic fibroblast growth factor [18], hepatocyte growth factor [40] and platelet factor 4 [19], but appreciably smaller than the binding site for RMCP-1 [23].

The high anionic charge density of heparin enables the polysaccharide to interact with a variety of proteins. However, because MC heparin is not normally accessible to several of these ligands, the physiological significance of many such interactions seems questionable. For LF it is clear that during an inflammatory reaction both LF and heparin PG secreted from activated neutrophils and MCs, respectively, should be present in the tissue. Thus interaction of LF with heparin is likely to occur *in vivo*.

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