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Distorted Secretory Granule Composition in Mast Cells with Multiple Protease Deficiency

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Mast cells (MCs) are versatile effector cells of the immune system, contributing both to adaptive and innate immune responses (1). A hallmark feature of MCs is their high content of cytoplasmic secretory granules, which constitute the dominating morphological criterion for identifying MCs as such. When MCs are activated they can undergo degranulation, a process in which the contents of the secretory granules are released to the extracellular space (2). The granules contain a panel of preformed compounds, including bioactive amines (e.g., histamine), preformed cytokines (e.g., TNF), a number of MC-specific proteases (3, 4), and proteoglycans of serglycin type (5, 6). Although in many cases the exact mechanisms by which MCs contribute in pathological settings are incompletely understood, it is widely recognized that the preformed granule compounds that are released upon MC degranulation are key actors in MC-mediated events (1–3). Hence, the MC secretory granules are of crucial importance for the biological actions of MCs, but despite this notion there is only limited knowledge of the mechanisms that regulate their composition.

Serglycin is composed of a small (∼17 kDa) core protein to which negatively charged glycosaminoglycans (GAGs) are attached, with the GAGs attached being either heparin/heparan sulfate (HS) or chondroitin sulfate (CS) type. Heparin/HS is built up by repeating hexuronic acid (HexA)-glucosamine (GlcN) disaccharide units, where the HexA residues can be either glucuronic acid or iduronic acid and where the GlcN residues can be either N-sulfated or N-acetylated. Additionally, the HexA residues may be 2-O-sulfated and the GlcN units can carry 6-O- and, rarely, 3-O-sulfate groups (7). CS chains are built up by repeating HexA-N-acetylgalactosamine (GalNAc) disaccharide units, in which the HexA residues can be 2-O-sulfated and the GalNAc residues may carry 4-O- and/or 6-O-sulfate groups (8). Importantly, both heparin/HS and CS exhibit a large extent of heterogeneity, both with regard to chain length and to the extent and pattern of sulfation (7, 8).

In previous studies we have shown that serglycin proteoglycan promotes the storage of a number of positively charged secretory granule–localized MC proteases, including chymases (mouse MC protease [mMCP]-4 and mMCP-5), tryptase (mMCP-6), and carboxypeptidase A3 (CPA3) (3, 4, 9, 10). A likely explanation for these observations is that negatively charged GAG chains attached to the serglycin core protein interact electrostatically with positive charge displayed by its binding partners, that is, the proteases (5, 6). Although these findings have established a role for negatively charged proteoglycans of serglycin type in regulating the storage of granule compounds in MCs, little is known of the dynamics in regulating MC secretory granule composition. For example, one important question is whether there is an interdependence of the...
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/ trypsin) 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−/− (12), whereas the WT C57BL/6 mice lack expression of MMP-6 (11), mMCP-4 being dependent on CPA3 for storage. Hence, at the protein level (13, 19), and we therefore decided to assess these effects in vivo–derived MCs, that is, BMMCs (data not shown). To ascertain that the corresponding protein products were absent, Western blot analysis was carried out. The absence of the corresponding proteases was also verified in multiple MC proteases.

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 all absent in corresponding tissue from the triple KO mice (Fig. 1A).

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 cytospin slides. Samples were fixed with 100% methanol and subsequently stained with toluidine blue solution (0.1% toluidine blue in 0.17 mM NaCl [pH 2]). Samples were rinsed with H2O, dried, and covered with VectorMount 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).

Disaccharide analysis

Samples were prepared as previously described followed by reverse-phase ion-pair (RP-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

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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 all absent in corresponding tissue from the triple KO mice (Fig. 1A). Previous studies have indicated that CPA3 and mMCP-5 show a strong interdependence at the protein level (13, 19), and we therefore decided to assess whether the levels of mMCP-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).
mMCP-4, mMCP-5, mMCP-6, and CPA3, and all of these proteases were either absent (mMCP-4, mMCP-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

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, mMCP-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
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 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).

FIGURE 4. Multiprotease deficiency causes defective granule integrity in MCs. Cytospin slides were prepared from peritoneal cell exudates of WT (A), mMCP-4−/− (B), mMCP-6−/− (C), CPA3−/− (D), and multiprotease-deficient (mMCP-4−/−; mMCP-6−/−; CPA3−/−) (E) mice. (F and G) Sections were prepared from ear skin tissue of WT (F) and multiprotease-deficient (G) mice. Cytospin slides and sections were stained with toluidine blue. In (F) and (G), MCs are indicated by arrows. Insets display enlarged images of individual MCs. Note the defective staining of MCs lacking multiple proteases. Original magnification ×400.
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 [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method (18) gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains. In agreement with the [$^{35}$S]sulfate labeling approach, the enzymatic/RPIP-HPLC method also gives detailed information of the disaccharide composition of the respective GAG chains.

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(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 (KO) mice and analyzed their contents and structure of heparin/HS. As seen in Fig. 7F, CS constituted the main type of GAG in whole ear skin tissue.
from both WT and multi-MC protease-deficient mice, accounting for ~91 and ~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). 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 multi-MC protease-deficient mouse strain additionally lack mMCP-5 at the protein level. Altogether, the generated mouse strain thus lacks a panel of stored

![Image of Figure 7](http://www.jimmunol.org/DownloadedFrom/6PROTEASE-DEFICIENT_MAST CELLS.png)

**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 multi-MC protease-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 multi-MC protease-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 multi-MC protease-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; 6S, HexA-GlcNAc(6-O-SO_3); 6S, HexA-GlcNAc(6-O-SO_3); 2S, HexA(2-O-SO_3)-GlcNAc; NS6S, HexA-GlcNSO_3(6-O-SO_3); NS2S, HexA(2-O-SO_3)-GlcNSO_3; 2S6S, HexA(2-O-SO_3)-GlcNAc(6-O-SO_3); NS6S2S, HexA(2-O-SO_3)-GlcNSO_3(6-O-SO_3). CS disaccharides (C, F): OS CS/Hya, nonsulfated disaccharides of CS or hyaluronan origin; 4S, HexA-GalNAc(4-O-SO_3); 6S, HexA-GalNAc(6-O-SO_3); 2S, HexA(2-O-SO_3)-GalNAc; 6S4S, HexA-GalNAc(4,6-di-O-SO_3); 4S2S, HexA(2-O-SO_3)-GalNAc(4-O-SO_3); 6S2S, HexA(2-O-SO_3)-GalNAc(6-O-SO_3); 6S4S2S, HexA(2-O-SO_3)-GalNAc(4,6-di-O-SO_3). 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-deacetylasparagine/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 through 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-/monosulfated 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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**Disclosures**

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