Serglycin proteoglycan is required for secretory granule integrity in mucosal mast cells

Tiago BRAGA*, Mirjana GRUJIC*, Agneta LUKINIUS†, Lars HELLMAN‡, Magnus ÅBRINK* and Gunnar PEJLER*¹

*Swedish University of Agricultural Sciences, Department of Molecular Biosciences, The Biomedical Centre, Box 575, 751 23 Uppsala, Sweden, †Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, 751 85 Uppsala, Sweden, and ‡Department of Cell and Molecular Biology, Uppsala University, The Biomedical Centre, Box 596, SE-751 24 Uppsala, Sweden

SG (serglycin) PGs (proteoglycans) are strongly implicated in the assembly of MC (mast cell) granules. However, this notion has mainly been on the basis of studies of MCs of the connective tissue subtype, whereas the role of SG PG in mucosal MCs has not been explored. In the present study, we have addressed the latter issue by using mice with an inactivated SG gene. Bone marrow cells were differentiated *in vitro* into the mucosal MC phenotype, expressing the markers mMCP (mouse MC protease) -1 and -2. Biosynthetic labelling experiments performed on these cells revealed an ~80 % reduction of ³⁵SO₄²⁻ incorporation into PGs recovered from SG^{-/-} cells as compared with SG^{+/+} counterparts, indicating that SG is the dominating cell-associated PG of mucosal MCs. Moreover, the absence of SG led to defective meta-chromatic staining of mucosal MCs, both *in vivo* and in the *in vitro*-derived mucosal MCs. Ultrastructural analysis showed

that granules were present in similar numbers in SG^{+/+} and SG^{-/-} cells, but that their morphology was markedly affected by the absence of SG, e.g. with electron-dense core formation only seen in SG^{+/+} granules. Analysis of the MC-specific proteases showed that mMCP-1 and mMCP-7 were completely independent of SG for storage, whereas mMCP-2 showed a partial dependence. In contrast, mMCP-4 and -6, and carboxypeptidase A were strongly dependent on SG for storage. Together, our data indicate that SG PG is of crucial importance for assembly of mature mucosal MC granules, but that the specific dependence on SG for storage varies between individual granule constituents.

Key words: chymase, mast cell, protease, proteoglycan, serglycin, tryptase.

INTRODUCTION

Due to their location close to the host/environment surface, MCs (mast cells) play a key role in the first line of defence against many invaders. For example, MCs have been shown to be important for combating infections caused by intestinal parasites [1–3] and bacteria [4,5]. In addition, it is now widely recognized that MCs contribute to adaptive immune responses in a variety of settings [6]. Although the specific phenotype of a MC population may vary to a large extent, dependent on exact tissue location and on exposure to stimuli, two major subclasses of MCs can be distinguished: MMCs (mucosal MCs) and CTMCs (connective tissue MCs) [7]. CTMCs are present, for example, in the skin, peritoneal cavity and intestinal submucosa, whereas MMCs are located in mucous membranes and in the intestinal lamina propria [7]. Previous studies suggest that these two subtypes have different functions. For example, MMCs, but not CTMCs, increase in number when a parasite infection occurs [8,9]. Moreover, MMCs are, in contrast with CTMCs, dependent on T-cells [10–12].

The different functional properties and tissue locations of CTMCs and MMCs are reflected by clearly distinct contents of secretory granule components, in particular as regards specific composition of proteases and PGs (proteoglycans) [13–15]. While the CTMC subclass is known to contain MC CPA (carboxypeptidase A), the chymases mMCP (mouse mast cell protease)-4 and mMCP-5, as well as the tryptases mMCP-6 and mMCP-7 [16–18], MMCs are instead characterized by a predominant expression of the chymases mMCP-1 and mMCP-2 [19,20]. Importantly,

however, the exact protease expression profile is dynamic, as shown by the fact that MCs can alter their expression of proteases over the course of a parasitic infection [21,22]. Moreover, *in vitro* exposure of BMMCs (bone-marrow-derived MCs) to different cytokines will affect the specific profile of protease expression [23,24]. In addition to differential expression of protease genes, the different MC subclasses display distinct PG contents. Early studies provided evidence that rat peritoneal MCs predominantly contained PGs of heparin type [25], whereas intestinal MMCs instead contain predominantly CS (chondroitin sulfate) of the ‘oversulfated’ CS-E type (containing both 4-O- and 6-O-sulfated GalNAc units) [26].

It has been assumed for a long time that the sulfated, and thereby negatively charged, MC PGs mediate storage of various basically charged secretory granule components [7]. Strong support for this notion came when two groups showed simultaneously that interference with the heparin biosynthesis in CTMCs, by targeting the gene for NDST-2 (N-deacetylase/N-sulfotransferase-2), caused dramatic defects in their storage of secretory granule proteases [27,28]. In contrast, BMMCs were affected only to a limited extent by the NDST-2 knockout, most likely because of their preferential synthesis of CS rather than heparin [28,29]. Noteworthy, however, is that the small amounts of heparin present in BMMCs appear to be essential for the processing of CPA and storage of mMCP-5, although granular morphology and mMCP-6 storage are not affected [28,29]. Moreover, because the MMCs do not contain heparin, they were not affected by the NDST-2 knockout, and the possible role of

Abbreviations used: BMMC, bone-marrow-derived mast cell; CPA, carboxypeptidase A; CS, chondroitin sulfate; CTMC, connective tissue mast cell; GAG, glycosaminoglycan; HPRT, hypoxanthine–guanine phosphoribosyltransferase; IL, interleukin; MC, mast cell; MMC, mucosal mast cell; mMCP, mouse mast cell protease; MMP, matrix metalloproteinase; NDST-2, N-deacetylase/N-sulfotransferase 2; PG, proteoglycan; RT, reverse transcription; SCF, stem cell factor; SG, serglycin; TEM, transmission electron microscopy; TGF, transforming growth factor.

¹ To whom correspondence should be addressed (email Gunnar.Pejler@bmc.uu.se).

PGs in secretory granule homeostasis in MMCs has therefore remained an open question.

In the PGs of both CTMCs and MMCs, it has been believed that the respective GAG (glycosaminoglycan) chains (CS or heparin) are attached to the same protein 'core', namely SG (serglycin). In a previous study, we targeted the SG gene and found, indeed, that CTMC granules showed similar, although more profound, defects to those previously observed after interference with NDST-2 [30]. In addition, we found that bone marrow cells differentiated into a CTMC-like phenotype were affected to a similar extent as *in vivo*-derived CTMCs [30]. In the present study, we used the SG^{-/-} mouse strain to specifically address the role of SG PG in MMCs. We show that SG PG is the dominating cell-associated PG in MMCs and that it is needed for assembly of mature secretory granules. However, the various MC proteases exhibit differential dependence on SG for storage, with mMCP-1, -2 and -7 being less dependent on SG for storage than mMCP-4, -5 and -6 and CPA.

EXPERIMENTAL

MMCs

In vitro-derived MMC-like cells were obtained by culturing femur and tibia bone marrow cells from SG^{+/+} and SG^{-/-} littermates as described previously [30]. For the majority of experiments, mice backcrossed to the C57BL/6 genetic background were used. For analysis of mMCP-7, mice were backcrossed for ten generations to the DBA-1 background. The cell cultures were maintained at a concentration of $\sim 0.5 \times 10^6$ cells/ml in medium supplemented with 1 ng/ml recombinant human TGF- β (transforming growth factor β) (Peprotech), 1 ng/ml mouse IL (interleukin) -3 (Peprotech), 5 ng/ml mouse IL-9 (Peprotech) and 50 ng/ml mouse SCF (stem cell factor) (Peprotech), according to a previously described protocol [31]. Medium and cytokines were changed every 2–3 days, for 14 days.

Staining procedures

Cells were collected on to cytospin slides and were stained with May–Grünwald–Giemsa [30]. The chloroacetate assay was performed on cytospin slides as follows: 0.5 g of pararosaniline base (Sigma) was dissolved in 20 ml of water and 2.5 ml of HCl during heating, followed by filtering. To 0.1 ml of this solution, 0.1 ml of 4% sodium nitrite (Merck) was then added to yield nitrosylated pararosaniline. Solution A (2.73 g of Na₂HPO₄ in 250 ml of water) and solution B (2.27 g of KH₂PO₄ in 250 ml of water) were prepared and mixed (41 ml of solution A and 9 ml of solution B) to prepare solution C. Cytospin slides were placed in a solution containing 10 mg of naphthol-ASD chloroacetate (Sigma), 1 ml of *N,N*-dimethylformamide (Sigma), 35 ml of solution C and 0.2 ml of nitrosylated pararosaniline solution (contents were combined in the order given, followed by filtering) for 40 min, after which they were washed in water for 5 min and air dried.

Small intestines were excised and fixed in either 4% (w/v) paraformaldehyde or Carnoy's fixative followed by embedding in paraffin, sectioning and staining with acidic Toluidine Blue.

Extraction of intestinal protein

Small intestines were excised from SG^{+/+} and SG^{-/-} mice, washed with PBS and stored at -70°C . After defrosting, tissues were first homogenized (using a PT1200 Polytron device; Kinematica AG) in 1.5 ml of low-salt PBS (pH 7.4; 0.14 M NaCl) containing 1 mM EDTA, 1% (v/v) Triton X-100,

0.14 M NaCl and a protease inhibitor cocktail {final concentrations: 2.3 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 10 mM EDTA, 0.2 mM bestatin, 0.03 mM pepstatin A and 0.03 mM E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]} (Sigma–Aldrich), followed by centrifugation at 17000 g for 20 min at 4°C . Supernatants were collected and pellets were homogenized in 500 μl of high-salt PBS (pH 7.4; 2 M NaCl) containing 1 mM EDTA, 1% (v/v) Triton X-100, 2 M NaCl and protease inhibitor cocktail as above. Samples were rotated for 30 min at 4°C and centrifuged at 13400 rev./min for 20 min at 4°C , and the resulting supernatants were collected.

Immunoblot analysis

Extracts from small intestines or BMMCs were analysed for the presence of mMCP-1, -2, -4, -5 and -6 and CPA by utilizing specific rabbit antisera, according to the methods described previously [30]. Immunoblot analysis for mMCP-7 was carried out with a rat antiserum towards recombinant mMCP-7.

Zymography

Samples from MMC-conditioned media or cell extracts were analysed by gelatin zymography as described previously [32]. MMP (matrix metalloproteinase) -2 and -9 were identified by comparison of their migration velocities with those of standard human MMP-2 and -9 present in conditioned medium from the HT-1080 cell line.

GAG isolation and analysis

BMMCs (20×10^6 cells) were biosynthetically labelled overnight with 0.32 mCi of carrier-free $^{35}\text{SO}_4^{2-}$ (Amersham Biosciences). Cells were pelleted by centrifugation at 300 g for 10 min, followed by the purification of cell-associated and secreted PGs as described previously [30]. Samples (10000 c.p.m.) of isolated ^{35}S -labelled GAGs were mixed with 0.5 mg of CS-A (containing 4-O-sulfated GalNAc residues; Sigma) and 0.45 mg of pig mucosal heparin (a gift from Ulf Lindahl, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden) and were analysed by anion-exchange chromatography on DEAE-Sephacel [30]. To release GAG chains from intact PGs, samples of purified PGs were treated with 0.5 M NaOH overnight, followed by neutralization with HCl and subsequent analysis on a Superose 6 column (Amersham Biosciences) eluted with 0.2 M NH₄HCO₃ at 0.25 ml/min. Fractions (0.5 ml) were collected and were analysed for ^{35}S -radioactivity by scintillation counting.

To determine the CS content, samples of labelled GAGs were treated with 0.05 unit of chondroitinase ABC (Seikagaku) in 20 μl of 0.05 M Tris/HCl, pH 8.0, 0.3 M NaCl and 1 mg/ml BSA, and incubated at 37°C for 15 h. Samples were analysed by gel chromatography on a Sephadex G-50 column (0.5 cm $\times$ 90 cm) (Amersham Biosciences), eluted with 0.2 M NH₄HCO₃. Fractions of 0.5 ml were collected and analysed for ^{35}S -radioactivity. For papain digestion, 5000 c.p.m. were evaporated to dryness and resuspended in 300 μl of 50 mM sodium acetate, pH 5.5, and 2 M NaCl. To each sample, 25 μl of 0.5 M cysteinium chloride, 25 μl of 0.5 M EDTA and 50 units of papain (Sigma) were added. Samples were incubated at 56°C for 18 h, with shaking. Reactions were stopped by heating for 5 min at 100°C , followed by centrifugation at 600 g for 5 min.

RT (reverse transcription)–PCR analysis

Total RNA was isolated using Total RNA Isolation NucleoSpin RNA II kit (Machery-Nagel) using the protocol provided by the manufacturer. For RT–PCR, Superscript II (Invitrogen) and

specific reverse primers were used in order to produce single-stranded cDNA. HPRT (hypoxanthine–guanine phosphoribosyl-transferase) was used as a housekeeping control. The primers used, except the primers for mMCP-7, were as described previously [30]. The primers for mMCP-7 were 5'-TGCCAATGAC-ACCTACTGGATGC-3' (forward) and 5'-CAGGAGTCATGT-CCTTCATTCC-3' (reverse), yielding a 512 bp product. The RT-PCR conditions were 35 cycles of 95 °C for 2 min, 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, then 72 °C for 10 min. PCR products were sequenced to confirm their identity with the proposed target sequence.

TEM (transmission electron microscopy)

Cells were fixed in 2 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, supplemented with 0.1 M sucrose for 10 h. Next, cells were post-fixed in 1 % (w/v) osmium tetroxide in the same buffer for 90 min, dehydrated in graded series of ethanol and embedded in the epoxy plastic Agar 100 (Agar Aids). Ultrathin sections were placed on Formvar-coated copper grids and contrasted with 2 % (w/v) uranyl acetate and Reynolds lead citrate. Analysis was performed in a Hitachi Electron Microscope at 75 kV.

RESULTS

To study the role of SG PG in MMCs, we recovered bone marrow cells from SG^{+/+} and SG^{-/-} animals and cultured them in medium containing TGF- β , SCF, IL-3 and IL-9, conditions that were shown previously to induce a homogeneous MMC-like phenotype with regard to protease content [31]. After 2 weeks of culture, SG^{+/+} and SG^{-/-} cells were recovered and their morphological and biochemical characteristics were compared. As shown in Figure 1(A) (left-hand panels), SG^{+/+} *in vitro*-derived MMC-like cells showed strong metachromatic granular staining with May–Grünwald–Giemsa, a typical feature of mature MCs. In contrast, SG^{-/-} cells were not stained. Instead, May–Grünwald–Giemsa-negative 'empty'-appearing vesicles were clearly visible (Figure 1A, right-hand panels); such vesicles were also visible, although to a lesser extent, in SG^{+/+} MCs. As is evident from Figure 1(A) (upper panels), cells of SG^{+/+} and SG^{-/-} genotypes were similar in terms of shape and size, indicating that the cell culture conditions gave rise to homogeneous cell populations. In order to address the *in vivo* relevance of these findings, tissue sections of small intestines were stained with Toluidine Blue. As shown in Figure 1(B), rare metachromatically stained cells were observed both in the mucosal (left-hand panel) and in the submucosal (right-hand panel) region of the small intestine from SG^{+/+} mice, indicating the presence of MMCs and CTMCs respectively. In contrast, Toluidine Blue-positive cells were undetectable in both the intestinal mucosa and submucosa of SG^{-/-} animals. These results indicate that the absence of SG PG causes dramatic defects in staining of MMCs with cationic dyes, both *in vivo* and in *in vitro*-derived MMC-like cells.

To analyse the importance of SG PGs for secretory granule organization in more detail, SG^{+/+} and SG^{-/-} cells were analysed by TEM. TEM analysis of both SG^{+/+} and SG^{-/-} cells revealed normal ultrastructure of membranes, mitochondria and other features, indicating optimal culturing conditions and preparation procedure. Proliferating cells were frequently seen in both cell cultures. Granules in SG^{+/+} cells were spherical and displayed diversity in the arrangement of substructures in the interior of each granule. Three main types of granules could be distinguished in SG^{+/+} cells: (i) granules containing small vesicles, most likely exosomes [33], that were filled with electron-dense material;

Figure 1 Morphology and phenotype of mucosal-type BMMCs

(A) Cytospin slides were prepared from bone marrow cells recovered from SG^{+/+} and SG^{-/-} mice after 15 days of culture in medium containing TGF- β /IL-3/IL-9/SCF. Slides were stained with May–Grünwald–Giemsa. Note that SG^{-/-} cells do not show metachromatic staining of their granules, and note also the May–Grünwald–Giemsa-negative vesicles (arrows) present in their cytoplasm, while SG^{+/+} cells display metachromatically stained granules. (B) Sections were prepared from small intestines of SG^{+/+} and SG^{-/-} animals and were stained with Toluidine Blue. Rare Toluidine Blue-positive cells were detected in the mucosal region (left-hand panel; arrow) and in the submucosa (right-hand panel; arrow) of SG^{+/+} mice. No Toluidine Blue-positive cells were found in sections prepared from SG^{-/-} animals (results not shown). (C) Chloroacetate esterase activity in SG^{+/+} (left-hand panel) and SG^{-/-} (right-hand panel) *in vitro*-derived MMC-like cells. Note that cells of both the SG^{+/+} and the SG^{-/-} genotypes show strong chloroacetate esterase activity.

(ii) granules containing small vesicles together with a grainy substance of moderate to high electron density; and (iii) granules containing grainy substance of high electron density, without other substructural elements (Figures 2A–2C). Generally, SG^{+/+} granules were divided into distinct compartments of low electron density (electron-translucent) and electron-dense cores (Figures 2A–2C). Notably, the granule types identified in the *in vitro*-derived MMCs resemble those identified previously in BMMCs developed in the presence of conditioned medium from Con A (concanavalin A)-stimulated splenocytes [34]. The granule morphology of SG^{-/-} cells was markedly different. First, granules of SG^{-/-} cells were slightly smaller than those in SG^{+/+} cells and often had an ovoid shape. Furthermore, most of the SG^{-/-} granules lacked the intragranular vesicles (exosomes) that were often present in SG^{+/+} counterparts, although solitary granules containing such vesicles could be observed. Another striking difference between SG^{+/+} and SG^{-/-} granules was that the granules of SG^{-/-} cells lacked the subdivision into electron-dense and translucent regions that was typical of the SG^{+/+} granules. Instead, SG^{-/-} granules were usually completely filled with material of intermediate to relatively high electron density. Moreover, the contents of the SG^{-/-} granules were of a more amorphous character and lacked the typical formation of grainy

Figure 2 TEM of $SG^{+/+}$ and $SG^{-/-}$ cells showing granules

(A) Representative micrograph from $SG^{+/+}$ *in vitro*-derived MMC-like cells demonstrating granules containing (i) many small electron dense vesicles (arrow), (ii) small vesicles together with grainy substance (arrowhead) or (iii) mainly grainy substance (broken arrow). Original magnification $\times 20\,000$; scale bar, $0.5\ \mu\text{m}$. (B) High magnification clearly revealed the grainy structure and the distinction of electron-lucent/electron-dense parts of the granules in the $SG^{+/+}$ cells. Original magnification $\times 75\,000$; scale bar, $0.1\ \mu\text{m}$. (C) A closer view of the co-existence in $SG^{+/+}$ cells of granules filled with small vesicles (arrows) and granules with an electron-dense core of a grainy material (arrowhead). Original magnification $\times 42\,000$; scale bar, $0.1\ \mu\text{m}$. (D) A representative micrograph of a $SG^{-/-}$ cell shows cytoplasm granules (arrows), which are mostly spherical, but some are ovoid. Most of the $SG^{-/-}$ granules were completely filled with an amorphous matrix of moderate electron density, although solitary granules contained small vesicles harbouring material of varying electron density (arrowhead). Original magnification $\times 18\,000$; scale bar, $0.5\ \mu\text{m}$. (E) The amorphous matrix of the $SG^{-/-}$ granules is obvious at high magnification. Original magnification $\times 75\,000$; scale bar, $0.1\ \mu\text{m}$. (F) Detail of the cytoplasm co-localization of the typical $SG^{-/-}$ cell granules with amorphous granule matrix (arrows) and granules containing small vesicles filled with material of different electron density (arrowhead). Note the incorporation/budding of a small vesicle (broken arrow). Original magnification $\times 42\,000$; scale bar, $0.1\ \mu\text{m}$.

electron-dense core that was seen in $SG^{+/+}$ granules (Figures 2D–2F). These results are clearly consistent with a role for SG in the granule maturation process, with the amorphous appearance of the $SG^{-/-}$ granules resembling the immature granules seen in other secretory-granule-containing cells, e.g. immature β -cells of the pancreas [35]. Furthermore, the subdivision of $SG^{+/+}$ granules into regions of high and low electron densities is clearly in line

Table 1 Incorporation of $^{35}\text{SO}_4^{2-}$ into PGs from *in vitro*-derived MMC-like cells

Results are expressed as c.p.m. of incorporated radioactivity/ 1×10^6 cells and are representative of three independent experiments.

Source of ^{35}S -labelled PGs	$SG^{+/+}$	$SG^{-/-}$
Secreted PGs	10 000	9000
Cell-associated PGs	107 000	22 000

with the granule morphology seen in more mature pancreatic β -cells [35]. Importantly, the lack of SG PG did not affect the cellular morphology, apart from its effects on secretory granules.

Next, experiments were undertaken to characterize the GAGs synthesized by the *in vitro*-derived MMC-like cells and to study the relative contribution of GAGs present in PGs of SG type to the total synthesis of GAGs. *In vitro*-derived MMC-like cells were biosynthetically labelled with $^{35}\text{SO}_4^{2-}$, and labelled GAGs were subsequently recovered both from the conditioned media (secreted GAGs) and from the cell layer. Quantification of the total amount of radioactivity in each pool showed that the cell layer of $SG^{+/+}$ cells contained ~ 5 -fold higher amounts of labelled material than the corresponding pool recovered from $SG^{-/-}$ cells (Table 1), indicating that SG is the main cell-associated PG in *in vitro*-derived MMC-like cells. In contrast, similar amounts of labelled GAGs were recovered in conditioned media from $SG^{+/+}$ and $SG^{-/-}$ cells, indicating that SG is not secreted to a major extent by the *in vitro*-derived MMC-like cells (Table 1).

To characterize further the nature of the GAGs, samples of ^{35}S -labelled material were subjected to anion-exchange chromatography along with internal standards of CS-A (low degree of sulfation; ~ 1 sulfate group/disaccharide) and heparin (highly sulfated; ~ 2.5 sulfate groups/disaccharide unit). As shown in Figures 3(A) and 3(D) respectively, most of the cell-associated and secreted material from $SG^{+/+}$ cells showed co-elution with standard CS-A, indicating a relatively low degree of sulfation. However, a small shoulder of material was eluted slightly later in the salt gradient. Cell-associated (Figure 3B) and secreted (results not shown) material from $SG^{-/-}$ cells displayed a similar pattern of charge density to the corresponding samples from $SG^{+/+}$ counterparts, indicating that ^{35}S -labelled macromolecules of SG and non-SG species have similar anionic charge densities. For a comparison, ^{35}S -labelled material from $SG^{+/+}$ and $SG^{-/-}$ bone marrow cells that had been differentiated *in vitro* into a more CTMC-like phenotype [30] were analysed. Interestingly, GAGs derived from $SG^{+/+}$ CTMC-like cells were eluted markedly later in the salt gradient as compared with the corresponding material derived from *in vitro*-derived MMC-like cells, indicating a substantially higher charge density (Figure 3C). Furthermore, GAGs from $SG^{-/-}$ CTMC-like cells were eluted clearly before the corresponding material recovered from $SG^{+/+}$ counterparts (Figure 3C). Together, these results indicate that SG PGs in CTMC-like cells have markedly higher charge densities than non-SG PGs, whereas SG and non-SG species in *in vitro*-derived MMC-like cells display similar anionic charge. Furthermore, these findings introduce the possibility that the cytokine mixture that preferentially induces a MMC-like phenotype directs the GAG synthesis, by unknown mechanisms, towards the assembly of lower-sulfated species as compared with CTMC GAGs. Since SG PG can accommodate GAGs of either heparin or CS type as side chains, it was important to investigate which class of GAG was attached to the SG core protein. Chondroitinase ABC digestion resulted in depolymerization of most of the labelled GAGs

Figure 3 Analysis and characterization of ^{35}S -labelled GAGs

$\text{SG}^{+/+}$ and $\text{SG}^{-/-}$ BMMCs were biosynthetically labelled with $^{35}\text{SO}_4^{2-}$, followed by isolation of ^{35}S -labelled GAGs from the cell layer and from conditioned media. Samples (10 000 c.p.m.) from the cell fractions of $\text{SG}^{+/+}$ (A; ■) and $\text{SG}^{-/-}$ (B; ■) *in vitro*-derived MMC-like cells, and from the conditioned medium of $\text{SG}^{+/+}$ cells (D; ■) were analysed by anion-exchange chromatography, and the elution positions of the ^{35}S -labelled macromolecules were compared with those of internal standards of unlabelled CS and pig mucosal heparin (HP; CS and HP were detected by the carbazole reaction; solid grey line). Note the co-elution of GAGs from *in vitro*-derived MMC-like cells with standard CS. (C) GAGs isolated from $\text{SG}^{+/+}$ (■) and $\text{SG}^{-/-}$ (◇) bone marrow cells (cell layer) that had been cultured under conditions that result in MCs of a CTMC-like phenotype [30] were analysed by anion-exchange chromatography along with internal standards of CS and heparin. Note the co-elution of GAGs from $\text{SG}^{+/+}$ cells with standard heparin and the markedly lower charge density of ^{35}S -labelled macromolecules from $\text{SG}^{-/-}$ cells. (E) ^{35}S -labelled GAGs recovered from $\text{SG}^{+/+}$ (□) or $\text{SG}^{-/-}$ (◆) *in vitro*-derived MMC-like cells were analysed on a Sephadex G-50 column, either before (solid line) or after (broken line) digestion with chondroitinase ABC. (F) Purified ^{35}S -labelled macromolecules recovered from the cell layer of $\text{SG}^{+/+}$ (□; solid line) or $\text{SG}^{-/-}$ cells (▲; solid line) were analysed by gel filtration on a Superose 6 column eluted with 0.2 M NH_4HCO_3 . Samples of $\text{SG}^{+/+}$ (□; dashed line) and $\text{SG}^{-/-}$ (◆; broken line) were also analysed after release of free GAG chains from the PGs by treatment with 0.5 M NaOH.

from $\text{SG}^{+/+}$ cells (Figure 3E). In addition, most of the GAGs from the $\text{SG}^{-/-}$ cells were depolymerized by chondroitinase ABC. Hence, most of the GAGs attached to both SG and non-SG species of PGs in *in vitro*-derived MMC-like cells are of the CS type.

To obtain further information on the macromolecular properties of the PGs, the purified ^{35}S -labelled macromolecules were analysed by size-exclusion chromatography on a Superose 6 column. In addition, material was analysed after treatment with 0.5 M NaOH to release GAG chains from the protein core. As shown in Figure 3(F), most of the labelled material from $\text{SG}^{+/+}$ cells was eluted as a single peak. Radiolabelled material from $\text{SG}^{-/-}$ cells was eluted somewhat later, indicating a lower molecular size. Alkali treatment of ^{35}S -labelled macromolecules from the $\text{SG}^{+/+}$ *in vitro*-derived MMC-like cells caused a marked right shift of the peak, indicating release of GAG chains bound to the core protein (Figure 3F). In contrast, alkali treatment of labelled material from $\text{SG}^{-/-}$ cells caused only a minor right shift of the

peak, indicating that the ^{35}S -labelled GAGs recovered from $\text{SG}^{-/-}$ cells were mainly in the form of free GAG chains. Noteworthy, the GAG chains derived from $\text{SG}^{-/-}$ cells were eluted before the corresponding GAGs present in $\text{SG}^{+/+}$ cells, indicating that GAGs attached to non-SG species have a higher average molecular mass than those connected to the SG protein core.

A typical feature of SG PGs is their resistance to protease treatment, explained by their small protein core that is inaccessible to proteolytic cleavage arising from dense arrangement of GAG chains [25,36]. In order to address whether this is a characteristic of SG PGs from *in vitro*-derived MMC-like cells, radiolabelled PGs were treated with papain, followed by gel-filtration analysis. Indeed, PGs from $\text{SG}^{+/+}$ cells were to a large extent (~70 %) resistant to treatment. Papain treatment of ^{35}S -labelled macromolecules from $\text{SG}^{-/-}$ cells caused a slight right shift, similar to that caused by alkali treatment, consistent with the notion that GAGs from $\text{SG}^{-/-}$ cells were mainly present in the form of free polysaccharide chains (results not shown).

Figure 4 mRNA and protein levels of MC proteases in *in vitro*-derived MMC-like SG^{+/+} and SG^{-/-} cells

(A) mRNA expression of MC proteases. Total RNA was isolated from SG^{+/+} and SG^{-/-} bone marrow cells after 15 days of culture in medium containing TGF- β /IL-3/IL-9/SCF, and was analysed by RT-PCR, using the primers specified in the Experimental section. Expression of the *HPRT* gene was used as housekeeping control. Lane M, molecular-mass markers. (B) Immunoblot analysis of MC proteases in extracts from *in vitro*-derived MMC-like cells. SG^{+/+} and SG^{-/-} BMNCs were cultured for 15 days in medium containing TGF- β /IL-3/IL-9/SCF. Cellular extracts were prepared and were analysed for the presence of the proteases indicated.

MC granules are typically characterized by strong chloroacetate esterase activity, attributable to their high content of chymase activity [37]. In a previous study, we showed that the chloroacetate esterase activity in CTMCs was strongly dependent on the presence of SG PG [30]. Experiments were therefore carried out to investigate whether MMC chloroacetate esterase activity was also dependent on the presence of SG PG. However, in striking contrast with CTMCs, chloroacetate esterase activities were similar in SG^{+/+} and SG^{-/-} *in vitro*-derived MMC-like cells (Figure 1C). Thus the protease(s) responsible for the chloroacetate esterase activity in *in vitro*-derived MMC-like cells does not appear to depend on SG. This implies further that the MMC proteases may display a different degree of SG-dependence as compared with the CTMC proteases. Experiments were therefore performed to compare the protease profiles of SG^{+/+} and SG^{-/-} MMCs, at both the mRNA and protein levels. RT-PCR analysis showed that the *in vitro*-derived MMC-like cells displayed clearly detectable transcripts for the MMC markers mMCP-1 and mMCP-2, indicating that the *in vitro* culture conditions employed indeed gave cells of MMC-like phenotype. Furthermore, transcripts were detected for mMCP-4 and mMCP-5, in agreement with a previous report [31], and for mMCP-6 and CPA (Figure 4A). Importantly, all of these protease genes were expressed in both SG^{+/+} and SG^{-/-} cells, indicating that the lack of SG did not affect the degree or direction of cellular differentiation (Figure 4A).

Next, experiments were carried out to study how the lack of SG affected the proteases at the protein level. Immunoblot analysis of cell extracts from *in vitro*-derived MMC-like cells revealed the presence of mMCP-1 antigen in cells of SG^{+/+} genotype. Interestingly, mMCP-1 protein was present in similar amounts in SG^{-/-} counterparts, indicating that this protease is not dependent on SG PG for storage (Figure 4B). Also, mMCP-2 protein was detected in SG^{+/+} cells, but in contrast with mMCP-1, mMCP-2 levels were somewhat reduced in SG^{-/-} cells, indicating a

Figure 5 Immunoblot analysis of MC proteases in intestinal extracts

Intestinal extracts were prepared from SG^{+/+} (+/+) and SG^{-/-} (-/-) mice by a two-step procedure, first with a low-salt (LS) buffer and subsequently with a high-salt (HS) buffer. Low- and high-salt extracts were analysed for the presence of the indicated proteases.

partial dependence on SG for storage. A much more pronounced dependence on SG PG for storage was seen for mMCP-4, mMCP-6 and CPA, with antigen levels being close to undetectable in cells of SG^{-/-} genotype (Figure 4B). mMCP-5 protein was detected in both SG^{+/+} and SG^{-/-} *in vitro*-derived MMC-like cells, but with a marked decrease of mMCP-5 levels in the absence of SG (Figure 4B).

To test whether these results could be confirmed *in vivo*, small intestines from SG^{+/+} and SG^{-/-} mice were homogenized using a two-step procedure, first with a low-salt buffer and then with a high-salt buffer. Immunoblot analysis of the corresponding extracts reproduced the patterns seen in the *in vitro*-derived MMC-like cells, i.e. mMCP-1 antigen was present in similar amounts in SG^{+/+} and SG^{-/-} tissue, mMCP-2 showed a partial reduction in SG^{-/-} tissue, whereas mMCP-6 and CPA were essentially undetectable in SG^{-/-} small intestine. Interestingly, the different MC proteases were recovered in different extraction pools. mMCP-1 and -2 were both recovered exclusively in the low-salt extract, indicating a low degree of electrostatic interaction with other tissue components (Figure 5). In contrast, a large part of the total mMCP-6 and CPA antigen required high-salt conditions for extraction, indicating tight electrostatic interactions with anionic components of the tissue (Figure 5).

The experiments described above were all performed on mice of the C57BL/6 background. This mouse strain expresses mMCP-1, -2, -4, -5 and -6 and CPA, but lacks the tryptase mMCP-7 owing to the presence of a premature stop codon in the mMCP-7 gene [38]. Thus it is not possible to investigate the dependence of mMCP-7 on SG using the C57BL/6 strain. In order to address this issue, we therefore backcrossed the SG^{-/-} strain to the DBA-1 genetic background for ten generations, and then analysed *in vitro*-derived MMC-like cells from this strain as well as ear skin tissue for mMCP-7 expression and dependence on SG for storage. As shown in Figure 6(A), *in vitro*-derived MMC-like cells from SG^{+/+} and SG^{-/-} mice of DBA-1 background showed expression of the mMCP-7 gene as determined by RT-PCR analysis. Moreover, mMCP-7 antigen was clearly detected in cell extracts from SG^{+/+} *in vitro*-derived MMC-like cells from DBA-1 mice as determined by immunoblot analysis, but, as expected, not in extracts obtained from corresponding cells of the C57BL/6 background (Figure 6B). mMCP-7 protein was also detected in extracts from SG^{-/-} cells of the DBA-1 background, with the level of mMCP-7 being similar to that in extracts from SG^{+/+} cells (Figure 6B). This indicates that mMCP-7 storage does not depend on SG. mMCP-7

Figure 6 mRNA and immunoblot analysis of mMCP-7

(A) *In vitro*-derived MMC-like cells were cultured from SG^{+/+} and SG^{-/-} mice of the DBA-1 genetic background. After 4 days of culture, total RNA was isolated and was analysed for mMCP-7 and HPRT (housekeeping control) mRNA expression by RT-PCR. (B) *In vitro*-derived MMC-like cells (taken after 15 days of culture) from SG^{+/+} and SG^{-/-} mice of the DBA-1 background were analysed for mMCP-7 antigen by immunoblot analysis. As a negative control, *in vitro*-derived MMC-like cells derived from C57BL/6 animals were also analysed for mMCP-7 antigen. (C) Ear pinnae were prepared from SG^{+/+} and SG^{-/-} mice of the DBA-1 background and were extracted with a high-salt buffer, followed by immunoblot analysis for mMCP-7 antigen.

antigen was also detected in extracts from ear skin tissue, again with the level of mMCP-7 being similar in extracts from SG^{+/+} and SG^{-/-} mice. The latter indicates that mMCP-7 storage is independent of SG also at the *in vivo* level. For a comparison, SG^{+/+} and SG^{-/-} *in vitro*-derived MMC-like cells from mice of the DBA-1 background were also analysed for mMCP-6 antigen. In accordance with results performed on cells from mice of the C57BL/6 background, mMCP-6 antigen levels were markedly reduced in SG^{-/-} cells (results not shown).

In addition to the MC-specific proteases (see above), MCs are known to express MMPs [39]. Since the potential dependence of the MMPs on PGs for storage or secretion has not been investigated previously, we chose to address this issue. Conditioned media, as well as cell extracts, were collected from SG^{+/+} and SG^{-/-} *in vitro*-derived MMC-like cells (of C57BL/6 background), and were analysed by gelatin zymography. Both MMP-2 and MMP-9 were clearly detected in conditioned medium from SG^{+/+} and SG^{-/-} cells. However, the levels were similar in medium obtained from SG^{+/+} and SG^{-/-} cells, indicating that the expression/secretion of MMP-2 and MMP-9 is not dependent on SG (results not shown). No detectable MMP activity was seen in the corresponding cell extracts, indicating that MMP-2 and -9 are not stored to any large extent in *in vitro*-derived MMC-like cells (results not shown).

DISCUSSION

A classical feature of MCs is their strong metachromatic staining with cationic dyes. These staining properties are dependent on the large amounts of negatively charged GAGs that are present in the MC secretory granules. However, different MC subclasses express different GAG species, i.e. heparin or CS. Moreover, the degree of sulfation of the respective GAG may vary significantly

depending on MC subclass and tissue location. Although this has been recognized for a long time, very little is known regarding the factors that regulate this diversity. In the present study, we have specifically addressed the PGs of MMCs. Detailed studies of this MC subclass have recently been made possible through the identification of cytokine mixtures that specifically promote the differentiation of bone marrow precursors into MCs of the MMC-like phenotype [31], whereas previous protocols have resulted mainly in MCs of a more CTMC-like phenotype [24]. However, it is important to stress that bone-marrow-derived CTMC-like cells differ in many aspects from the CTMCs found *in vivo*. For example, *in vitro*-derived CTMC-like cells synthesize mainly GAGs of the CS type, whereas *in vivo*-derived CTMCs produce predominantly heparin [25,27]. Strikingly, however, the GAGs recovered both from *in vivo*-derived CTMCs and from *in vitro*-derived counterparts display conspicuously high charge density [25,29]. Thus, although the major GAGs of *in vivo*- and *in vitro*-derived CTMCs are of different species, their degree of sulfation is conserved.

In contrast with the CTMC PGs, relatively little has been known concerning the characteristics and function of the PGs present in MMCs. In an early report, it was shown that intestinal MMCs of rats infected with *Nippostrongylus brasiliensis* synthesized large amounts of oversulfated CS [26]. However, the nature of the MMC PG, i.e. to which protein core the CS chains are attached, and the role of the MMC PGs in granule homeostasis have not previously been clarified. In the present study, we have shown that MMCs with an inactive SG gene show an ~80% reduction in the amount of ³⁵S-labelled PGs recovered from the cell layer, indicating strongly that SG is the major cell-associated PG in *in vitro*-derived MMC-like cells. In CTMC-like BMMCs, the lack of SG caused a similar reduction of cell-associated PGs [30], thus suggesting that SG accounts for most of the cellular PGs in both of these MC classes. However, it is important to stress that the lack of SG did not result in a complete lack of cell-associated PGs in either MMC- or CTMC-like BMMCs, indicating that PGs of species other than SG are expressed. To date, the possible expression and cellular location of PG species other than SG in MCs have not been well studied. However, considering that the absence of SG resulted in an essentially complete lack of granular staining with cationic dyes, it is likely that SG accounts for most of the secretory granule PGs. Therefore we may suggest that non-SG PGs expressed by MCs are mostly cell-surface-associated species. In contrast with the effect on cell-associated PGs, the absence of SG did not influence the level of secreted PGs. The most probable explanation for this observation is that the PGs secreted by the *in vitro*-derived MMC-like cells are of non-SG type.

We show here that bone marrow cells cultured with TGF- β /SCF/IL-3/IL-9 synthesize PGs of relatively low charge density. This is in sharp contrast with the PGs synthesized by bone marrow cells that have been driven into a more CTMC-like phenotype. Although both populations of BMMCs synthesize mainly SG PGs with attached CS chains, the latter cell population synthesizes PGs of markedly higher anionic charge density. This indicates that the level of sulfation of a SG PG is highly dependent on the specific cytokine environment. Possibly, the regulation of sulfation density is achieved by altering the levels or activities of the sulfotransferases responsible for introducing the 4-O- and 6-O-sulfate groups into GalNAc residues in CS. However, the mechanisms involved in the regulation of these enzymatic activities in MCs remain to be studied. Nevertheless, the distinct anionic charge densities of the PGs, depending on whether the MCs have been differentiated into a MMC- or CTMC-like phenotype, strongly support the fact that the different culture conditions have resulted in distinct MC subtypes.

Considering the major effects on granule morphology seen in CTMCs following the knockout of either NDST-2 or SG, it may be speculated that SG PG may actually participate in the formation of secretory vesicles. However, the ultrastructural studies presented here clearly show that granules are formed irrespectively of the presence or absence of SG. Importantly, however, the granule morphology differed markedly in SG^{+/+} and SG^{-/-} *in vitro*-derived MMC-like cells. In SG^{+/+} cells, granules contained distinct areas of low and high electron density, with the electron-dense material assembled into a grainy and condensed dense core. In contrast, SG^{-/-} granules lacked this substructural division, with amorphous material occupying the entire granule space. We may therefore suggest that SG PG is not necessary for the intracellular transport of compounds into the granule, but that SG is needed for the condensation of granular constituents. Most likely, the mechanism of condensation involves electrostatic interactions between basically charged domains of the respective granule compounds and the anionic GAGs attached to the SG protein core.

During the studies of CTMCs, it was found that all of the characteristic CTMC secretory granule proteases expressed in the C57BL/6 strain, i.e. mMCP-4, -5 and -6 and CPA, were strongly dependent on SG PG for storage [30]. Importantly, the possible dependence of the MMC proteases on SG for storage has not been investigated previously, and we therefore addressed this issue here. Furthermore, the possible dependence of mMCP-7 on SG for storage has not been investigated previously. The reason for this is that previous studies on the SG knockout strain have been conducted on mice of C57BL/6 background, a strain with a defective mMCP-7 gene [38]. We show that, in contrast with mMCP-4, -5 and -6 and CPA, mMCP-1 and mMCP-7 are independent of SG for storage, whereas mMCP-2 is only partially dependent on SG. The most likely explanation for these different degrees of dependence on SG for storage lies within the cationic properties of the respective proteases. Most of the CTMC proteases (mMCP-4, -5 and CPA) display high net positive charges (Table 2) and it has been shown by molecular modelling that the basic residues of mMCP-4 and -5 are contained within two separate and defined patches [40]. Possibly, this organization of basic charge may promote multivalent and thereby strong interactions with anionic PGs. In contrast, mMCP-1 has a markedly lower net charge (Table 2) and, moreover, mMCP-1 lacks one of the two basic clusters present in mMCP-4 and -5. The partial dependence of mMCP-2 on SG for storage is intriguing. Interestingly, however, the net positive charge of mMCP-2 is intermediate between those of mMCP-1 compared with mMCP-4/mMCP-5/CPA (Table 2), thus providing a likely explanation for its intermediate dependence on SG for storage. In a recent study, we investigated the SG-dependence of various components of the lytic granules of cytotoxic T-lymphocytes for storage [41]. Also in the same study, we found a distinct correlation between exposure of positively charged patches on the molecular surface and dependence on SG for storage. We showed that granzyme B was strongly dependent on SG for storage, and that this may depend on a defined basic patch on the surface of the molecule. In contrast, granzyme A was independent of SG for storage, despite its high net positive charge (Table 2), possibly due to a less defined basic region on the molecular surface [41].

At first view, the strong dependence of mMCP-6 on SG for storage may seem contradictory, considering its net negative charge (Table 2). However, mMCP-6 displays a contiguous cluster of histidine residues at the molecular surface and, at the acidic pH prevalent in secretory granules, these histidine residues are positively charged and engage in tight electrostatic interactions with GAGs [42]. Interestingly, we show in the present study that the second major secretory trypsin in murine MCs, mMCP-7,

Table 2 Net charge of selected proteases and their dependence on SG for storage

Net charges were calculated for mature proteins, i.e. without activation peptide.

Protease	Net charge (pH 7.0)	SG-dependence for storage	Reference
mMCP-1	+4.7	—	Present study
mMCP-2	+7.2	(+)	Present study
mMCP-4	+16.7	+	Present study and [30]
mMCP-5	+13.9	+	Present study and [30]
mMCP-6	-2.2	+	Present study and [30]
mMCP-7	-6.2	—	Present study
CPA	+18.5	+	Present study and [30]
Granzyme A	+15.3	—	[41]
Granzyme B	+18.9	+	[41]

appears to be independent of SG PGs for storage. In line with such a differential dependence of mMCP-6 and -7 on SG PG, it has been shown that mMCP-6 is preferentially retained at the surface of degranulated MCs following passive anaphylaxis, possibly through a firm association with SG PG even after exocytosis, whereas mMCP-7 readily diffuses away from the degranulated MCs into the circulation [43]. In further support for a weaker interaction of mMCP-7 than mMCP-6 with SG PG, the histidine cluster that was shown to mediate interaction between mMCP-6 and heparin is not fully conserved in mMCP-7 [42]. Moreover, a lysine/arginine patch that has been identified on the surface of mMCP-6 by molecular modelling, and has been suggested to engage in the interactions with GAGs, is absent in mMCP-7 [43].

On the basis of the findings of the present study and on those presented previously [30,41], we may suggest a general model for the role of SG PG in the organization of secretory granule. As is evident from the present study, the actual secretory vesicles are assembled independently of SG PG. Moreover, transport of secretory compounds into the granules does not appear to depend on SG PG. However, the maturation of the secretory granule with electron-dense core formation is dependent on SG PG. Certain granular constituents, e.g. mMCP-4 and -6, CPA and granzyme B, are highly dependent on SG for storage [30,41], indicating their presence within the electron-dense cores. In contrast, the lack of SG dependence seen for granzyme A, mMCP-1 and mMCP-7 suggests that these compounds are not included within the electron-dense cores. It is reasonable to assume that compounds not interacting with SG are not retained in the granule to the same extent as the components that are tightly associated with SG. Indeed, it has been shown that mMCP-1 is constitutively secreted from MMCs, clearly consistent with such a notion [44,45]. Taken together, SG may thus regulate the composition of secretory granules by binding to certain compounds, whereas others are independent of SG for storage. The fate of the SG-dependent proteases in cells lacking SG is an intriguing issue. Although this issue is still not completely resolved, recent evidence indicates that the fate may differ between different proteases and may include preferential degradation or exocytosis [46].

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