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SERGLYCIN PROTEOGLYCAN IS REQUIRED FOR SECRETORY GRANULE  
INTEGRITY IN MUCOSAL MAST CELLS

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## ABSTRACT

Serglycin (SG) proteoglycans (PGs) are strongly implicated in the assembly of mast cell (MC) granules. However, this notion is mainly based on studies of MCs of the connective tissue subtype, whereas the role of SG PG in mucosal MCs has not been explored. In this study we have addressed the latter issue by using mice with an inactivated SG gene. Bone marrow cells were *in vitro* differentiated into mucosal MC phenotype, expressing the markers mouse MC protease 1 (mMCP-1) and -2. Biosynthetic labelling experiments performed on these cells revealed an ~80% reduction of  $^{35}\text{SO}_4^{2-}$  incorporation into PGs recovered from  $\text{SG}^{-/-}$  cells as compared to  $\text{SG}^{+/+}$  counterparts, indicating that SG is the dominating cell-associated PG of mucosal MCs. Moreover, the absence of SG led to defective metachromatic 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  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  cells but that their morphology was markedly affected by the absence of SG, e.g. with electron dense core formation only seen in  $\text{SG}^{+/+}$  granules. Analysis of the MC-specific proteases showed that mMCP-1 and mMCP-7 were completely independent on SG for storage, whereas mMCP-2 showed a partial dependence. In contrast, mMCP-4, -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.

## INTRODUCTION

Due to their location close to the host/environment surface, mast cells (MCs)<sup>1)</sup> play a key role in the first line defence towards 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: mucosal MCs (MMCs) and connective tissue MCs (CTMCs)[7]. CTMCs are, e.g., present 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 to 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 proteoglycans (PGs) [13-15]. While the CTMC subclass is known to contain MC carboxypeptidase A (CPA), the chymases mouse mast cell protease-4 (mMCP-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 that MCs can alter their expression of proteases during the course of a parasite infection [21, 22]. Moreover, *in vitro* exposure of bone marrow-derived MCs (BMMCs) 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 of the “oversulphated” CS-E type (containing both 4-O- and 6-O-sulphated GalNAc units) [26].

It has been assumed for as long time that the sulphated, 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 N-deacetylase/N-sulfotransferase-2 (NDST-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 due to 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 is 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 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 glycosaminoglycan (GAG) chains (CS or heparin) are attached to the same protein “core”, namely serglycin (SG). In a recent study we targeted the SG gene and found, indeed, that CTMC granules showed similar, although more profound, defects as 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]. Here we used the SG<sup>-/-</sup> 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 granule. 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, 6 and CPA.

## EXPERIMENTAL

*MMCs.* *In vitro*-derived MMC-like cells were obtained by culturing femur and tibia bone marrow cells from SG<sup>+/+</sup> and SG<sup>-/-</sup> 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 10 generations to DBA-1 background. The cell cultures were maintained at a concentration of  $\sim 0.5 \times 10^6$  cells/mL in medium supplemented with recombinant human TGF- $\beta$  (1 ng/mL; Peprotech, London, UK), mouse IL-3 (1 ng/mL; Peprotech), mouse IL-9 (5ng/mL; Peprotech) and mouse stem cell factor (SCF; 50 ng/mL; 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 onto cytopsin slides and were stained with May Grünwald/Giemsa [30]. The chloroacetate assay was performed on cytopsin slides as follows: 0.5 g of pararosaniline base (Sigma) were dissolved in 20 mL H<sub>2</sub>O and 2.5 mL of HCl during heating, followed by filtering. To 0.1 mL of this solution was then added 0.1 mL of 4% sodium nitrite (Merck) to yield nitrosylated pararosanilin. Solution A (2.73 g Na<sub>2</sub>HPO<sub>4</sub> in 250 mL H<sub>2</sub>O) and solution B (2.27 g KH<sub>2</sub>PO<sub>4</sub> in 250 mL H<sub>2</sub>O) were prepared and mixed (41 mL A and 9 mL B) to prepare solution C. Cytopsin slides were placed in a solution containing 10 mg Naphtol-ASD chloroacetate (Sigma), 1 mL N,N-dimethylformamide (Sigma), 35 mL solution C and 0.2 mL nitrosylated pararosanilin solution (contents were combined in the order given, followed by filtering) for 40 min, after which they were washed in H<sub>2</sub>O for 5 minutes and air dried.

Small intestines were excised and fixed in either 4% 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<sup>+/+</sup> and SG<sup>-/-</sup> mice, washed with PBS and stored at -70°C. After defrosting, tissues were first homogenized (using a PT1200 Polytron device; Kinematica AG; Lucerne, Switzerland) in 1.5 mL of low salt PBS buffer (pH 7.4; 0.14 M NaCl) containing 1 mM EDTA, 1% Triton X-100, 0.14 M NaCl and a protease inhibitor cocktail (final concentrations: 2.3 mM AEBSF, 10 mM EDTA, 0.2 mM Bestatin, 0.03 mM pepstatin A, 0.03 mM E-64) (Sigma-Aldrich), followed by centrifugation (13,400 rpm, 4°C, 20 min). Supernatants were collected and pellets were homogenized in a high salt PBS buffer (pH 7.4; 2 M NaCl) containing 1 mM EDTA, 1% Triton X-100, 2 M NaCl and protease inhibitor cocktail as above. Samples were rotated for 30 min. at 4°C, centrifuged (13,400 rpm, 4°C, 20 min) and the resulting supernatants were collected.

*Immunoblot analysis.* Extracts from small intestines or bone marrow-derived MMCs were analyzed for the presence of mMCP-1, -2, -4, -5, -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 analyzed by gelatin zymography as described elsewhere [32]. Matrix metalloprotease 2 (MMP-2) and MMP-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.* Bone marrow-derived MMCs ( $20 \times 10^6$  cells) were biosynthetically labelled overnight with 0.32 mCi of carrier-free  $^{35}\text{S}$ -sulphate (Amersham Biosciences). Cells were pelleted by centrifugation, followed by the purification of cell-associated and secreted PGs as described previously [30]. Samples (10,000 cpm) of isolated  $^{35}\text{S}$ -labelled GAGs were mixed with 0.5 mg of CS-A (containing 4-O-sulphated GalNAc residues; Sigma) and 0.45 mg of pig mucosal heparin (gift from Ulf Lindahl, Uppsala University, Sweden) and were analyzed 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 over night, followed by neutralization with HCl and subsequent analysis on a Superose 6 column (Amersham Biosciences, Uppsala, Sweden) eluted with 0.2 M  $\text{NH}_4\text{HCO}_3$  at 0.25 ml/min. Fractions (0.5 ml) were collected and were analyzed for  $^{35}\text{S}$ -radioactivity by scintillation counting.

To determine the CS content, samples of labelled GAGs were treated with 0.05 units of chondroitinase ABC (Seikagaku, Tokyo, Japan) in 20  $\mu\text{L}$  of 0.05 M Tris-HCl (pH 8.0)/0.3 M NaCl/1 mg/mL bovine serum albumin, and incubated at  $37^\circ\text{C}$  for 15 h. Samples were analyzed by gel chromatography on a Sephadex G-50 column (0.5 x 90 cm) (Amersham Biosciences), eluted with 0.2 M  $\text{NH}_4\text{HCO}_3$ . 0.5 mL fractions were collected and analyzed for  $^{35}\text{S}$ -radioactivity. For papain digestion, 5000 cpm were evaporated to dryness and resuspended in 300  $\mu\text{L}$  of 50 mM sodium acetate (pH 5.5)/ 2M NaCl. To each sample, 25  $\mu\text{L}$  0.5M cysteinium chloride, 25 $\mu\text{L}$  0.5M EDTA and 50 U papain (Sigma) were added. Samples were incubated for 18h, at  $56^\circ\text{C}$  with shaking. Reactions were stopped by heating for 5 min. at  $100^\circ\text{C}$ , followed by centrifugation (5 min, 600 g).

*RT-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. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as a house keeping control. The primers used, except the primers for mMCP-7, were as described previously [30]. The primers for mMCP-7 were: 5'-TGC CAA TGA CAC CTA CTG GAT GC- 3' (forward) and 5'-CAG GAG TCA TGT CCT TCA TTC C- 3' (reverse; yielding a 512 bp product). RT-PCR was performed using the following steps: 95°C (2 min), 95°C (30 s), 60°C (30 s), 72°C (1 min), 35 times to step 2, 72°C (10 min). PCR products were sequenced to confirm their identity with the proposed target sequence.

*Transmission electron microscopy (TEM).* Cells were fixed in 2% 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% osmium tetroxide in the same buffer for 90 min., dehydrated in graded series of ethanol and embedded with the epoxy plastic Agar 100 (Agar Aids, Stansted, UK). Ultrathin sections were placed on Formvar-coated copper grids and contrasted with 2% 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- $\beta$ , stem cell factor (SCF), IL-3 and IL-9, conditions that were previously shown to induce a homogenous MMC-like phenotype as regards 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 Fig. 1A (left 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 (Fig. 1A: right panel); such vesicles were also visible, although to a lesser extent, in  $SG^{+/+}$  MCs. As is evident from Fig. 1A (upper panels), cells of  $SG^{+/+}$  and  $SG^{-/-}$  genotype were similar in terms of shape and size, indicating that the cell culture conditions gave rise to homogenous 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 Fig. 1B, rare metachromatically stained cells were observed both in the mucosal (left panel) and in the submucosal (right 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 both in 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 analyze the importance of SG PGs for secretory granule organization in more detail,  $SG^{+/+}$  and  $SG^{-/-}$  cells were analyzed 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<sup>+/+</sup> 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<sup>+/+</sup> cells: i) granules containing small vesicles, most likely exosomes [33], that were filled with electron dense material, ii) granules containing small vesicles together with a grainy substance of moderate to high electron density, or iii) granules containing grainy substance of high electron density, without other substructural elements (Fig. 2 A-C). Generally, SG<sup>+/+</sup> granules were divided into distinct compartments of low electron density (electron translucent) and electron dense cores (Fig. 2 A-C). Notably, the granule types identified in the *in vitro*-derived MMCs resemble those previously identified in BMDCs developed in the presence of conditioned medium from Con A-stimulated splenocytes [34]. The granule morphology of SG<sup>-/-</sup> cells was markedly different. Firstly, granules of SG<sup>-/-</sup> cells were slightly smaller than those in SG<sup>+/+</sup> cells and often had an ovoid shape. Furthermore, most of the SG<sup>-/-</sup> granules lacked the intragranular vesicles (exosomes) that were often present in SG<sup>+/+</sup> counterparts, although solitary granules containing such vesicles could be observed. Another striking difference between SG<sup>+/+</sup> and SG<sup>-/-</sup> granules was that the granules of SG<sup>-/-</sup> cells lacked the subdivision into electron dense- and translucent regions that was typical for the SG<sup>+/+</sup> granules. Instead, SG<sup>-/-</sup> granules were usually completely filled with material of intermediate to relatively high electron density. Moreover, the contents of the SG<sup>-/-</sup> granules were of a more amorphous character and lacked the typical formation of grainy electron dense core that was seen in SG<sup>+/+</sup> granules (Fig. 2 D-F). These results are clearly consistent with a role for SG in the granule maturation process, with the amorphous appearance of the SG<sup>-/-</sup> granules resembling the immature granules seen in other secretory granule-containing cells, e.g. immature  $\beta$ -cells of the pancreas [35]. Further, the

subdivision of SG<sup>+/+</sup> granules into regions of high and low electron densities is clearly in line with the granule morphology seen in more mature pancreatic  $\beta$ -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 <sup>35</sup>SO<sub>4</sub><sup>2-</sup>, 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<sup>+/+</sup> cells contained ~5-fold higher amounts of labelled material than the corresponding pool recovered from SG<sup>-/-</sup> 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<sup>+/+</sup> and SG<sup>-/-</sup> cells, indicating that SG is not secreted to a major extent by the *in vitro*-derived MMC-like cells. (Table 1).

To further characterize the nature of the GAGs, samples of <sup>35</sup>S-labelled material were subjected to anion exchange chromatography along with internal standards of CS-A (low degree of sulfation; ~1 sulphate groups/disaccharide) and heparin (highly sulphated; ~2.5 sulphate groups/disaccharide unit). As shown in Fig 3A and D, respectively, most of the cell-associated and secreted material from SG<sup>+/+</sup> cells showed co-elution with standard CS-A, indicating a relatively low degree of sulfation. However, a small shoulder of material eluted slightly later in the salt gradient. Cell-associated (Fig. 3B) and secreted (not shown) material from SG<sup>-/-</sup> cells displayed a similar pattern of charge density as the corresponding samples from SG<sup>+/+</sup> counterparts, indicating that <sup>35</sup>S-labelled macromolecules of SG- and non-SG species have

similar anionic charge densities. For a comparison,  $^{35}\text{S}$ -labelled material from  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  bone marrow cells that had been *in vitro* differentiated into a more CTMC-like phenotype [30] were analyzed. Interestingly, GAGs derived from  $\text{SG}^{+/+}$  CTMC-like cells 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 (Fig. 3C). Further, GAGs from  $\text{SG}^{-/-}$  CTMC-like cells were eluted clearly before the corresponding material recovered from  $\text{SG}^{+/+}$  counterparts (Fig. 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. Further, 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 sulphated species as compared to 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 that was attached to the SG core protein. Chondroitinase ABC digestion resulted in depolymerization of most of the labelled GAGs from  $\text{SG}^{+/+}$  cells (Fig. 3E). In addition, most of the GAGs from the  $\text{SG}^{-/-}$  cells were depolymerised by chondroitinase ABC. Hence, most of the GAGs attached both to SG- and non-SG species of PGs in *in vitro*-derived MMC-like cells are of CS type.

To obtain further information on the macromolecular properties of the PGs, the purified  $^{35}\text{S}$ -labelled macromolecules were analyzed by size exclusion chromatography on a Superose 6 column. In addition, material was analyzed after treatment with 0.5 M NaOH to release GAG chains from its protein core. As shown in Fig. 3F, most of the labelled material from  $\text{SG}^{+/+}$  cells eluted as a single peak. Radiolabelled material from  $\text{SG}^{-/-}$  cells eluted somewhat later, indicating a lower molecular size. Alkali treatment of  $^{35}\text{S}$ -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 (Fig. 3F). In contrast, alkali treatment of labelled material from  $SG^{-/-}$  cells caused only a minor right shift of the peak, indicating that the  $^{35}S$ -labelled GAGs recovered from  $SG^{-/-}$  cells were mainly in the form of free GAG chains. Noteworthy, the GAG chains derived from  $SG^{-/-}$  cells eluted before the corresponding GAGs present in  $SG^{+/+}$  cells, indicating that GAGs attached to non-SG species have a higher average molecular weight 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 due to dense substitution with 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  $SG^{+/+}$  cells were to a large extent (~70%) resistant to treatment. Papain treatment of  $^{35}S$ -labelled macromolecules from  $SG^{-/-}$  cells caused a slight right shift, similar to that caused by alkali treatment, consistent with the notion that GAGs from  $SG^{-/-}$  cells were mainly present in the form of free polysaccharide chains (results not shown).

MC granules are typically characterized by strong chloroacetate esterase activity, attributable to their high content of chymase activity [37]. In a previous report 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 if also MMC chloroacetate esterase activity was dependent on the presence of SG PG. However, in striking contrast to CTMCs, chloroacetate esterase activities were similar in  $SG^{+/+}$  and  $SG^{-/-}$  *in vitro*-derived MMC-like cells (Fig. 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 further implies that the MMC proteases may display a different degree of SG-dependence as compared to the CTMC proteases. Experiments

were therefore performed to compare the protease profiles of  $SG^{+/+}$  and  $SG^{-/-}$  MMCs, both at 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. Further, transcripts were detected for mMCP-4 and mMCP-5, in agreement with a previous report [31], and for mMCP-6 and CPA (Fig. 4A). Importantly, all of these protease genes were expressed both in  $SG^{+/+}$  and  $SG^{-/-}$  cells, indicating that the lack of SG did not affect the degree or direction of cellular differentiation (Fig. 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 (Fig. 4B). Also mMCP-2 protein was detected in  $SG^{+/+}$  cells but in contrast to mMCP-1, mMCP-2 levels were somewhat reduced in  $SG^{-/-}$  cells, indicating a 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 (Fig. 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 (Fig. 4B).

To test if these results could be confirmed *in vivo*, small intestines from  $SG^{+/+}$  and  $SG^{-/-}$  mice were homogenized by a two step procedure, first with a low salt- 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 (Fig. 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 (Fig. 5).

The experiments described above were all performed on mice of C57BL/6 background. This mouse strain expresses mMCP-1, -2, -4, -5, -6 and CPA but lacks the tryptase mMCP-7 due 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 DBA-1 genetic background for 10 generations, and then analyzed *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 Fig. 6A, *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 C57BL/6 background (Fig. 6B). mMCP-7 protein was also detected in extracts from  $SG^{-/-}$  cells of DBA-1 background, with the level of mMCP-7 being similar as in extracts from  $SG^{+/+}$  cells (Fig. 6B). This indicates that mMCP-7 storage does not depend on SG. mMCP-7 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 on SG also at the *in vivo* level. For a comparison,  $SG^{+/+}$  and  $SG^{-/-}$  *in vitro*-derived MMC-like cells from mice of DBA-1 background were also analyzed for mMCP-6 antigen. In accordance with

results performed on cells from mice of C57BL/6 background, mMCP-6 antigen levels were markedly reduced in SG<sup>-/-</sup> cells (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<sup>+/+</sup> and SG<sup>-/-</sup> *in vitro*-derived MMC-like cells (of C57BL/6 background), and were analyzed by gelatin zymography. Both MMP-2 and MMP-9 were clearly detected in conditioned medium from SG<sup>+/+</sup> and SG<sup>-/-</sup> cells. However, the levels were similar in medium obtained from SG<sup>+/+</sup> and SG<sup>-/-</sup> 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 (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 this 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 MMC-like phenotype [31], whereas previous protocols have mainly resulted in MCs of 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 CS type, whereas *in vivo*-derived CTMCs produce predominantly heparin [25, 27]. Strikingly though, 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 to 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 oversulphated CS [26]. However, the nature of the MMC PG, i.e. which protein core to which the CS chains are attached to, and the role of the MMC PGs in granule homeostasis has previously not been

clarified. Here we show that MMCs with an inactive SG gene show an ~80% reduction in the amount of  $^{35}\text{S}$ -labelled PGs recovered from the cell layer, strongly indicating 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 either in MMC- or CTMC-like BMMCs, indicating that PGs of other species than SG are expressed. To date, the possible expression and cellular location of PG species other than SG in MCs has 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 to 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- $\beta$ /SCF/IL-3/IL-9 synthesize PGs of relatively low charge density. This is in sharp contrast to the PGs synthesized by bone marrow cells that have been driven into a more CTMC-like phenotype. Although both populations of BMMCs synthesize predominantly 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-sulphate 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 MMC- or CTMC-like phenotype strongly support that the different culture conditions 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 though, 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, -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. Further, 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 to mMCP-4, -5, -6 and CPA, mMCP-1 and mMCP-7 are independent on 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 versus mMCP-4/mMCP-5/CPA (Table 2), thus providing a likely explanation for its intermediate dependence on SG for storage. In a recent report we studied the SG-dependence of various components of the lytic granules of cytotoxic T lymphocytes for storage [41]. Also in that 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 on 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 His residues at the molecular surface and, at the acidic pH prevalent in secretory granules, these His residues are positively charged and engage in tight electrostatic interactions with GAGs [42]. Interestingly, we show here that the second major secretory tryptase in murine MCs, mMCP-7,

appears to be independent on 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 His cluster that was shown to mediate interaction between mMCP-6 and heparin is not fully conserved in mMCP-7 [42]. Moreover, a Lys/Arg 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].

Based on the findings presented here 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 this work, 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, -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 on 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 indicate that the fate may differ between different proteases and may include preferential degradation or exocytosis [46].



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## FOOTNOTES

<sup>1)</sup> The following abbreviations are used: MC, mast cell; PG, proteoglycan; SG, serglycin; mMCP-, mouse mast cell protease; CPA, carboxypeptidase A; TEM, transmission electron microscopy; BMMC, bone marrow-derived mast cell; CS, chondroitin sulphate; NDST, N-deacetylase/N-sulphotransferase; MMC, mucosal mast cell; CTMC, connective tissue type mast cell; GAG, glycosaminoglycan; HPRT, hypoxanthine guanine phosphoribosyl transferase; MMP, matrix metalloprotease

Table 1. Incorporation of  $^{35}\text{SO}_4^{2-}$  into PGs from *in vitro*-derived MMC-like cells. Results are expressed as cpm incorporated/ $1 \times 10^6$  cells. Results are representative of three independent experiments.

<u>Source of <math>^{35}\text{S}</math>-labelled PGs</u>	<u>SG<sup>+/+</sup></u>	<u>SG<sup>-/-</sup></u>
Secreted PGs	10,000	9,000
Cell-associated PGs	107,000	22,000



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

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

<sup>1)</sup> Net charges were calculated for mature proteins, i.e. without activation peptide.

FIGURE LEGENDS

**Fig. 1. Morphology and phenotype of mucosal type bone marrow-derived MCs.** (A)

Cytospin slides were prepared from bone marrow cells recovered from SG<sup>+/+</sup> and SG<sup>-/-</sup> mice after 15 days of culture in medium containing TGF- $\beta$ /IL-3/IL-9/SCF. Slides were stained by May Grünwald/Giemsa. Note that SG<sup>-/-</sup> 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<sup>+/+</sup> cells display metachromatically stained granules. (B) Sections were prepared from small intestines of SG<sup>+/+</sup> and SG<sup>-/-</sup> animals and were stained with toluidine blue. Rare toluidine blue-positive cells were detected in the mucosal region (left panel; *arrow*) and in the submucosa (right panel; *arrow*) of SG<sup>+/+</sup> mice. No toluidine-positive cells were found in sections prepared from SG<sup>-/-</sup> animals (not shown). (C) Chloroacetate esterase activity in SG<sup>+/+</sup> (left panel) and SG<sup>-/-</sup> (right panel) *in vitro*-derived MMC-like cells. Note that cells of both SG<sup>+/+</sup> and SG<sup>-/-</sup> genotype show strong chloroacetate esterase activity.

**Fig.2. Transmission electron microscopy.** (A) Representative micrograph from SG<sup>+/+</sup> *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 (*dashed arrow*). Original mag. X 20,000, bar = 0.5  $\mu$ m. (B) High magnification clearly revealed the grainy structure and the distinction of electron lucent/electron dense parts of the granules in the SG<sup>+/+</sup> cells. Original mag. X 75,000, bar = 0.1  $\mu$ m; (C) A closer view of the coexistence in SG<sup>+/+</sup> cells of granules filled with small vesicles (*arrows*) and granules with an electron dense core of a grainy material (*arrowhead*). Original mag. X 42,000, bar = 0.1  $\mu$ m. (D) A representative micrograph of a SG<sup>-/-</sup> cell shows cytoplasm granules (*arrows*), mostly spherical but some ovoid. Most of the SG<sup>-/-</sup> granules were completely filled with an amorphous matrix of

moderate electron density, although solitary granules contained small vesicles harboring material of varying electron density (*arrowhead*). Original mag. X 18,000, bar = 0.5  $\mu\text{m}$ . (E) The amorphous matrix of the  $\text{SG}^{-/-}$  granules is obvious at high magnification. Original mag. X 75,000, bar = 0.1  $\mu\text{m}$ . (F) Detail of the cytoplasm co-localization of the typical  $\text{SG}^{-/-}$  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 (*dashed arrow*). Original mag. X 42,000, bar = 0.1  $\mu\text{m}$ .

**Fig. 3. Analysis and characterization of  $^{35}\text{S}$ -labelled GAGs.**  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  bone marrow-derived MCs were biosynthetically labelled with  $^{35}\text{SO}_4^{2-}$ , followed by isolation of  $^{35}\text{S}$ -labelled GAGs from the cell layer and from conditioned media. Samples (10,000 cpm) 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 analyzed by anion exchange chromatography and the elution positions of the  $^{35}\text{S}$ -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 CTMC-like phenotype [30] were analyzed 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  $^{35}\text{S}$ -labelled macromolecules from  $\text{SG}^{-/-}$  cells. (E)  $^{35}\text{S}$ -Labelled GAGs recovered from  $\text{SG}^{+/+}$  (□) or  $\text{SG}^{-/-}$  (◆) *in vitro*-derived MMC-like cells were analyzed on a Sephadex G-50 column, either before (solid line) or after (dashed line) digestion with chondroitinase ABC. (F) Purified  $^{35}\text{S}$ -labelled macromolecules recovered from the cell layer of  $\text{SG}^{+/+}$  (▲; solid line), or  $\text{SG}^{-/-}$  cells (□; solid line) were analyzed by gel filtration on a Superose 6

column eluted with 0.2 M  $\text{NH}_4\text{HCO}_3$ . Samples of  $\text{SG}^{+/+}$  ( $\square$ ; dashed line) and  $\text{SG}^{-/-}$  ( $\blacklozenge$ ; dashed line) were also analyzed after release of free GAG chains from the PGs by treatment with 0.5 M NaOH.

**Fig. 4. mRNA and protein levels of MC proteases in *in vitro*-derived MMC-like  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  cells.** (A) mRNA expression of MC proteases. Total RNA was isolated from  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  bone marrow cells after 15 days of culture in medium containing TGF- $\beta$ /IL-3/IL-9/SCF, and was analyzed by RT-PCR, using the primers specified under "Experimental Procedures". Expression of the HPRT gene was used as house keeping control. (B) Immunoblot analysis of MC proteases in extracts from *in vitro*-derived MMC-like cells.  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  bone marrow cells were cultured for 15 days in medium containing TGF- $\beta$ /IL-3/IL-9/SCF. Cellular extracts were prepared and were analyzed for the presence of the proteases indicated.

**Fig. 5. Immunoblot analysis of MC proteases in intestinal extracts.** Intestinal extracts were prepared from  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  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 analyzed for the presence of the indicated proteases.

**Fig. 6. mRNA and immunoblot analysis of mMCP-7.** (A) *In vitro*-derived MMC-like cells were cultured from  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  mice of DBA-1 genetic background. After 4 days of culture, total RNA was isolated and was analyzed for mMCP-7 and HPRT (house keeping control) mRNA expression by RT-PCR. (B) *In vitro*-derived MMC-like cells (taken after 15 days of culture) from  $\text{SG}^{+/+}$  and  $\text{SG}^{-/-}$  mice of DBA-1 background were analyzed for mMCP-7 antigen by immunoblot analysis. As a negative control, *in vitro*-derived MMC-like cells derived from

C57BL/6 animals were also analyzed for mMCP-7 antigen. (C) Ear pinna were prepared from SG<sup>+/+</sup> and SG<sup>-/-</sup> mice of DBA-1 background and were extracted with a high salt buffer, followed by immunoblot analysis for mMCP-7 antigen.

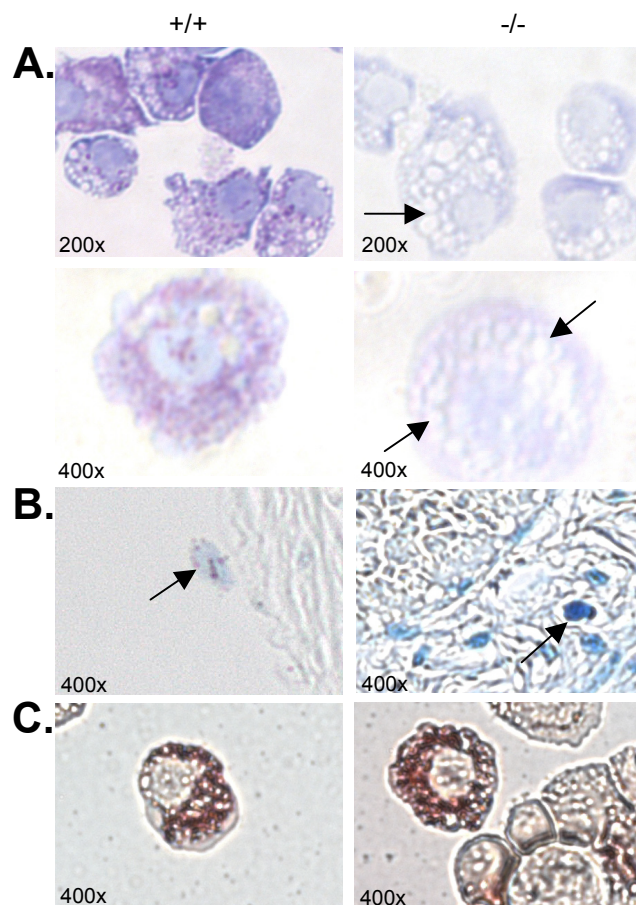


Fig. 1

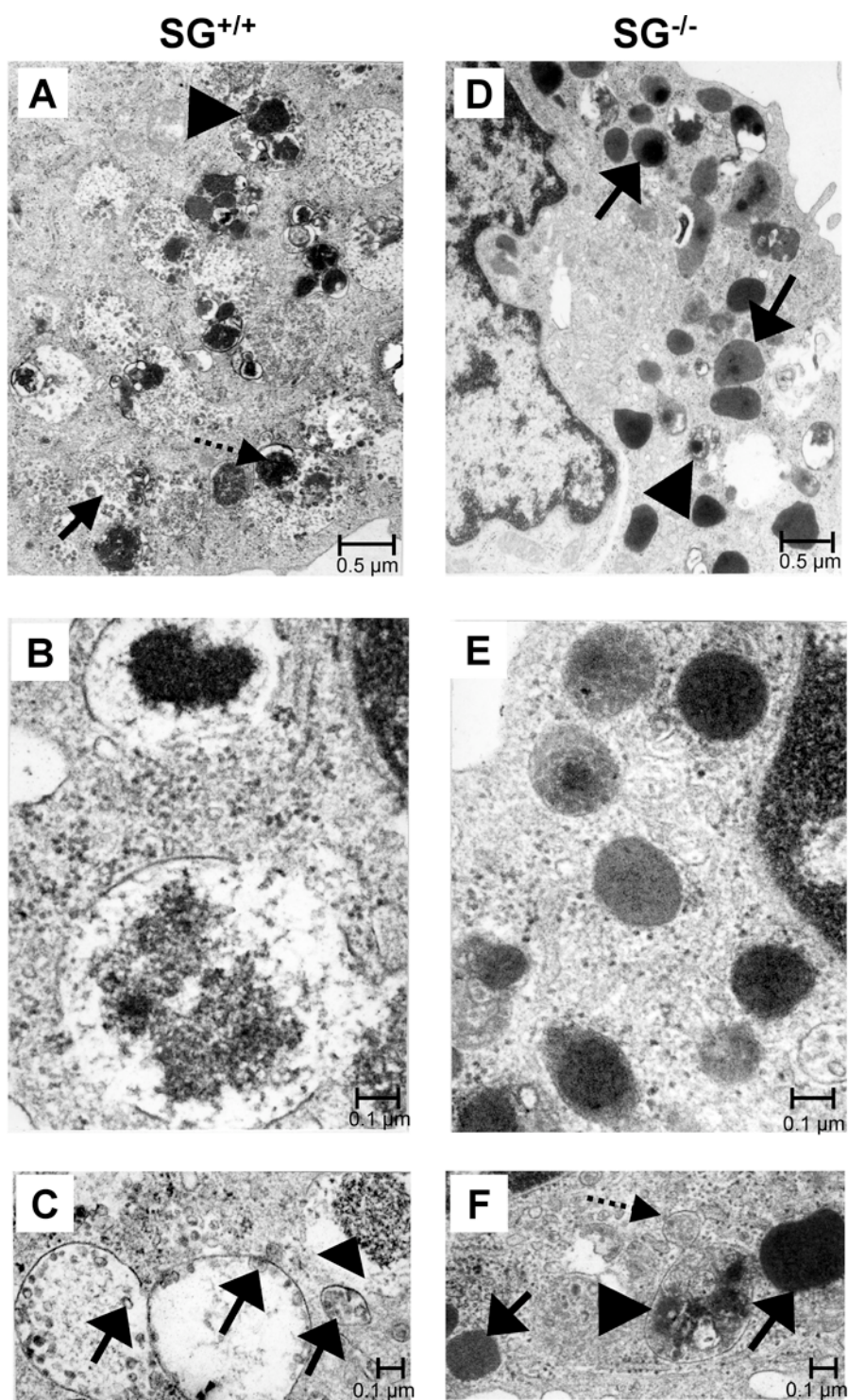


Fig. 2

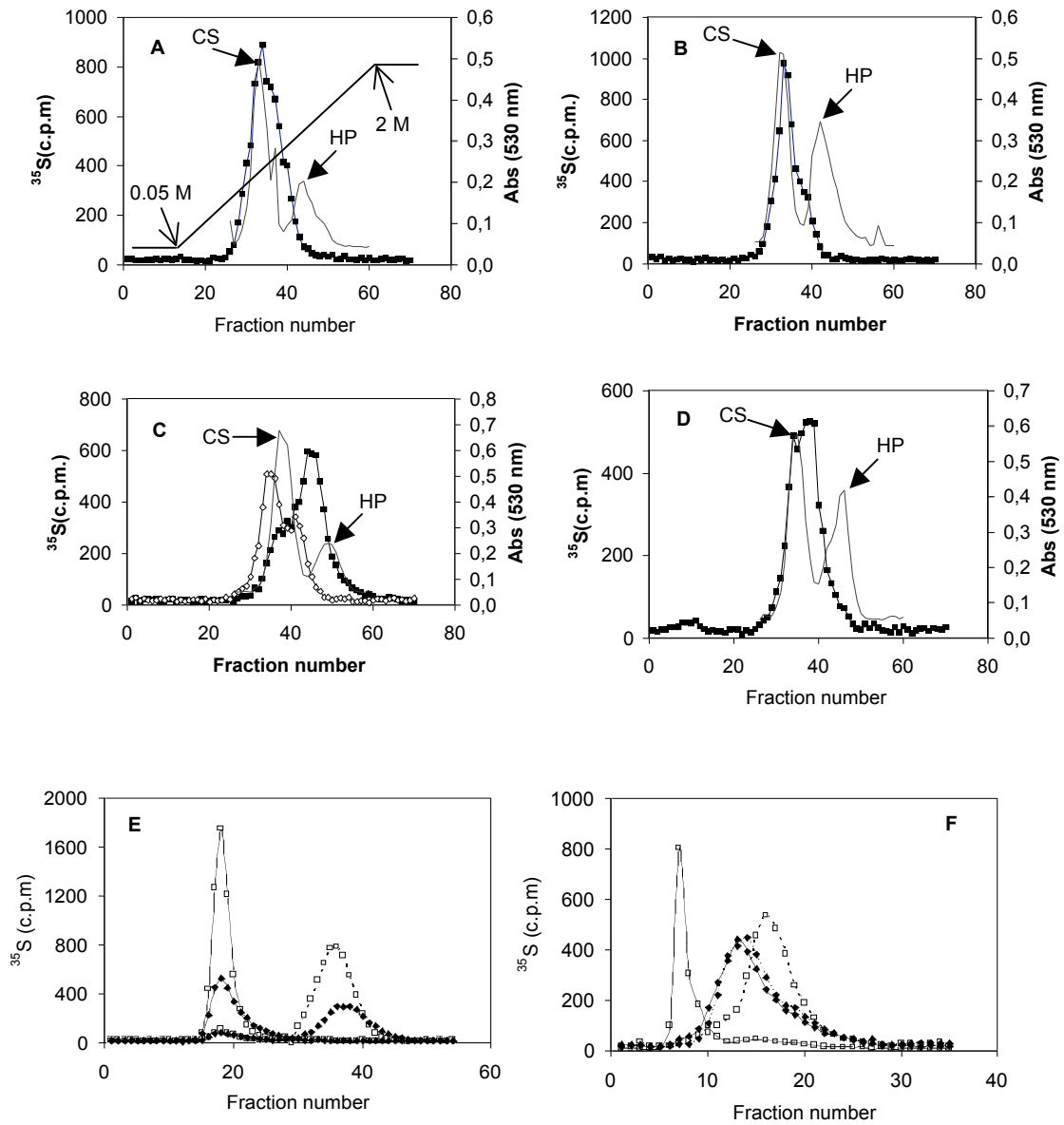


Fig. 3



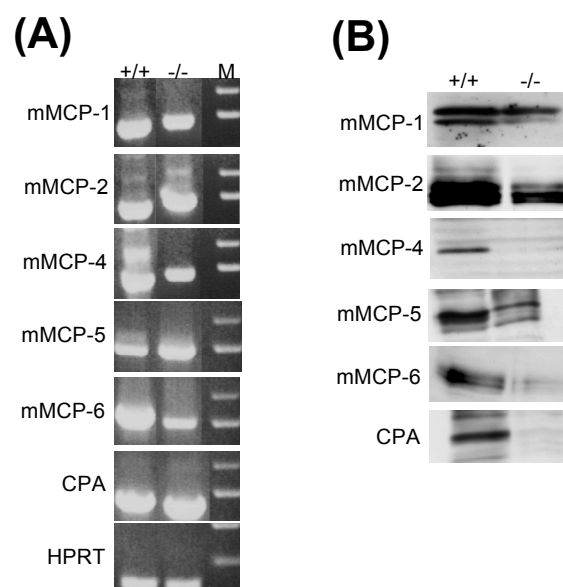


Fig. 4

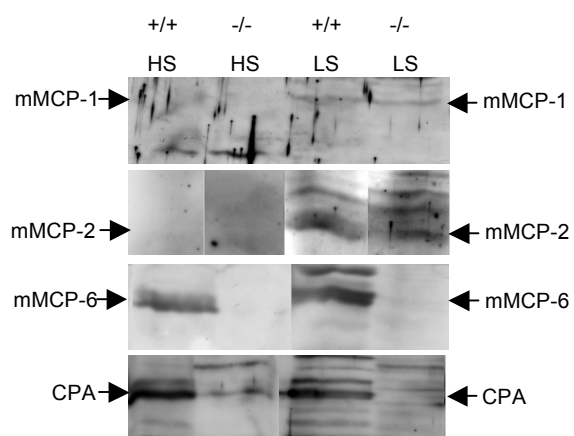


Fig. 5

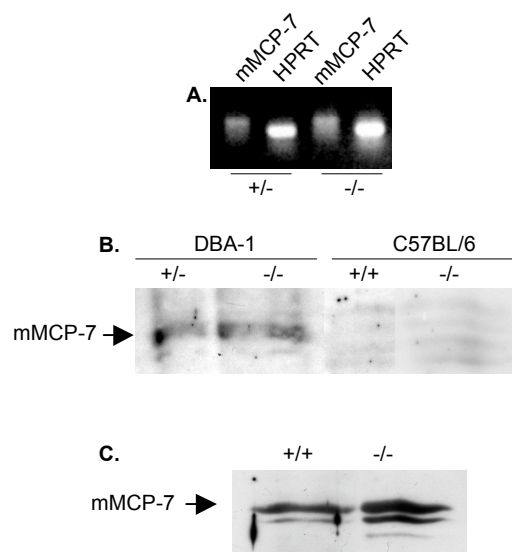


Fig. 6