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Distorted Secretory Granule Composition in Mast Cells with Multiple Protease Deficiency

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Mast cells are characterized by an abundance of secretory granules densely packed with inflammatory mediators such as bioactive amines, cytokines, serglycin proteoglycans with negatively charged glycosaminoglycan side chains of either heparin or chondroitin sulfate type, and large amounts of positively charged proteases. Despite the large biological impact of mast cell granules and their contents on various pathologies, the mechanisms that regulate granule composition are incompletely understood. In this study, we hypothesized that granule composition is dependent on a dynamic electrostatic interrelationship between different granule compounds. As a tool to evaluate this possibility, we generated mice in which mast cells are multideficient in a panel of positively charged proteases: the chymase mouse mast cell protease-4, the tryptase mouse mast cell protease-6, and carboxypeptidase A3. Through a posttranslational effect, mast cells from these mice additionally lack mouse mast cell protease-5 protein. Mast cells from mice deficient in individual proteases showed normal morphology. In contrast, mast cells with combined protease deficiency displayed a profound distortion of granule integrity, as seen both by conventional morphological criteria and by transmission electron microscopy. An assessment of granule content revealed that the distorted granule integrity in multiprotease-deficient mast cells was associated with a profound reduction of highly negatively charged heparin, whereas no reduction in chondroitin sulfate storage was observed. Taken together with previous findings showing that the storage of basic proteases conversely is regulated by anionic proteoglycans, these data suggest that secretory granule composition in mast cells is dependent on a dynamic interrelationship between granule compounds of opposite electrical charge. The Journal of Immunology, 2013, 191: 3931–3938.

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; CPA, carboxypeptidase A; MMP, matrix metalloproteinase; mMCP, mouse mast cell protease; PCMB, peritoneal cell–derived mast cell; RFIP, reverse-phase ion-pair; TEM, transmission electron microscopy; WT, wild-type.

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various granule compounds such that a reduction of positive charge will affect the granule composition in an analogous fashion as does the reduction in negative charge density. To address this issue, in this study we generated mice deficient in multiple positively charged proteases that are stored within MC granules, that is, mMCP-4, mMCP-5, mMCP-6, and CPA3. Indeed, we show that the absence of multiple MC proteases results in severe effects on granule integrity, as reflected by distorted granule staining properties and ultrastructure, and that these effects are associated with defective storage of heparin within granules. Collectively, our findings suggest that MC granule composition is a result of a dynamic electrostatic relationship between oppositely charged compounds.

**Materials and Methods**

**Reagents**

The chromogenic substrates S-2288 (for detection of trypsin-like activity/tryptase) and S-2586 (for detection of chymotrypsin-like activity/chymase) were from Chromogenix (Milano, Italy), and M-2245 (for detection of CPA activity) was from Buchem (Bubendorf, Switzerland).

**Mice**

Mice triple-deficient in mMCP-4, mMCP-6, and CPA3 were generated by intercrossing mMCP-4−/− (11), mMCP-6−/− (12), and CPA3−/− mice (13), all on a C57BL/6J genetic background. The mMCP-6−/− strain used is mMCP-7−/− sufficient (14). Mice were genotyped by PCR using the following primer combinations: mMCP-4 gene (forward, 5′-CGA GGT CAA CTG TAC TCT 3′; reverse, 5′-GGT GAT CTC CAG ATG GGC CAT GTA AGG GCG-3′), mMCP-6 gene (forward primer, 5′-GTA CTT GTC CTC ATG CAG GGT-3′; reverse, 5′-GAC CAT GAT GTG ATG GCG CTT ATG-3′), and CPA3 gene (forward primer, 5′-CCA ACT AAC TCC CTT TGT GCT CC-3′; reverse 2, 5′-GTC CGG ACA CGC TGA ACT TG-3′), and CPA3 gene (forward primer, 5′-GTA CTT GTC CTC ATG CAG GGT-3′; reverse, 5′-GAC CAT GAT GTG ATG GCG CTT ATG-3′), and CPA3 gene (forward primer, 5′-CCA ACT AAC TCC CTT TGT GCT CC-3′; reverse 2, 5′-GTC CGG ACA CGC TGA ACT TG-3′). WT and mMCP-4−/− CPA3−/− deficient mice were on a C57BL/6J genetic background. Eight- to 10-wk-old mice were used in all experiments. All experiments were approved by the Local Ethics Committee.

**Cell culture**

Bone marrow–derived MCs (BMMCs) (15) and peritoneal cell–derived MCs (PCMCs) (16, 17) were isolated and cultured as previously described.

**Western blot analysis**

Western blot analysis was performed as described (15).

**Analysis of β-hexosaminidase and protease activities**

Sample preparation and measurements of enzymatic activities were performed as described (15).

**Cell morphology**

For toluidine blue staining, peritoneal cells or PCMCs were collected onto cytosin slides. Samples were fixed with 100% methanol and subsequently stained with toluidine blue solution (0.1% toluidine blue in 0.17 M NaCl [pH 2]). Samples were rinsed with H2O, dried, and covered with Vector-Mount permanent mounting medium (Vector Laboratories, Burlingame, CA). Ear tissue was fixed in 4% paraformaldehyde/PBS, embedded in paraffin, sectioned, and stained with toluidine blue. Transmission electron microscopy (TEM) was performed as previously described (9).

**Proteoglycan analysis**

Proteoglycan analysis was performed as described with some modifications (15). Briefly, 105 PCMCs (0.55 × 106 cells/ml) in sulfate-free DMEM medium were labeled biosynthetically with 12.5 μCi/ml of Na2[35S]SO4 (PerkinElmer, Waltham, MA). After 24 h the samples were harvested, centrifuged, and the supernatants (corresponding to the medium fraction) were applied to 0.3 M DEAE Sephacel columns (GE Healthcare, Uppsala, Sweden). Pelleted cells were lysed with 500 μl 0.5% Triton X-100 in PBS/2 M NaCl and subsequently diluted to 10 ml with 0.5% Triton X-100.

DNA was removed by centrifugation (2000 × g, 10 min, 4˚C) and smaller DNA fragments were further digested by 300 U DNAase I from bovine pancreas (Sigma–Aldrich). The lysed cells were subsequently applied to DEAE Sephacel columns. Bound proteoglycans were eluted with PBS/3 M NaCl/0.5% Triton X-100, and 10 μl of each fraction (300 μl/fraction) was mixed with 2 ml OptiPhase HiSafe 3 scintillation mixture and analyzed by scintillation counting. Fractions containing [35S]-labeled proteoglycans were pooled, desalted through PD10 columns (GE Healthcare), and treated with 0.5 M NaOH overnight at 4˚C to release free GAG chains from the proteoglycans. The samples were neutralized with 4 M HCl, desalted on PD10 columns, and concentrated. A portion of GAG chains were treated with HNO3 (pH 1.5) to degrade heparin/HS chains (15). HNO3-treated or untreated GAGs (10,000 cpm in 100 μl) were mixed with 150 μg blue dextran (marker of void volume; Sigma–Aldrich) and applied to a Superdex 200 column (GE Healthcare). The column was eluted with 1 M NaCl/0.05 M Tris-HCl (pH 7.4) at 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed by scintillation counting.

For anion-exchange chromatography, GAG samples were mixed with 2.25 mg standard pig mucosal heparin and 0.5 mg standard chondroitin sulfate A. Samples (10,000 cpm in 1 ml) were applied to a HiTrap DEAE FF column (GE Healthcare) that was washed with 50 mM NaAc/50 mM LiCl (pH 4) and then eluted at 0.5 ml/min with a linear gradient of LiCl (from 50 mM to 3 M) in 50 mM NaAc (pH 4). Twenty-five microliters of each fraction was assayed with the carbazole method to detect the unlabeled standard heparin and CS; the rest (475 μl) was analyzed by scintillation counting.

**Disaccharide analysis**

Samples were prepared as previously described followed by reverse-phase ion-pair (RPIP)-HPLC analysis (18).

**Statistical analysis**

The raw data were exported to the Microsoft Office Excel 2007 software program, where an unpaired t test was performed. Results shown are from individual experiments, representative of at least two individual experiments. A p value ≤0.05 was considered statistically significant.

**Results**

**Generation of mice with deficiency in multiple MC proteases**

To study the role of positively charged proteases in granule dynamics, we chose to generate mice with a triple deficiency in chymase, tryptase, and CPA3 by intercrossing mMCP-4−/− (11), mMCP-6−/− (12), and CPA3−/− (13) mice. The triple deficiency in mMCP-4, mMCP-6, and CPA3 was confirmed by genotyping (data not shown). To ascertain that the corresponding protein products were absent, Western blot analysis was carried out. The analysis was performed on extracts from ear skin tissue and peritoneal cells, with both of these tissues being rich in MCs. As expected, mMCP-4, mMCP-6, and CPA3 were readily detected in WT peritoneal cell extracts, and mMCP-6 and CPA3 were detectable in ear tissue, but they were absent in corresponding tissue from the triple KO mice (Fig. 1A). Previous studies have indicated that CPA3 and MCP-5 show a strong interdependence at the protein level (13, 19), and we therefore decided to assess whether the levels of MCP-5 protein were affected in the triple KO mice. As seen in Fig. 1B, mMCP-5 was detected in ear tissue and in peritoneal cell extracts from WT mice, but was strongly diminished in tissue from triple KO animals, in agreement with mMCP-5 being dependent on CPA3 for storage. Hence, at the protein level, the generated mouse strain has a quadruple deficiency in mMCP-4, mMCP-5, mMCP-6, and CPA3, that is, all of the proteases that are known to be stored in complex with serglycin in MCs (9, 10).

The absence of the corresponding proteases was also verified in MCs cultured from bone marrow, that is, BMMCs (data not shown). To study the effects of the protease deletion in MCs with a phenotype that closely resembles that of in vivo–derived MCs, we also derived MCs by expanding the peritoneal MC population, that is, PCMCs (16). As shown in Fig. 1, PCMCs contained high levels of...
mMCP-4, mMCP-5, mCP-6, and CPA3, and all of these proteases were either absent (mMCP-4, mCP-6, CPA3; Fig. 1A) or strongly reduced (mMCP-5; Fig. 1B) in PCMCs developed from multiprotease-deficient mice.

MC proteases account for the major part of the total proteolytic activities in skin, peritoneum, and in cultured MCs

To evaluate the functional consequences of the multiple protease deficiency, we first assessed the effect of multiple MC protease deficiency on total proteolytic activities in tissues and cultured MCs. As shown in Fig. 2A, trypsin-like activity (measured with S-2288) was readily detected in extracts of total peritoneal cells and ear tissue (Fig. 2B), as well as in extracts from cultured BMMCs and PCMCs from WT mice (Fig. 2C, 2D). In corresponding samples from skin, peritoneal cells, and BMMCs from multiprotease-deficient mice, a profound reduction of trypsin-like activity was seen. However, robust residual trypsin-like activity was seen in multiprotease-deficient PCMCs. Possibly, the high residual trypsin-like activity in multiprotease-deficient MCs may be explained by the intact expression of mMCP-7, a serglycin-independent tryptase, in the mMCP-6−/− mouse strain (12). Indeed, immunoblot analysis verified the presence of mMCP-7 protein in PCMCs from the multiprotease-deficient mice (data not shown). Chymotrypsin-like activity (measured with S-2586) was clearly detected in ear tissue, peritoneal cells, and PCMC extracts from WT mice but was virtually undetectable in corresponding tissues/cells from multiprotease-deficient animals (Fig. 2). Furthermore, whereas CPA-like activity (measured with M-2245) was clearly detected in WT tissues/cells, CPA-like activity was undetectable in corresponding samples from multiprotease-deficient mice (Fig. 2). Taken together, these results indicate that the serglycin-dependent MC proteases account for a major part of the total trypsin-like, chymotrypsin-like, and CPA-like proteolytic activities in ear skin tissue, the peritoneal cavity, and in cultured MCs.

The absence of multiple MC proteases does not affect the ability of MCs to degranulate

To evaluate whether the multiple protease deficiency affected the ability of MCs to degranulate, we stimulated PCMCs with calcium ionophore and measured the release of β-hexosaminidase. As seen in Fig. 3, β-hexosaminidase release was similar in WT and multiprotease-deficient PCMCs, suggesting that the simultaneous absence of mMCP-4, mMCP-5, mCP-6, and CPA3 did not affect MC functionality in terms of their ability to degranulate. The baseline levels of β-hexosaminidase as well as residual levels were similar in WT and multiprotease-deficient PCMCs.
β-hexosaminidase levels following degranulation were slightly higher in multiprotease-deficient versus WT MCs (Fig. 3).

**The absence of multiple MC proteases results in distorted granule integrity**

A widely established method to judge granule integrity is to stain MCs with cationic dyes such as toluidine blue. In mature MCs, such dyes produce strong metachromatic staining, and we have shown that the metachromatic staining is critically dependent on the presence of serglycin with sulfated heparin side chains within granules (10, 20, 21). As expected, WT MCs in both peritoneum (Fig. 4A) and in ear skin (Fig. 4F) stained strongly with toluidine blue. Furthermore, peritoneal MCs deficient in individual proteases showed normal staining properties (Fig. 4B–D). However, when simultaneously lacking mMCP-4, mMCP-5, mMCP-6, and CPA3, peritoneal MCs exhibited a markedly reduced toluidine blue staining, indicating that the combined protease deficiency caused distorted granule composition. The numbers of peritoneal MCs were similar in WT and multiprotease-deficient mice. Strongly defective granular staining was also seen in multiprotease-deficient skin MCs of the ear tissue (Fig. 4G). In fact, the effect of the multiprotease deficiency on MC morphology was even more pronounced in ear tissue in comparison with the peritoneal cavity.

To extend these findings we also performed ultrastructural analysis using TEM. As depicted in Fig. 5, granules in WT peritoneal MCs were filled with highly electron dense material that was homogeneously distributed within the granules. In contrast, the granules of multiprotease-deficient MCs had a patchy, profoundly less homogeneous appearance, and demonstrated an overall reduction in electron density. Hence, the simultaneous absence of mMCP-4, mMCP-5, mMCP-6, and CPA3 affects the granules at the ultrastructural level.

**The absence of multiple MC proteases results in defective storage of heparin in cultured MCs**

A potential explanation for the reduced metachromatic staining (see Fig. 4) is that multiple-protease deficiency causes a reduction in proteoglycan content of MCs. To assess this possibility we first labeled PCMCs biosynthetically with [35S]sulfate, which will be incorporated as either O-sulfate- or N-sulfate groups in the GAG side chains of the corresponding proteoglycans. After a 24-h labeling period, proteoglycans were purified. As judged by total [35S]sulfate incorporation, there was a trend toward a decrease in the amount of cell-associated proteoglycans in multiprotease-deficient MCs accompanied by a trend toward increased proteoglycan secretion (not shown), but neither of these observations reached statistical significance. After liberation of free GAG chains from corresponding proteoglycans by alkali treatment, a further analysis showed that essentially all of the [35S]-labeled GAGs from both WT and multiprotease-deficient cells were depolymerized by nitrous acid (pH 1.5) (Fig. 6A). This shows that the sulfated GAGs belong to the heparin/HS family rather than being of CS type (CS is not degraded by nitrous acid [pH 1.5]). Furthermore, the GAG chains from WT and multiprotease-deficient cells were of similar size (Fig. 6A) and had approximately equal anionic charge density (Fig. 6B). Notably, the GAGs from both WT and multiprotease-deficient cells eluted at similar positions on anion exchange chromatography as did standard heparin (Fig. 6B). This indicates that the GAGs produced are of high negative charge density, which is a characteristic feature of heparin as opposed to HS. Hence, the sensitivity to depolymerization by nitrous acid in combination with the high negative charge density classifies the [35S]-labeled GAGs produced by PCMCs as heparin (rather than HS).
Importantly, note that the biosynthetic labeling procedure will only detect those proteoglycans that are synthesized during the time frame of the labeling period, but will not account for those that have accumulated in MCs over time. Hence, there is a possibility that the biosynthetic labeling approach will not reflect the total content of stored proteoglycans in MCs. To provide a more complete picture of the stored proteoglycans of WT versus multiprotease-deficient MCs, we therefore employed an alternative method based on enzymatic digestion of unlabeled GAGs with either heparin lyases or chondroitinase ABC, followed by RPIP-HPLC analysis for detection and quantification of formed disaccharides (18). In addition to providing a measurement of total GAG content, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [35S]sulfate labeling approach, the enzymatic/ RPIP-HPLC approach revealed that heparin/HS is a dominating GAG synthesized by both WT and multiprotease-deficient PCMCs as shown by the high content of disaccharide species characteristic for GAGs of heparin/HS type (Fig. 7A). Notably, among the various heparin/HS disaccharide species, there was a striking dominance of the trisulfated disaccharide HexA(2-O-SO$_3^-$)-GlcNSO$_3^-$ (6-O-SO$_3^-$) (denoted NS6S2S). A high content of this highly sulfated disaccharide is a characteristic feature of heparin as opposed to HS (7). Importantly, the high content of the NS6S2S disaccharide species is in agreement with the high overall negative charge density as judged by anion exchange chromatography (see Fig. 6B). Significant amounts of the disulfated HexA-GlcNSO$_3^-$ (6-O-SO$_3^-$) (NS6S) species (NS6S) were also recovered, whereas low or undetectable levels of HexA2-O-SO$_3^-$)-GlcNSO$_3^-$ (NS2S) and of various monosulfated or nonsulfated species were seen (Fig. 7A).

When comparing the disaccharide profiles of WT versus multiprotease-deficient MCs, it was evident that the multiprotease deficiency caused a reduction in the total heparin content, as reflected by a profound reduction of the NS6S2S and NS6S disaccharide species (Fig. 7A). However, there were no significant differences in the relative distribution of the various disaccharide variants when comparing heparin from WT versus multiprotease-deficient MCs (Fig. 7B), indicating that the multiple protease deficiency did not affect the structural properties of the heparin. Hence, these findings indicate that the multiprotease deficiency in MCs causes a profound reduction in the amount, but not structure, of heparin stored within the secretory granules.

Although heparin was identified as the major GAG produced by the PCMCs, a smaller portion of CS was also recovered (~16% of total GAG content) (Fig. 7C). However, there was no reduction in the amount of CS when comparing WT and multiprotease-deficient MCs (Fig. 7C). The CS disaccharide composition was similar in CS from WT and multiprotease-deficient MCs (Fig. 7C), being strongly dominated by the monosulfated HexA(4S)-GalNAc (4S) and disulfated HexA-GalNAc(4,6-di-O-SO$_3^-$) (6S4S) disaccharide species.

The absence of multiple MC proteases results in defective storage of MC heparin in vivo

Next, we investigated whether the multiprotease deficiency causes similar effects on the storage of MC heparin in vivo as those observed in cultured PCMCs. For this purpose we prepared extracts of whole ear skin tissue from WT and multiprotease-deficient animals and analyzed their contents and structure of heparin/HS and CS using the enzymatic/RPIP-HPLC method. Importantly, note that this approach accounts for all of the GAGs present in the ear skin, that is, not only GAGs produced by MCs. As seen in Fig. 7F, CS constituted the main type of GAG in whole ear skin tissue.
from both WT and multiprotease-deficient mice, accounting for \(\sim 91\) and \(\sim 97\)% of the total GAG content, respectively. However, significant amounts of heparin/HS disaccharides were also detected (Fig. 7D, 7E). Among these, similar to PCMCs, the trisulfated NS6S2S was the dominating species and NS6S was also present in appreciable amounts (although considerably lower than NS6S2S) (Fig. 7D). However, differing from PCMCs, significant amounts of various non- and monosulfated disaccharide species and NS2S were observed (Fig. 7D).

A comparison of the GAG content of ear skin from WT versus multiprotease-deficient animals showed that the CS content and disaccharide composition was indistinguishable (Fig. 7F). In contrast, the multiprotease deficiency caused a profound reduction in the amounts of heparin/HS-derived disaccharides (Fig. 7D, 7E). In particular, the multiprotease deficiency caused a dramatic reduction in those disaccharides that were shown to be characteristic for MCs (see data for PCMCs), that is, NS6S2S (82% reduction) and NS6S (83% reduction) (Fig. 7D), whereas the levels of NS2S and various non- or monosulfated disaccharides were unaffected (Fig. 7D). These findings indicate that the absence of multiple MC proteases causes a profound reduction in the amount of stored heparin in MCs in vivo.

Discussion
In this study, to our knowledge we describe for the first time the generation of a mouse strain simultaneously lacking multiple proteases stored within MCs of the connective tissue type, that is, mMCP-4, mMCP-6 and CPA3. Previous studies have shown that CPA3 and mMCP-5 show a strong interdependence for storage within MCs; that is, the knockout of CPA3 causes a profound impairment of mMCP-5 storage (at the protein level) and vice versa (13, 19). Consequently, MCs of the multiprotease-deficient mouse strain additionally lack mMCP-5 at the protein level. Altogether, the generated mouse strain thus lacks a panel of stored proteins.

FIGURE 7. Effect of multi-MC protease deficiency on content and structure of GAGs. GAGs were isolated from PCMCs (A–C) and ear skin tissue (D–F) from WT and multiprotease-deficient mice. GAGs were depolymerized to disaccharide size by treatment with chondroitinase ABC and heparinase I, II, and III, respectively. Disaccharides were separated by RPIP-HPLC. (A and D) The disaccharide composition of heparin/HS recovered from PCMCs (A) and ear skin (D) of WT and multiprotease-deficient (KO) mice as indicated. Disaccharide composition is given as nanomoles per 10^6 cells (PCMCs) or per milligram wet tissue (ear skin). (B and E) Percentage of disaccharide variants in heparin/HS from PCMCs (B) and ear skin (E). (C and F) The disaccharide composition of CS recovered from PCMCs (C) and ear skin (F) of WT and multiprotease-deficient (KO) mice as indicated. The disaccharide composition is given as nanomoles per 10^6 cells (PCMCs) or per milligram wet tissue (ear skin). Heparin/HS disaccharides (A–D): NAc, HexA-GlcNAc; NS, HexA-GlcNSO_3_2; 6S, HexA-GlcNSO_3_2(6-O-SO_3_2); 2S, HexA(2-O-SO_3_2)-GlcNAc; NS6S, HexA-GlcNSO_3_2(6-O-SO_3_2); NS2S, HexA(2-O-SO_3_2)-GlcNSO_3_2; 2S6S, HexA(2-O-SO_3_2)-GlcNAc(6-O-SO_3_2); NS6S2S, HexA(2-O-SO_3_2)-GlcNSO_3_2(6-O-SO_3_2). CS disaccharides (C, F): OS CS/Hya, nonsulfated disaccharides of CS or hyaluronan origin; 4S, HexA-GalNAc(4-O-SO_3_2); 6S, HexA-GalNAc(6-O-SO_3_2); 2S, HexA(2-O-SO_3_2)-GalNAc; 6S4S, HexA-GalNAc(4,6-di-O-SO_3_2); 4S2S, HexA(2-O-SO_3_2)-GalNAc(4-O-SO_3_2); 6S2S, HexA(2-O-SO_3_2)-GalNAc(6-O-SO_3_2); 6S4S2S, HexA(2-O-SO_3_2)-GalNAc(4,6-di-O-SO_3_2). Mean ± SEM; n = 3. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
proteases, as a result of both genetic targeting and posttranslational effects. Importantly, note that all of the genetically targeted proteases (i.e., mMCP-4, mMCP-6, and CPA3) show an MC-specific expression pattern (4, 22). Hence, any effects of the multiprotease deficiency can be attributed to effects related to MCs. We anticipate that the multiprotease-deficient strain generated in this study will be an important tool to assess the global effects of the MC proteases.

Previous studies have shown that the storage of mMCP-4, mMCP-5, mMCP-6, and CPA3 is strongly dependent on serglycin, whereas the storage of mMCP-7 and of the mucosal MC protease mMCP-1 is serglycin-independent (9, 10, 20, 21). The mice generated in this study thus lack all of the MC-specific proteases that are known to be dependent on serglycin for storage. Most likely, the dependence of these proteases on serglycin is explained by electrostatic interactions between the GAG chains of serglycin and the respective proteases. In support of this notion, serglycin expressed in a MC context has a remarkably high anionic charge density and, conversely, the serglycin-dependent MC proteases display high positive surface charges and bind strongly to GAGs, in particular to heparin, in purified systems (23–26). Moreover, MCs lacking N-deacetylase/N-sulfotransferase 2, an enzyme required for sulfation of the heparin chains attached to the serglycin core protein, have similar defects in protease storage as those seen in serglycin−/− MCs (21, 27, 28). In further support for an electrostatic mechanism, there is a strong positive correlation between the net positive charge of various MC chymases and their extent of dependence on serglycin for storage (22).

A major finding in this study was that the simultaneous absence of serglycin-dependent proteases caused a major distortion of the secretory granule integrity. This was indicated by the profound reduction in toluidine blue staining of multiprotease-deficient MCs and the defective granule ultrastructure as seen by TEM. In contrast to the multiprotease-deficient MCs, MCs with single protease deficiencies exhibited normal granule morphology. This suggests that effects on granule integrity due to the lack of individual proteases can be rescued by compensation provided by other proteases, whereas no compensatory mechanism is available that can rescue granule integrity when all of the serglycin-binding proteases are absent simultaneously.

Because toluidine blue is known to stain anionic compounds such as proteoglycans, a likely explanation for the reduced toluidine blue staining was that the protease deficiency results in effects on the secretory granule proteoglycans. In agreement with this notion, we show that the simultaneous absence of serglycin-binding proteases in MCs causes a profound reduction in stored heparin, as seen both in cultured MCs and in vivo. Importantly, the reduction in heparin was not a consequence of a gross effect on total GAG synthesis, as there was no reduction in the amounts of GAGs of CS type in multiprotease-deficient MCs. Hence, the deletion of the various serglycin-dependent proteases causes a specific defect in heparin storage. Although we at present cannot explain the differential effect on heparin versus CS, one possibility would be that the CS recovered from the cultured MCs may be derived from proteoglycans located in cellular compartments distinct from granules, for example, at the cell surface. Alternatively, as supported by experimental data (21, 23, 27, 28), the MC proteases may interact preferentially with heparin versus CS. A reduction in the levels of proteases may therefore have a more limited effect on CS as compared with the effects on heparin storage. Intriguingly, whereas the enzymatic/RPIP-HPLC method revealed a large reduction of stored heparin in multiprotease-deficient versus WT MCs, such an effect was not apparent when biosynthetically labeling the cells with [35S]sulfate. This may at first glance appear as a contradiction. Importantly, however, note that the biosynthetic labeling approach only accounts for those GAGs that are produced within the limited time frame of the labeling experiment, whereas the enzymatic/RPIP-HPLC approach accounts for all of the GAGs that have accumulated in MCs as they mature during several weeks. An important conclusion is thus that the actual synthesis of heparin is not influenced by the multiple protease absence, as reflected by the short time frame [35S]sulfate labeling approach. Rather, the reduced total heparin content, as shown by the enzymatic/RPIP-HPLC approach, is likely due to long-term effects of the multiprotease deficiency on the ability of MCs to store heparin.

Interestingly, although CS synthesis in cultured MCs was undetectable using the [35S]sulfate labeling approach, CS disaccharides in appreciable amounts were detected when applying the enzymatic/RPIP-HPLC methodology. A likely explanation for this seeming discrepancy is that CS might accumulate in MCs at early stages of maturation, that is, at stages preceding the time point when the [35S]sulfate labeling was performed. Another potential explanation would be that CS synthesis in MCs occurs at a slower rate than does the synthesis of heparin. Hence, the short time frame of the [35S]sulfate labeling procedure would predominantly detect biosynthetically labeled heparin rather than CS.

When comparing the heparin/HS disaccharide profiles of PCMCs versus skin tissue, some notable differences were observed. In the heparin/HS from PCMCs, there was a clear dominance of NS6S2S and NS6S, suggesting that these two species are signature disaccharides characteristic for mature MCs. In skin tissue, NS6S2S and NS6S were also found in high amounts but, in contrast to PCMCs, appreciable amounts of various non- and monosulfated species as well as NS2S were also found. Because the GAGs isolated from skin extract represent a mixture of GAGs from all of different cell types found in the skin, we cannot with certainty establish the cellular sources of the respective disaccharide species. However, because NS6S2S and NS6S were profoundly reduced in skin from the multiprotease-deficient mice, and considering that high expression of these two disaccharide units is characteristic for MCs, it appears reasonable that NS6S2S and NS6S found in skin tissue are MC derived. In contrast, the levels of the various non-monomosulfated species and NS2S were not affected by the multiprotease deficiency, and it thereby appears conceivable that these latter disaccharide species arise from low-sulfated HS proteoglycans produced by non-MCs (e.g., fibroblasts).

A likely explanation for the reduction in MC heparin due to the multiple protease absence is that the storage of heparin is dependent on electrostatic interactions with the positively charged proteases. According to such a scenario, a reduction in positive electric charge (displayed by the MC proteases) would thus disturb the electric charge balance within granules, leading to impaired storage of heparin and thereby a distortion of the granule composition. Conversely, previous studies have demonstrated that a reduction in negative charge, either due to the lack of serglycin (9, 10) or sulfated heparin (20, 21, 27), causes severe defects in protease storage. In line with these findings is also a previous report showing that MCs lacking histamine exhibit reduced storage of granule proteases and proteoglycans (29). Although not experimentally proven, a likely explanation for the latter findings may be that a reduction in histamine (positively charged) has a downstream adverse effect on the storage of negatively charged serglycin proteoglycans, and that the reduced serglycin storage in turn results in impaired storage of the positively charged proteases.

Taken together, the present findings together with previous observations suggest that granule composition in MCs is dependent on a dynamic interaction between various differently charged granule
compounds. According to this model, a reduction in either negatively or positively charged granule constituents would cause impaired storage of compounds of opposite charge.

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References


