Serglycin Is Essential for Maturation of Mast Cell Secretory Granule*

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To address the biological function of the scarcely studied intracellular proteoglycans, we targeted the gene for serglycin (SG), the only known committed intracellular proteoglycan. SG−/− mice developed normally and were fertile, but their mast cells (MCs) were severely affected. In peritoneum there was a complete absence of normal fertile, but their mast cells (MCs) were severely affected. Furthermore, peritoneal cells and ear tissue from SG−/− animals were devoid of the various MC-specific proteases. However, mRNA for the proteases was present in SG+/−, SG−/−, and SG−/− tissues, indicating that SG is essential for the storage, but not expression, of the MC proteases. Experiments, in which the differentiation of bone marrow stem cells into mature MCs was followed, showed that secretory granule maturation was compromised in SG−/− cells. Moreover, SG−/− and SG+/- cells, but not SG−/- cells, synthesized proteoglycans of high anionic charge density. Taken together, we demonstrate a key role for SG proteoglycan in MC function.

Proteoglycans (PGs) constitute a heterogeneous group of glycoproteins that all contain one or more glycosaminoglycan (GAG) chains attached to their respective “core” protein (1, 2). Most cell types express PGs and, often, the same cell type expresses more than one type of PG. PGs are present on the cell surface of most cells, and it is now established that cell surface PGs have an essential role in acting as co-receptors for various growth factors (3–6). Moreover, PGs are present in various extracellular locations, including cartilage (7) and basement membranes (8). In addition, they can be found intracellularly (9).

PGs have emerged from being considered as relatively inert structural components of connective tissue into the center stage of biology, having an enormous impact on a wide array of biological processes. Particular progress in understanding the function of this group of molecules has arisen from gene targeting studies. In such studies the strategies can be divided into two major directions: (i) targeting of the gene coding for a PG core protein or (ii) targeting of enzymes involved in the biosynthesis of the GAG side chains (reviewed in Ref. 10). Thus, in previous studies the biological functions of heparan sulfatase (HS)/heparin PGs have been approached by targeting the genes for different enzymes involved in HS/heparin biosynthesis, i.e. EXT-1 (an HS polymerase (11)), N-deacetylase/N-sulfotransferases (NDSTs) (12–14), C5-epimerase (15), 2-O-sulfotransferase (16), and 3-O-sulfotransferases (17). Indeed, the lethality induced by several of these gene knockouts confirms a vital importance for HS/heparin in various physiological processes. By using the alternative approach, i.e. targeting specific PG core proteins, important functions have been ascribed to the cell surface PGs and the extracellular matrix PGs (glypican-3 (18), syndecan-1 (19), syndecan-4 (20, 21), agrin (22), dystroglycan (23), and perlecain (24, 25)), whereas the targeting of glypic-an-2 did not result in any noticeable phenotypic consequences (26).

Remarkably, although many studies have addressed the biological function of the various cell surface PGs and the extracellular PGs, the intracellular PGs have so far received less attention. PGs that are found inside cells can either be “part time” intracellular PGs, i.e. being present in the intracellular space (e.g. during uptake or secretion processes) or be destined for an intracellular compartment, i.e. being a “committed” intracellular PG. The committed intracellular PGs are predominantly found in the secretory granule of hematopoietic cells such as mast cells (MCs), cytotoxic T-lymphocytes, neutrophils, and platelets where they have an important function in binding to various secretory granule compounds, thus facilitating their storage (12, 13). The PGs found in hematopoietic cell types are generally thought to be of serglycin (SG) type. Indeed, cDNAs encoding the SG core protein have been cloned from MCs (27, 28), and SG mRNA has been detected in a variety of other hematopoietic cell lineages (reviewed in Ref. 9). In addition, SG expression has been reported in nonhematopoietic cell types, such as endothelial cells (29) and pancreatic acinar cells (30), as well as in ES cells (31).

Although the core protein of PGs in secretory granules of hematopoietic cells may be the same, the GAG chain moieties of the PGs differ markedly between different cell types and can also differ between subtypes of SG-containing cells. In connective tissue-type MCs, the GAG side chains are predominantly of heparin type, having a [GlcUA/IdoUA-GlcNAc]n carbohydrate backbone structure with most of the GlcNAc units being N-sulfated. In addition, the GlcUA/IdoUA units may carry 2-O-sulfate groups, and the GlcNAc units can be O-sulfated, at the 6- and 3-positions (32). Most importantly, the connective tissue-type MC is the only cell type that has the capability to synthesize the highly sulfated GAG that is denoted “heparin,”
whereas a multitude of other cells synthesise HS, i.e., lower sulfated species of the same general structure. Most interestingly, whereas the connective tissue-type MCs predominantly synthesise GAG chains of heparin type, the mucosal MC subtype synthesizes mainly chondroitin sulfate (CS) chains (33). CS chains have a [GlcUA-GalNAc] backbone structure carrying O-sulfate groups at the 4- and/or 6-position of the amino sugar. As shown in the text, it is the 4- and 6-sulfated variant (oversulfated CS; CS-E) that dominates in MCs (33, 35, 36). In cell types other than MCs, lower sulfated GAG species of CS type are predominantly attached to the secretory granule PG core proteins (9).

To gain insight into the biological function of the committed intracellular PGs, here we have targeted the gene for SG by homologous recombination in ES cells and generated SG−/− mice. We show that SG has a crucial role in the formation of mature MC secretory granules.

EXPERIMENTAL PROCEDURES

Reagents—The chromogenic peptide substrates S-2586 and S-2288 were from Chromogenix (Mo Lndal, Sweden). The CPA substrate M-2245 (N4-methylphenoxazinomethylene)-Phe-OH) was from Bachem (Bubendorf, Switzerland). The key anti-rabbit Ig conjugated to horseradish peroxidase was purchased from Amersham Biosciences. Anti-sera toward recombinant CPA, m MCP-5, and m MCP-6 were raised in rabbits. The antisera toward m MCP-4 was a gift from Lars Hellman (Uppsala University, Uppsala, Sweden).

Gene Targeting and Construct Design—A complete genomic DNA sequence contiguous covering the SG gene was extracted from the Ensembl database (Sanger Institute). Based on the corresponding SG cDNA sequence, exons 1–3 were identified in the genomic DNA sequence. The SG gene is spanning over ~15 kb including regulatory sequences (48). We decided to make a classical knockout by replacing exon 1 and the regulatory sequences with a neomycin-resistant cassette. A PCR-based strategy was employed by using a PCR enzyme with proofreading activity (LA-PCR, Takara Bio Inc., Otsu, Shiga, Japan). We amplified a 3.3-kb fragment, which has been shown to be essential for transformation, by long-range PCR using primers I and II (primer I is outside the construct and forward, 5′-GCT TCG AGT GAG TTG GGA TTA TGA AGT CTA AAG GGC C-3′; and reverse primer 5′-ATC TCG AGA ATT CCT GCC TTT CCC TCT AGC TCA CAA AC-3′; the additional restriction sites are indicated in boldface) and a 4.3-kb downstream targeting arm starting at 3,800 bp and ending 500 bp upstream of the start site (fragment A, forward primer 5′-GAG TCG AGT GAG TTG GGA TTA TGA AGT CTA AAG GGC C-3′ and reverse primer 5′-GAG TCG AGA ATT CCT GCC TTT CCC TCT AGC TCA CAA AC-3′; the additional restriction sites are indicated in boldface) and a 4.3-kb downstream targeting arm located in intron 1 at 200 bp downstream of exon 1 (fragment C, forward primer 5′-GCG GAT CCA AGC TTG TCA GGA TGG AAA AGG GTT C-3′; and reverse primer 5′-ATC TCG AGC GCC CAG AAG GAT GGT CTA TAT TGG-3′). The downstream arm, fragment C, was cloned into the BamHI and NotI site of the pNeo vector, and the A fragment was cloned into the EcoRI and SmaI site (modifying the XhoI site for blunt end cloning) by long-range PCR using primers I and II (primer I is outside the construct and forward, 5′-GCT CAA TTC ACA TTT CAC GCC GGC C-3′; and reverse primer (protection of promoter region of exon 1) 5′-GCC ACA AGG GAA CAT TGC CAG C-3′ and primer II). In all experiments throughout this study, animals with a mixed genetic background of C57BL/6J and 129SvJ were used. To compensate for possible genetic variation in the F1 generation, littersmates of all three genotypes were analyzed.

Peritoneal Cells—Peritoneal cells from (mice, 12–18 weeks old) of SG−/−, SG−/−, and SG−/− mice were collected by peritoneal washing with 10 ml of cold phosphate-buffered saline (PBS) (pH 7.4).

Staining Protocols of Cells—Cells were collected onto object glasses by cytopsin (700 rpm, 5 min). A standard procedure for May Grunwald/ Giemsa staining was used. Briefly, cells were fixed in methanol (5 min) and then washed once in H2O, before staining in May Grunwald solution (15 min) followed by Giemsa (10 min), with washing steps between and after staining. The chloroacetate esterase assay was performed according to the instructions provided by the manufacturer (Sigma).

Analysis of Protease Activities in Tissues and BMMCs—Ear extracts were collected by perfusing the left ear, followed by homogenization using a PT1200 Polytron device (Kinematica AG, Littau-Lucerne, Switzerland). After homogenization, Trion X-100 was added to give a final concentration of 0.5%. Extracts were centrifuged (10,000 × g, 4 °C, 20 min), and the supernatants were recovered, and samples from the supernatants were used for activity measurements. Peritoneal cells and BMMCs were solubilized in lysis buffer (PBS/1 mM NaCl, 0.5% Trition X-100; 100 μl lysis buffer/1 × 106 cells). Ten μl of the peritoneal cell, BMMC, or ear extracts were mixed with 90 μl of H2O, followed by the addition of 20 μl of 1.8 mM solutions in (H2O) of chromogenic substrates for either chymotrypsin-like proteases (5–2586), trypsin-like proteases (S-2288), or CPA (M-2245). The absorbance was measured at 405 nm with a Titrinos spectrophotometer (Flow Laboratories), and initial reaction velocities were determined with the Delta Soft 3 software.

Western Blot Analysis—Samples of ear tissue extracts, solubilized peritoneal cells, or bone marrow-derived MCs (BMMCs) were mixed with 3× SDS-PAGE sample buffer containing 5% β-mercaptoethanol. Samples (30 μl) of these mixtures were subjected to SDS-PAGE on 12% gels. Proteins were subsequently blotted onto nitrocellulose membranes, followed by blocking with 5% milk powder in PBS (1 h, room temperature). Next, the membranes were incubated with antibodies toward the proteases, diluted 1:400 in TBS, 2% bovine serum albumin, 0.1% Tween 20, at 4 °C overnight. After washing the membranes extensively with TBS, blots were incubated with anti-rabbit Ig conjugated to horseradish peroxidase (diluted 1:5000 in TBS, 0.1% Tween 20). After 45 min of incubation at room temperature, the membranes were washed extensively with TBS, 0.1% Tween 20. The membranes were developed with the ECL system (Amersham Biosciences) according to the protocol provided by the manufacturer.

Bone Marrow-derived MCs (BMMCs)—BMMCs were obtained by culturing femur and tibia bone marrow cells from SG−/−, SG−/−, and SG−/− littermates (males, 18 weeks old) in Dulbecco's modified Eagle's medium (SVA, Uppsala, Sweden) supplemented with 10% heat-inactivated fetal calf serum (Biotech Line AS), 50 μg/ml gentamycine sulphate (SVA, Uppsala, Sweden), 2 mM l-glutamine (SVA, Uppsala, Sweden), and 50 μl WEHI-3B conditioned media (Littau-Lucerne, Switzerland). After homogenization, Triton X-100 was added to give a final concentration of 0.5%. Extracts were centrifuged (10,000 l, 11000 g, 5 min). Four fractions (11000 g, 5 min) were collected, and 2 μl of each fraction were analyzed for 35S radioactivity by scintillation counting.

Glycosaminoglycan Isolation and Analysis—30 × 106 BMMCs were biosynthetically labeled overnight with 0.32 μCi of carrier-free 35S-sulfate (Amersham Biosciences). Cells were pelleted by centrifugation for 10 min at 300 × g (4 °C) and were solubilized (at 4 °C) by adding 1 ml of PBS, 2 mM NaCl, 0.5% Triton X-100. After 30 min of incubation, 1 mL of the labeled macromolecules were purified as follows. The solubilised wet weight was diluted with 20 volumes of a second incubation buffer (1 M NaCl, 0.1% Triton X-100, pH 8.0). After washing the column with 4 ml of 50 mM Tris-Cl, 1 mM NaCl, 0.1% Triton X-100 (pH 8.0), and 4 ml of 50 mM NaAc, 0.15 mM NaCl, 0.1% Triton X-100 (pH 4.0), the columns were eluted with 50 mM NaAc, 2 mM EDTA, and the dialyzed samples were collected. The fractions were analyzed for 35S radioactivity by scintillation counting. Fractions containing radioactive material were subjected to papain digestion. To 1100 μl of sample, 25 μl of 0.5 M cyanide chloride, 25 μl of 0.5 M EDTA, and 56 units of papain (Sigma) was added. The mixtures were incubated for 19 h at 56 °C with constant shaking, and the papain reaction was stopped by heating the samples at 95 °C. Five samples were then centrifuged (350 × g, 10 min, at room temperature), and the supernatants were desalted on PD-10 columns. Before loading the samples, the columns had been washed with 25 μl of H2O, loaded with 50 μg of pig mucosal heparin (gift from U. Lindahl, Uppsala, Sweden) and then washed once in H2O, before staining in May Grunwald solution (15 min) followed by Giemsa (10 min), with washing steps between and after staining. The chloroacetate esterase assay was performed according to the instructions provided by the manufacturer (Sigma).
Samples (10,000 cpm) of isolated 35S-labeled GAGs were mixed with 22.5 µl of 2 M LiCl, 0.5 mg of CS-A (Sigma), and 0.45 mg of pig mucosal heparin and were diluted with H2O to 1 ml. The samples were applied to a DEAE-Sephaloc, coupled to a high performance liquid chromatography system. Anion exchange chromatography was performed with a gradient of increasing concentration of LiCl, from 0.05 to 2 M, in 50 mM NaAc (pH 4.0), at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and were analyzed for uronic acid content, using the carbazole assay, and for 35S radioactivity.

For determination of CS content, samples (~40,000 cpm) were treated with 0.5 units of chondroitinase ABC (Seikagaku, Tokyo, Japan) in a total volume of 250 µl of 0.05 M Tris-HCl (pH 8.0), 0.03 M NaAc, 100 µg/ml bovine serum albumin and incubated at 37 °C for 15 h. Samples were then analyzed by gel chromatography on columns (0.5 × 90 cm) of Sephadex G-50 (Amersham Biosciences), eluted with 2 µM NH4HCO3. Fractions (0.5 ml) were collected and were analyzed for 35S radioactivity.

**RESULTS**

**Gene Targeting of Serglycin**—A genomic contig spanning the mouse SG gene locus was extracted from Ensembl data base (Sanger Institute) and analyzed for suitable restriction sites to be used for cloning of the targeting construct. After examination of the sequence, we decided to use a PCR-based approach for the construct design (Fig. 1A). The upstream and downstream arms indicated were amplified directly from genomic DNA of 129SVJ ES cell origin and subcloned into a neomycin-containing cassette. The target construct, thus deleting exon 1 and upstream regulatory sequences, was used for homologous recombination in 129SVJ ES cells, and among the resulting clones we found four that had recombined at the correct site. Homologous integration of the target construct was confirmed using long range PCR with three different primer combinations, which all yielded products of expected sizes: 4 (primers I and II), 5.5, 7 kb (primers I and IV); the 5.5-kb band corresponds to wt and the 7-kb band corresponds to the presence of the neo cassette), and 5 kb (primers III and V) (Fig. 1A). Two ES cell clones, 75 and 239, were selected and used for blastocyst injection. A total of six chimeric mice were obtained, and four of these gave germ line transmission. Genotyping of the agouti offspring, using primers I and II, showed that an expected 50% of the animals carried the mutation (an example of genotyping is shown in Fig. 1B). F1 SG−/− animals were crossed, and the litters were genotyped with primers I and IV. SG−/− offspring was obtained at the expected Mendelian frequency of 25%. For routine genotyping we developed a short range PCR protocol utilizing three primers where the analysis was performed directly on the crude tail extracts. With this protocol we detected all three genotypes in one reaction, as exemplified by the genotyping shown in Fig. 1C. To confirm the targeting of the SG gene, the expression of SG in spleen was examined by RT-PCR. As expected, the SG transcript was detected in SG+/+ and SG−/− cells but was absent in SG−/− cells (Fig. 1D). The SG−/− mice are viable and fertile and show no altered phenotypic or behavioral characteristics when compared with their SG+/+ or SG−/− littermates.

**Effect of SG Knockout on MC Numbers and Morphology**—The peritoneum contains a mixture of cells, typically composed of ~2–4% MCs with the remainder of cells being macrophages and lymphocytes in approximately equal proportions. To study the possible effects of the SG knockout on the leukocytes present in the peritoneum, peritoneal cells were recovered from SG+/+ or SG−/− mice as well as from SG−/− littermate animals and were stained with May Grünwald/Giemsa. Fig. 2A shows the presence of macrophages, lymphocytes, and densely stained MCs in peritoneal populations obtained from SG+/+ mice. SG−/− peritoneal cell populations were similar to the SG+/+ cells (not shown). In the SG−/− cell populations, the macrophages and lymphocytes showed normal morphology and were present in approximately equal amounts as in the SG+/+ and SG−/− counterparts (Fig. 2A). However, the peritoneal cell population from SG−/− animals showed a complete absence of the strongly stained MCs that are apparent in wt and heterozygote populations. Instead, cells that appeared to contain large and seemingly empty vesicles were observed. Possibly, these cells may thus represent MCs devoid of granule content. This cell population constituted only ~0.2% of the total cellular content of the peritoneal cell population.

As an alternative way to identify MCs, we employed the chloroacetate esterase assay, which will predominantly stain for the chymases that are present in large amounts in MCs (see below). As shown in Fig. 2B, MCs from SG−/− mice stained strongly for chloroacetate esterase (macrophages were nega-
Gene Targeting of Serglycin

Gene Targeting of the SG Gene

The SG gene encodes a gastric serine protease called serglycin, which is expressed in the serous acinar cells of the stomach. In the peritoneal cavity, SG is expressed by mast cells (MCs), which are involved in numerous biological processes, including allergy, inflammation, and immunity.

The SG gene is targeted by homologous recombination in embryonic stem (ES) cells. The targeting construct consists of a Neo cassette inserted at the 5′ end of the gene, flanked by homologous arms and flanked by a second cassette of a different selectable marker. The targeting construct is designed to delete the entire coding sequence of the SG gene.

To generate SG-deficient mice, the targeting construct is linearized and introduced into ES cells. Clones that harbor the desired homologous recombination event are identified by PCR. The targeted ES clones are then injected into blastocysts to generate chimeric mice.

Offspring from the chimeric mice are then crossed to mice carrying a germ line mutation to select for germline transmission of the targeted allele. The targeted allele is confirmed by PCR using primers located on the homologous arms.

The SG-null mice show a reduction in stored protease activities at the level of activity but also at the protein level, the level of MCs in mouse predominantly express the chymases, mouse MC protease 4 (mMCP-4) and mMCP-5, the tryptases mMCP-6 and -7 as well as CPA, the mucosal MCs express the chymases mMCP-1 and -2 but no tryptases or CPA. Moreover, the protease expression profile is dependent on the degree of MC maturation (reviewed in Refs. 38-40).

First, we assessed whether the SG knockout affected the level of protease activities in the total peritoneal cell population as well as in ear tissue, a connective tissue that is rich in MCs. Peritoneal cell extracts and ear tissue homogenates were prepared, and the level of trypsin-like, chymotrypsin-like, and CPA activities were measured. From Table I it is evident that all of these protease activities were detected in both peritoneal cells and in ear tissue derived from SG+/− and SG++/− animals. In contrast, peritoneal extracts and ear tissue homogenates from SG-null animals displayed a striking reduction in all of these activities. In fact, all of these protease activities were virtually undetectable in both peritoneal cells and in ear tissue derived from SG+/− and SG++/− animals. Table I shows the level of protease activities in the total peritoneal cell population as well as in ear tissue homogenates of SG-null animals.

Effect of the SG Knockout on MC Proteases—Next, we investigated whether the SG knockout affected the protease activities of MCs. The level of protease activities was measured in peritoneal cell extracts and ear tissue homogenates of SG-null animals. The results showed a striking reduction in all of these activities. In fact, all of these protease activities were virtually undetectable in both peritoneal cells and in ear tissue derived from SG-null animals.

In conclusion, the SG knockout results in a drastic reduction in stored protease activities at the level of activity but also at the protein level, the level of MCs in mouse predominantly express the chymases, mouse MC protease 4 (mMCP-4) and mMCP-5, the tryptases mMCP-6 and -7 as well as CPA, the mucosal MCs express the chymases mMCP-1 and -2 but no tryptases or CPA. Moreover, the protease expression profile is dependent on the degree of MC maturation (reviewed in Refs. 38-40).

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respectively. Note the absence of densely stained MCs in the SG presence of macrophages (green), lymphocytes (black), and MCs (red), respectively. Note the absence of densely stained MCs in the SG−/− population and the presence in SG−/+ populations of cells containing large empty vesicles. B, cytoospin slides were also assayed for choroacetate esterase activity, a chymase-dependent activity. In wt MCs strong staining was observed, and even empty vesicle-containing cells showed strong staining (left panel). For comparison, NDST-2−/− peritoneal cells were also analyzed (boxed center panel). A reduced but strong staining was observed, and both empty vesicle-containing and granulated MCs were stained in the NDST-2 knockout. In the right panel SG−/+ cells are shown. Notably the empty vesicle-containing cells show no staining. As a negative control of the assay macrophages are shown (lower left and right panel). C, expression of c-Kit. c-Kit (CD117) antigen was detected by immunostaining of cytoospin slides prepared from SG−/+ and SG−/− peritoneal cells, using a monoclonal antibody. No staining was observed when an isotype-matched control antibody was used (not shown). Insets in C show enlarged images.

if the MC proteases were affected at the mRNA expression levels, total RNA was prepared from ear tissue and peritoneal cells and was analyzed for the presence of protease transcripts using RT-PCR. As shown in Fig. 3B, transcripts for mMCP-2, -4, -5, -6, and CPA were detected in both SG−/+ and SG−/− tissues, indicating that the MC protease genes are transcribed despite the defective SG gene (Fig. 3B). In fact, the mMCP-2 and -5 genes appeared to be transcribed at a somewhat higher rate in the SG knockout than in the SG−/+ controls.

Effect of the SG Knockout on MC Granule Maturation—To investigate the mechanism that leads to the MC defects described above, we chose to follow the in vitro differentiation of bone marrow stem cells into MCs, the so-called bone marrow-derived MCs. BMMCs are readily generated by culturing bone marrow stem cells in the presence of IL-3 and can be induced to mature further by addition of SCF (41–43). Bone marrow cells were prepared from SG−/+ and SG−/− animals as well as from littermate SG−/+ animals and were cultured together with WEHI-3B-conditioned medium as a source of IL-3. Cells were taken from the cultures at various time points and were used for investigation of time-dependent changes in morphology during MC differentiation (Fig. 4). Bone marrow cells from all three genotypes appeared very similar at day 0 (see Fig. 4; not shown for the SG−/− cells). Note the presence of neutrophil-like cells with similar morphology in SG−/+ and SG−/− populations, indicating that this class of leukocytes is not affected to a major degree by the lack of SG. Cells taken at early time points (1–2 weeks) showed the presence of numerous empty vesicles, reminiscent of the morphology seen in vivo in the SG−/− peritoneal population (see Fig. 2). This type of morphology was seen in bone marrow-derived cells taken from all genotypes. During prolonged culture in the presence of IL-3, these vesicles appeared to become filled with content in SG−/+ and SG−/− cells, but cells from SG−/− animals did not show this gradual filling of vesicles (see cells stained at 3–5 weeks; Fig. 4). In addition, after 5 weeks of culture the BMMCs were assayed for choroacetate esterase activity. SG−/+ and SG−/− cells showed strong staining (see inset in Fig. 4; not shown for SG−/+/) in the whole cytoplasm, whereas the SG−/− cells showed a reduced intensity of staining, with most of the staining in the perinuclear region (see inset in Fig. 4). A likely explanation for the latter finding is that esterase-expressing proteases are synthesized but the lack of SG PGs leads to defective storage in secretory granules.

To investigate if the SG−/− cells, after 4 weeks of culture with IL-3, could respond to MC growth factors, we added SCF to the culture medium. The SG−/+ and SG−/− BMMCs did not show any marked morphological changes (Fig. 5A and data not shown) upon additional stimulation of SCF. In contrast, the SG−/− cells responded vividly by forming similar empty vesicles as those seen after ~1 week of culture with IL-3, indicating initiation of granule formation. However, SCF stimulation of the SG−/− cells did not lead to further granule maturation (Fig. 5A). Furthermore, we examined whether SCF stimulation affected the expression of the SCF receptor, c-Kit. From Fig. 5B it is clear that most of the cells in SG−/+ and SG−/− IL-3-driven BMMCs expressed only very low levels of c-Kit on their surface. However, the addition of SCF resulted in a clear expression of c-Kit in both the SG−/+ and SG−/− bone marrow-derived cells (Fig. 5B).

To investigate further the phenotype of the BMMCs of different SG genotypes, we analyzed for MC protease levels. BMMCs from SG−/+ and SG−/− animals contained clearly detectable levels of trypsin-like as well as CPA activities (Table I). Moreover, they were positive for mMCP-5, -6, and CPA as assessed by immunoblot analysis (Fig. 6A) but did not contain detectable mMCP-4 antigen (not shown), in agreement with the lack of chymotrypsin-like activity (Table I). In contrast, the bone marrow-derived cells from SG-null animals were virtually devoid of protease activities (Table I) and antigen (Fig. 6A).

However, RT-PCR and Northern blot analysis showed that the SG−/− bone marrow-derived cells expressed mRNA for mMCP-2, -4, -5, -6, and CPA (Fig. 6, B and C). In the absence of SCF addition, it appeared as if transcript levels, as judged by PCR analysis, for mMCP-2, -4, -5 and -6 were somewhat higher in SG−/+ cells as compared with the SG−/+ counterparts (Fig. 6B). A higher level of mMCP-4 transcript in SG−/+ cells than in SG−/+ or SG−/− cells that had not been treated with SCF was also supported by Northern blot analysis, although mMCP-6 levels were similar in all genotypes (Fig. 6C). Addition of SCF to bone marrow-derived cells induced an increase in the mRNA levels for mMCP-4 and mMCP-6, as judged by Northern blot analysis (Fig. 6C). Furthermore, PCR analysis indicated that SCF stimulation also resulted in increased levels of mMCP-2 and -5 transcripts in SG−/+ and SG−/− cells, although there was no apparent increase in the transcription of the CPA gene upon SCF addition (Fig. 6B). The increased expression of mMCP-4 in response to SCF is in agreement with previous studies (44) demonstrating that this protease is only expressed at late stages of MC differentiation.
Experiments were performed in order to study how the inactivation of the SG gene affected the synthesis of sulfated PGs in IL-3-driven bone marrow cells. To this end, cells were labeled with "S\(^{35}\)SO\(_4\)\(^2\)" followed by isolation of radiolabeled intracellular and secreted GAG side chains. SG\(^{+/+}\) as well as SG\(^{+/-}\) cells showed a marked incorporation of "S\(^{35}\) label into intracellular GAGs. In contrast, cells derived from SG\(^{-/-}\) animals incorporated only small amounts of "S\(^{35}\) radioactivity into intracellular macromolecules (Table II). Similar amounts of "S\(^{35}\)-labeled GAGs were recovered from the conditioned cell culture media obtained from SG\(^{+/+}\), SG\(^{+/-}\), and SG\(^{-/-}\) cells (Table II). Further experiments were carried out to examine the charge densities of the GAGs isolated from the different SG genotypes. Anion exchange chromatography analysis of "S\(^{35}\)-labeled intracellular GAGs from SG\(^{+/-}\) (not shown) and SG\(^{-/-}\) (Fig. 7A) BMMCs revealed the presence of mainly highly charged species that showed co-elution with standard pig mucosal heparin. These highly charged GAG species were also evident in GAGs isolated from conditioned medium (Fig. 7B). In conditioned medium from SG\(^{+/-}\) (not shown) and SG\(^{-/-}\) (Fig. 7B) cells, we also detected a GAG population of lower anionic charge density, eluting as a shoulder before the highly charged material. In contrast, only the lower sulfated population of GAG was present in bone marrow cells derived from SG\(^{-/-}\) animals, both intracellularly and in the conditioned medium (Fig. 7A and B). Digestion of the radiolabeled GAGs with chondroitinase ABC resulted in nearly total depolymerization into low molecular weight components, indicating that the majority of the GAGs synthesized by the BMMCs were of CS type (Fig. 7C).

**DISCUSSION**

In this study, we performed a targeted inactivation of the gene for SG. Our results show that the SG inactivation results in major defects in connective tissue-type MCs, including severely affected morphology and compromised ability to store MC secretory granule proteases. The inactivation of SG thus results in an MC phenotype that shows some similarities with the phenotype induced by inactivating the gene for NDST-2 (12, 13). However, important differences between the NDST-2\(^{-/-}\) and SG\(^{-/-}\) strains are apparent. First, the NDST-2 knockout targets the GAG chains without affecting the actual core protein. Thus, the targeting of NDST-2 does not provide any information with regard to the involved core protein. Based on the high level of SG expression in MCs, it has been assumed that SG is the dominating PG found in the secretory granule.

**Table I**

|          | Ear tissue (n = 6\(^a\)) | Peritoneal cells (n = 2\(^b\)) | IL-3-driven bone marrow cells (n = 2\(^b\)) |
|----------|--------------------------|-------------------------------|---------------------------------------------|
|          | +/+                      | +/−                          | +/+                          | +/+                      | +/−                          | +/−                          | +/−                          |
| Trypsin-like activity | 40 ± 5.5                 | 35 ± 2.8                     | 10 ± 0.2                     | 38 ± 0.7                 | 10 ± 0.6                     | 100.0 ± 2.0                 | 90.7 ± 2.0                   |
| Chymotrypsin-like activity | 21 ± 5.0                 | 15 ± 2.8                     | 15 ± 3.0                     | 5.7 ± 3.7                 | 2.2 ± 1.4                     | 1.8 ± 0.16                 | 0.57 ± 0.3                   |
| CPA activity | −24 ± 7.0                | −33 ± 6.1                    | ND\(^c\)                      | −24 ± 8.3                 | −17 ± 1.4                    | ND\(^c\)                      | −2.8 ± 1.9                   |

\(^a\) Activities are given as dpm/μg (mean ± S.D.).
\(^b\) Activities are given as dpm/μl (mean ± 10\(^b\) cells).
\(^c\) ND, not determined.

**Fig. 3.** Immunoblot and expression analysis of MC proteases. A. Ear tissue homogenates and peritoneal cell extracts were prepared from SG\(^{+/+}\) and SG\(^{-/-}\) animals and were analyzed by using specific antisera toward mMCP-4, -5, -6, and CPA. B. mRNA levels for MC proteases. Total RNA was prepared from peritoneal (Perit.) cells and ear tissues and was analyzed by RT-PCR using the primers specified under “Experimental Procedures.” ND, not determined.

**Fig. 4.** Morphology and phenotype of bone marrow-derived cells. Bone marrow cells were recovered from SG\(^{+/+}\) and SG\(^{-/-}\) littermates and were cultured in the presence of WEHI-3B-conditioned medium as a source of IL-3. Cytospin slides were prepared at the time points indicated and were stained by May Grunwald/Giemsa. Note the empty vesicles present in both SG\(^{+/+}\) and SG\(^{−/−}\) cells at time points up to 3 weeks (3w) of culture (black arrows). Note also that the empty vesicles are absent in SG\(^{+/+}\) cells after 5 weeks (5w) of culture and are replaced by the metachromatic granule (white arrow; not present in SG\(^{−/−}\) cells). At 5 weeks, chloroacetate esterase activity was measured in situ (see insets).
However, it has not been possible to exclude a role for other types of PGs in MC secretory granule organization. Hence, the present study firmly establishes a crucial role for SG PGs for secretory granule integrity in MCs. Another important aspect is that the SG PGs can have either CS or HS/heparin chains attached to the core protein. In fact, it has been shown that MC PGs can be hybrids, containing both CS and HS chains (45). Thus, targeting of NDST-2 will affect HS/heparin synthesis, while being completely ineffective with regard to interference with the biosynthesis of CS-containing SG PGs. As a consequence, a defective NDST-2 will not result in major effects on cell types in which CS chains are predominantly attached to

**TABLE II**

Incorporation of $^{35}$SO$_4^{2-}$ into BMMC GAGs

| Source of $^{35}$SO$_4^{2-}$-labeled GAGs | +/+ | +/- | -/- |
|----------------------------------------|-----|-----|-----|
| Cell fraction                          |     |     |     |
| Experiment 1                           | 290,000 | 320,000 | 46,000 |
| Experiment 2                           | 370,000 | 280,000 | 27,000 |
| Medium fraction                        |     |     |     |
| Experiment 1                           | 28,000  | 46,000  | 26,000  |
| Experiment 2                           | 38,000  | 26,000  | 23,000  |

*Bone marrow cells were cultured in IL-3-containing medium and were biosynthetically labeled with $^{35}$SO$_4^{2-}$ as described under “Experimental Procedures.” Cell fractions and conditioned media were recovered, and GAGs present in the respective compartments were isolated by anion exchange chromatography and papain digestion, followed by quantification of incorporated $^{35}$S radioactivity.*

**Fig. 5.** Phenotypic characterization of bone marrow-derived cells. A, effect of SCF on bone marrow-derived cells. Bone marrow cells from SG$^{-/-}$ and SG$^{+/-}$ littermates were first incubated for 4 weeks (4w) with WEHI-3B-conditioned medium, after which SCF was added to the culture medium. Cytospin slides were prepared after 7 days and were stained with May Grunwald/Giemsa. B, c-Kit expression. Bone marrow cells were cultured for 4 weeks with WEHI-3B-conditioned medium and were further cultured for 1 week (1w), either in the absence (upper panel) or presence (lower panel) of SCF. Cytospin slides were prepared and were immunostained with an anti-c-Kit monoclonal antibody.

**Fig. 6.** Immunoblot and expression analysis of MC proteases in bone marrow-derived cells. A, immunoblot analysis for MC proteases. Extracts were prepared from bone marrow cells after 4 weeks of culture with WEHI-3B-conditioned medium and were analyzed for the presence of mMCP-5, -6, and CPA antigen, using specific antisera. B and C, mRNA levels for MC proteases. Total RNA was prepared from bone marrow cells that had been cultured for 4 weeks with WEHI-3B-conditioned medium and then further cultured for 7 days in the absence or presence of SCF. RT-PCR was performed using the primers specified under “Experimental Procedures.” C, Northern blot analysis of mMCP-4 and mMCP-6 transcripts. ND, not determined.

**Fig. 7.** Characterization of $^{35}$S-labeled GAGs. Bone marrow cells from SG$^{+/-}$, SG$^{+/-}$, and SG$^{-/-}$ littermates were cultured with WEHI-3B-conditioned medium (containing IL-3) for 4 weeks followed by the addition of $^{35}$SO$_4^{2-}$ to the cell cultures. After overnight labeling the conditioned media and cell fractions were recovered, followed by purification of $^{35}$S-labeled GAGs as described under “Experimental Procedures.” A and B, $^{35}$S-labeled GAGs isolated from SG$^{+/-}$ (●), SG$^{+/-}$ (▲), and SG$^{-/-}$ (○) cells (A) and medium fractions (B) were analyzed by anion exchange chromatography on a DEAE-Sephacel column. Arrows indicate the elution positions of standard CS and heparin, respectively. C, digestion of $^{35}$S-labeled GAGs by chondroitinase ABC. A sample (~40,000 cpm) of $^{35}$S-labeled GAGs from the cell fraction of SG$^{+/-}$ cells was digested with chondroitinase ABC, followed by separation on a Sephadex G-50 column. ●, undigested sample; □, digested sample.
the SG core protein. In mature mouse connective tissue-type MCs, the highly sulfated heparin chains constitute, by far, the dominating GAG species, although it has been reported that oversulfated CS is also present (33, 46). It is therefore expected that specific interference with HS/heparin synthesis (by targeting NDST-2) will give a marked effect on this MC population. In this context it is important to note that the GAG composition of human MCs is markedly different from that of rodents, with human MCs containing heparin and CS in an ~2:1 ratio (46). Thus, human MCs are expected to be highly dependent on SG for granule organization, whereas a lack of NDST activity may be compatible with secretory granule integrity.

In contrast to the mature connective tissue-type MCs in the peritoneum and ear tissue, for example, MCs derived by in vitro differentiation of bone marrow stem cells predominantly express GAG chains of CS type, although it has been reported that SCF stimulation of BMMCs can induce an increase in heparin synthesis (42). Consequently, the inactivation of SG is expected to have a much more profound effect on BMMCs than was caused by the inactivation of NDST-2, because the lack of SG should affect both CS and heparin biosynthesis. Indeed, the present study shows that the targeting of SG resulted in dramatic defects also for the BMMCs, whereas BMMCs from the NDST-2 knockout mice show normal morphology and granule maturation (13, 47). The implication of these findings is that SG is the predominant PG accommodating both CS and heparin chains in the MC secretory granule.

Another important difference between the NDST-2 and the SG knockouts was found when peritoneal MCs were stained for chloroacetate esterase activity. In agreement with the study by Humphries et al. (13), chloroacetate esterase-positive MCs were present in peritoneal cell populations from NDST-2−/− mice, and the number of positive cells was ~50% that found in wt cells. In contrast, no chloroacetate esterase-positive cells were found in peritoneal cells from SG−/− mice. A likely reason for this difference could be that the NDST-2 inactivation does not result in a complete lack of GAGs present in secretory granule. Possibly, CS synthesis may occur to some extent or nonsulfated [GlcUA-GlcNAc] chains could be present, and such GAG species may thus accommodate proteases expressing chloroacetate esterase activity. In the absence of the SG core protein, however, there may be a complete lack of GAG chains capable of mediating storage of any proteases with chloroacetate esterase activity.

We also decided to examine how the lack of SG affects the maturation of secretory granule during the MC differentiation process. For this purpose we took advantage of an established protocol for differentiation of bone marrow stem cells into MCs by culturing in medium containing IL-3. When bone marrow-derived cells were examined at 1 week of culture, we did not see any metachromatic staining, indicating poor granule maturation. At this stage of differentiation, we instead noticed the presence of cells containing vesicles that appeared to be empty. Notably, these cells showed some resemblance to the empty vesicle-containing cells seen in vivo in SG−/− peritoneal cell populations. It is thus possible that granule formation is initiated at this stage by forming these vesicles but that the granule content is too low to be stained by cationic dyes. It is of high interest to note that cells from SG−/− and SG−/− animals appeared relatively similar in terms of morphology after 1 week of culture, including the presence of empty vesicles, indicating that the early stages of granule maturation are not affected by the lack of SG. After ~3 weeks of culture in IL-3-containing medium, cells from SG−/− mice showed an increased metachromatic staining, indicating that the vesicles were being filled with PGs and other granule components. At this stage of MC differentiation, there was a sharp decrease in the number of empty vesicles observed in the SG−/− cells. A likely explanation for this finding is that, in the absence of incoming granule material, the initiated granule will revert, accompanied by major morphological changes. We also observed, by using the chloroacetate esterase assay, that MCs containing similar types of empty vesicles were found in the peritoneum of SG−/− mice. It thus appeared that this type of morphology is not exclusive for MCs that are lacking granule PGs. We may thus hypothesize that the empty vesicle-containing cells observed in vivo in the peritoneum of SG−/− mice may represent MCs at an early stage of differentiation, where the cells have initiated granule maturation but before the time point when the lack of SG causes de-routing of the morphology. The fate of these cells in vivo is not certain. One possibility would be that the low number of this cell type in vivo is a result of apoptosis as a consequence of the defects in granule maturation. This would undoubtedly lead to a markedly lower expression of MC protease transcripts in SG-null mice. However, we do not detect any major reduction in mRNA levels for mMCP-4, -5, -6, and CPA in BMMCs, ear tissue, or peritoneal cells from SG−/− animals as compared with SG+/+ or SG−/−, strongly arguing against this possibility. Hence, it is likely that these transcripts arise from cells that in terms of protease expression profiles should be identified as MCs but lack typical MC characteristics in terms of morphology and c-Kit expression.

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