Serglycin-deficient Cytotoxic T Lymphocytes Display Defective Secretory Granule Maturation and Granzyme B Storage*

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Cytotoxic T lymphocytes eliminate infected and tumor cells mainly by perforin/granzyme-induced apoptosis. Earlier studies suggested that serglycin/proteoglycans form macromolecular complexes with granzymes and perforin in the cytotoxic granule. Serglycin/proteoglycans may also be involved in the delivery of the cytolytic machinery into target cells. We have developed a serglycin-deficient mouse strain, and here we studied the importance of serglycin/proteoglycans for various aspects of cytotoxic T lymphocyte function. 35SO4 radiolabeling of serglycin-deficient cells demonstrated a dramatic reduction of incorporated label as compared with wild type cells, indicating that serglycin is by far the dominating proteoglycan species produced by the cytotoxic T lymphocyte. Moreover, lack of serglycin resulted in impaired ability of cytotoxic T lymphocytes to produce secretory granule of high electron density, although granule of lower electron density were produced both in wild type and serglycin-deficient cells. The serglycin deficiency did not affect the mRNA expression for granzyme A, granzyme B, or perforin. However, the storage of granzyme B, but not granzyme A, Fas ligand, or perforin, was severely defective in serglycin-deficient cells. Serglycin-deficient cells did not display defects in late cytotoxicity toward target cell lines. Taken together, these results point to a key role for serglycin in the storage of granzyme B and for secretory granule maturation but argue against a major role for serglycin in the apoptosis mediated by cytotoxic T lymphocytes.

Cytotoxic T lymphocytes (CTLs)2 and natural killer (NK) cells are key players of the immune response where they eliminate harmful cells, e.g. pathogen-infected cells and tumor cells, by initiation of apoptosis (reviewed in Refs. 1 and 2). CTL-mediated cytotoxicity is mediated by either the Fas ligand (FasL) or the perforin/granzyme pathway, where the Fas-dependent pathway is typical for self-reactive lymphoid cells and that the granzymes are delivered through this route (reviewed in Ref. 11). However, this model has been challenged by the finding that granzyme B can enter target cells autonomously (12) and through the identification of granzyme receptors on the surface of target cells (13, 14). Furthermore, it was recently reported that granzymes could be delivered into target cells without plasma pore formation (15).

Granzyme-mediated cytotoxicity is critically dependent on perforin, the only known membrane-disrupting protein in rodent cells. It was initially thought that perforin induces pore formation in the plasma membrane of target cells and that the granzymes are delivered through this route (reviewed in Ref. 11). However, this model has been challenged by the finding that granzyme B can enter target cells autonomously (12) and through the identification of granzyme receptors on the surface of target cells (13, 14). Granzymes are synthesized as preproenzymes and are processed into the active form by the sequential action of signal peptides and dipetidyl peptidase I (5). Of the various granzymes, granzymes A and B are the most abundant granzymes in mice and humans (2). Granzyme A induces a caspase-independent apoptotic pathway by cleaving the linker histone H1 and lamin (6, 7), and by destroying the SET complex (8). Granzyme B has been shown to be important for the rapid induction of apoptosis (9) and has been shown to induce apoptosis through both caspase-dependent and independent pathways (10).

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Proteoglycans (PGs) constitute a heterogeneous group of molecules, which are all composed of a protein core to which one or more glycosaminoglycan (GAG) chains are attached. It has been known for a considerable time that PGs are components of the secretory granule of cytotoxic cells. In 1985 it was reported that NK cells contained a protease-resistant chondroitin sulfate PG species that was released upon incubation of the NK cells with target cells, and the possibility that these PGs are involved in the packaging of cytolytic granule compounds was discussed (16, 17). Indeed, a subsequent report suggested that both perforin and granzyme A interacted with PGs at acidic pH, although the interaction with perforin with PGs was weakened at neutral pH (18). More recently it was shown that both granzyme B and perforin in cell extracts from YT cells co-precipitated with a PG species (15). Importantly, it was demonstrated that granzyme B was present as a complex with a PG after exocytosis and that the PG/granzyme B complex retained capacity to induce apoptosis (15). Along the same line, it has been shown that granzyme B in complex with free chondroitin 4-sulfate (chondroitin sulfate A) induces apoptosis in Jurkat cells (19).

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2 The abbreviations used are: CTL, cytotoxic T lymphocyte; NK, natural killer; Fasl, Fas ligand; PG, proteoglycan; GAG, glycosaminoglycan; SG, serglycin; Ac, acetyl; pNA, paranitroanilide; ConA, concanavalin A; PBS, phosphate-buffered saline; RT, reverse transcription; HPRT, hypoxanthine guanine phosphoribosyl transferase; TBS, Tris-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonic acid.
It is widely believed that it is PGs of the serglycin (SG) species that are localized to the CTL/NK cell granule. This notion is largely based on the observed resistance of PGs in NK granule to protease digestion, taking into account that the small SG core protein (~17 kDa (20, 21)) is densely substituted with GAGs that may protect the protein core from proteolytic attack (22). Moreover, it has been shown that SGPGs purified from monocyte (15) and CTL (23) cell lines indeed interact with perforin and granzyme B in purified systems. It has also been reported that SG mRNA is present in cytotoxic T cell lines (24). However, these findings do not provide any formal proof as regards the identity of the PG species prevalent in CTL/NK cell cytoplasmic granule. For example, it has not been possible to assess the relative contribution of SG versus other PG species in organization of cytolytic granule. Furthermore, although several reports have indicated an interaction between SGPGs and cytolytic compounds in vitro (see above), it is not known whether SG is the in vivo binding partner of, e.g. granzymes and perforin. We have recently developed a mouse strain with a targeted inactivation of the SG gene (25), and in the present study we used this strain to study the role of SG in CTLs. We show that SGPG is the dominating PG species expressed by CTLs and that SG is necessary for storage of granzyme B and for formation of electron dense cytolytic granule. In contrast, SG is not essential for storage of granzyme A, perforin, or FasL and is not necessary for cytolytic activity of CTLs.

**MATERIALS AND METHODS**

Reagents—The chromogenic peptide substrate Ac-IEPD-pNA and methyl-ο-mannopyranoside were from Sigma. ConcanaValin A (ConA) was purchased from Amersham Biosciences. [methyl-3H]Thymidine, Na2SO4, donkey anti-rabbit Ig and anti-mouse Ig, both antibodies conjugated to horseradish peroxidase, were purchased from Amersham Biosciences. monoclonal anti-human granzyme A antibody was purchased from BD Biosciences (Stockholm, Sweden), antiserum toward granzyme B and FasL were from Lab Vision Corp. (Fremont, CA), and the antiserum toward perforin was from Nordic BioSite AB (Stockholm, Sweden).

Mice—Mice deficient in SG as described previously (25), were backcrossed to C57BL/6J (animals in present study were at the N4 to N6 generation with a mixed genetic background of C57BL/6J (~95–99%) and 129SVJ (~1–5%)). Age-matched littermates (8–18 weeks old) from intercrosses of heterozygote N4 to N6 animals were used in all experiments. Production of Cytotoxic T Lymphocytes—CTLs were generated from blood or spleen of either SG+/+ or SG+/− mice (8–18 weeks old). Blood samples were taken from the euthanized littermate mice, and to block coagulation, an equal volume of 20 mM citrate (8–18 weeks old). Blood samples were taken from the euthanized littermate mice, and to block coagulation, an equal volume of 20 mM citrate (8–18 weeks old). Blood samples were then pelleted by centrifugation for 10 min at 300 g (4 °C) and solubilized (at 4 °C) by adding 1 ml of PBS/2 mM NaCl/0.5% Triton X-100. After 30-min incubation, 35S-labeled macromolecules were purified as described before (Abrink et al. (25)). Anion exchange chromatography, chondroitinase ABC digestion, and Sephadex G-50 chromatography were described previously (Abrink et al. (25)).

**RT-PCR Analysis**—Total RNA from CTLs and from the CTLL-2 cell line (gift from Thomas Tötterman, Rudbeck Laboratory, Uppsala, Sweden) was isolated using KIT NucleoSpin RNAII (Macherey-Nagel, Düren, Germany) according to the protocol provided by the manufacturer. Superscript II (Invitrogen) and specific reverse primers were used to produce single-stranded cDNA for granzyme A, granzyme B, perforin, syndecan-1–4, glypican-1–6 (as a control for the validity of syndecan and glypican primers, total RNA of day 14–16 whole embryos was prepared and subjected to RT-PCR), and hypoxanthine guanine phosphoribosyl transferase (HPRT). HPRT expression was used as a housekeeping control. The PCR reaction was 40 cycles with annealing and melting temperatures of 65 and 96 °C, respectively. The sequences of the PCR primers used are specified in TABLE ONE. The identity of amplified bands was verified by DNA sequencing. PCR products were purified from the agarose gel by QIAquick Gel Extraction kit (VWR International AB, Stockholm, Sweden) and were sequenced using the ABI PRISM BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and specific primer. The sequencing reaction was 25 cycles with annealing, polymerization, and melting temperatures of 50 °C, 60 °C, and 95 °C, respectively. Furthermore, DNA was precipitated with EtOH, resuspended in Template Suppression Reagent (Applied Biosystems).

**Western Blot Analysis**—Samples of 0.15–1.2 × 10⁶ CTLs or CTLL-2 were solubilized in 1 × SDS-PAGE sample buffer containing 5% β-mercaptoethanol. Samples corresponding to equal numbers of cells were subjected to SDS-PAGE on 10% gels. Proteins were subsequently blotted onto nitrocellulose membranes, followed by blocking with 5% milk powder in TBS/0.1% Tween 20 (1 h, room temperature). Next, the membranes were incubated with monoclonal Abs or antisera, diluted 1:200–1:250 in TBS/2% bovine serum albumin/0.1% Tween 20, at 4 °C overnight. After washing the membranes extensively with TBS/0.1% Tween 20, the membranes were incubated with anti-rabbit or anti-
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Table ONE

Sequences of PCR primers used
The primers used for amplification of granzyme A, granzyme B, and perforin were taken from Kelso et al. (44). PCR primers for glypican-1–6 and syndecan-1–4 were designed for the purpose of this study. All PCR primers were designed to span exon/intron boundaries to avoid amplification of genomic DNA.

| Product size | bp |
|--------------|----|
| 5'-ATT GGA GGA GAC ACG GTT GTT-3' | 582 |
| 5'-GCC TCG CAA AAT ACC ATC ACA-3' | 582 |
| 5'-ACA TGG CCT TAC TTT CGA TCA-3' | 468 |
| 5'-CGC CAT ATA TCT GAT TGG TTT-3' | 667 |
| 5'-GCT CCC TGC TAC ACT GCC ACT-3' | 633 |
| 5'-AGG GCT GTA AGG ACC GAG ATG-3' | 656 |
| 5'-GCC AGT GTC CCC AAA AGC CAT GTA T-3' | 598 |
| 5'-AGG GTT CCA CCC AGC AGA AGA A-3' | 633 |
| 5'-GCA ACT GCT AAG CCT TGA CGG AAG G-3' | 270 |
| 5'-GAA GCT GGT CCA GCC CAC CAT A-3' | 817 |
| 5'-CAT CAG ACA GCA GAT CAT GGC TCT C-3' | 384 |
| 5'-GTC CCT TGG TCA CAG AAA AGC TCA C-3' | 391 |
| 5'-GCA ACT GCT ATT CAG ACA ACT C-3' | 547 |
| 5'-GTT CCT TGG TGA TCT GTG GTG T-3' | 444 |
| 5'-GCA ACT GCT ATT CAG ACA ACT C-3' | 348 |
| 5'-AGC TTC TGC TGA ACT TCC ACT TGC T-3' | 360 |

*fw, forward primer; rev, reverse primer.

As targets, various tumor cell lines were used: EL4 (syngeneic, H-2b haplotype, a gift from Lars Hellman, Uppsala University, Uppsala, Sweden) and three allogeneic cell lines, P815 (H-2d haplotype, LGC Promochem, Borås, Sweden), J558 (H-2d haplotype, a gift from Lars Hellman), and YAC-1 (H-2a haplotype, LGC Promochem). Target cells were labeled overnight with 5 μCi/ml of [3H]thymidine. Before setting the cytotoxic assay, the targets were washed with medium and the cell concentration was adjusted to 105 cells/ml with culture medium containing 0.1 M methyl-D-mannopyranoside. Next, 100 μl of effector cells and 100 μl of target cells (giving an effector/target ratio of 30:1 or 60:1) were transferred into a 96-well round-bottom microtiter plate. To measure spontaneous target cell lysis (S), 100 μl of target cells was transferred to a plate as the latter (100 μl of target cells only) was also prepared for analysis of total counts (T). Plates were covered with a lid and centrifuged at 150 × g (room temperature) for 3 min. All of the plates, except the plate that was used for determination of total counts, were then left in a CO2 incubator. After various time points (4, 7, and 21 h), cells were harvested using a microtiter plate harvester and the amount of 3H radioactivity bound to the filters was quantified by liquid scintillation counting. The plate used for determination of total counts was harvested immediately after the centrifugation step. The cytotoxic activity of CTL was calculated as: % cytotoxicity = [(S - E)/S] × 100, where % spontaneous lysis = [(T - S)/T] × 100, T is total counts; S is spontaneous release, and E is experimental release.

Cytotoxicity Assays—The JAM test was performed according to the protocol described by Matzinger (28). Spleen cells of SG+/+ and SG−/− littermate mice (mixed genetic background of C57/Bl6 and 129SvJ mice, both with H-2b haplotype) were stimulated with 2 μg/ml ConA for 3 days. CTLs were then thoroughly washed with medium (see above, production of CTLs) and resuspended in fresh medium containing 0.1 M d-mannopyranoside at a concentration of 6 × 106 cells/ml.

Mouse Ig, both 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.

Measurement of Granzyme B Activity—Granzyme B activity was determined as previously described by Ewen et al. (27). Briefly, 3 × 106 CTL, stimulated with 5 μg/ml ConA for 3 days, were washed with PBS and lysed in 50 μl of lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.2, 1% (v/v) Triton X-100) for 15 min on ice with occasional shaking. The lysates were centrifuged for 1 min at 10,000 × g, 4 °C. 5-μl samples from the resulting supernatant (corresponding to 3 × 103 cells) were added to 96-well flat-bottomed plates, followed by the addition of 95 μl of a reaction buffer (50 mM Hapes, pH 7.5, 10% (w/v) sucrose, 0.05% (w/v) CHAPS, and 5 mM dithiothreitol) and 10 μl of N-Ac-IEPD-pNA (200 μM, dissolved in Me2SO). Plates were covered and incubated at 37 °C. Released paranitroanilide (pNA) was recorded at 405 nm using a Titertek Multiscan spectrophotometer (Flow Laboratories).

As targets, various tumor cell lines were used: EL4 (syngeneic, H-2b haplotype, a gift from Lars Hellman, Uppsala University, Uppsala, Sweden) and three allogeneic cell lines, P815 (H-2d haplotype, LGC Promochem, Borås, Sweden), J558 (H-2d haplotype, a gift from Lars Hellman), and YAC-1 (H-2a haplotype, LGC Promochem). Target cells were labeled overnight with 5 μCi/ml of [3H]thymidine. Before setting the cytotoxic assay, the targets were washed with medium and the cell concentration was adjusted to 105 cells/ml with culture medium containing 0.1 M methyl-D-mannopyranoside. Next, 100 μl of effector cells and 100 μl of target cells (giving an effector/target ratio of 30:1 or 60:1) were transferred into a 96-well round-bottom microtiter plate. To measure spontaneous target cell lysis (S), 100 μl of target cells was transferred to a plate as the latter (100 μl of target cells only) was also prepared for analysis of total counts (T). Plates were covered with a lid and centrifuged at 150 × g (room temperature) for 3 min. All of the plates, except the plate that was used for determination of total counts, were then left in a CO2 incubator. After various time points (4, 7, and 21 h), cells were harvested using a microtiter plate harvester and the amount of 3H radioactivity bound to the filters was quantified by liquid scintillation counting. The plate used for determination of total counts was harvested immediately after the centrifugation step. The cytotoxic activity of CTL was calculated as: % cytotoxicity = [(S - E)/S] × 100, where % spontaneous lysis = [(T - S)/T] × 100, T is total counts; S is spontaneous release, and E is experimental release.
Molecular Modeling of Mouse Granzyme A and B—The closest homologs of mouse granzyme A and B in the PDB structural data base were identified using BLAST (29) and used to construct homology models for the mouse proteins. The model for human granzyme A (pdb id 1op8 (30); 70% identities) was used to model mouse granzyme A, for granzyme B the model for rat granzyme B (pdb id 1f18 (31); 80% identities) was used. Homology models were generated using SOD (32) and the molecular graphics program O (33). To allow comparison of surface charge distribution, side chains of charged residues not modeled in the crystallographic structures were added in standard rotamer conformations. Side-chain rotamers were adjusted where necessary to avoid steric clashes. Surface representations were generated using PYMOL (www.pymol.org).

RESULTS

Morphology of Concanavalin A-activated CTLs—CTLs were generated from spleen and blood of both SG+/− and SG−/− animals, by culturing splenocytes and blood mononuclear cells in the presence of concanavalin A (ConA) for 5 days. Cytospin slides of non-stimulated (NS) and ConA-stimulated spleen cells were stained with Giemsa. As shown in Fig. 1A, activation of spleen cells with ConA caused a marked change in morphology, including dramatically enlarged cytoplasm. However, no obvious differences in morphology between SG+/− and SG−/− cells were observed at the light microscope level. This is in sharp contrast to the effect of the SG knock-out on mast cells, where SG inactivation was associated with dramatic morphological changes that were observable by the MayGrunewald/Giemsa stain (25). To get better insight into the possible effect of SG on CTL morphology we used transmission electron microscopy.

Transmission electron microscopy analysis of both SG−/− and SG+/− CTLs (Fig. 1B) revealed accurately processed spherical cells. In both genotypes mitochondria, free ribosomes, transport vesicles, and nuclei demonstrated an apparently normal ultrastructure. Some of the cells showed deep plasma membrane invaginations that appeared as translucent “holes” in the cytoplasm. In contrast, a clear difference between genotypes was detected in the ultrastructure of the cytotoxic granule. The CTLs derived from the SG−/− mice contained mostly heteromorphous cytotoxic granule with an electron-dense, irregular core in a transparent space but also some spherical electron-translucent granule filled with an amorphous matrix (Fig. 1B), whereas SG−/− CTLs possessed exclusively spherical electron-translucent granule with an amorphous matrix (Fig. 1B).

Effects of the SG Knock-out on the Synthesis of Sulfated PGs in CTLs—Although numerous studies have suggested that PGs of the SG species is a major component of cytolytic granule in CTLs (see the introduction), there is no definitive evidence for this notion. It was therefore imperative to assess the relative contribution of SG to the total amount of PGs synthesized by CTLs. For this purpose we labeled CTLs with [35S]SO4 to account the total amount of PGs synthesized by CTLs. For this purpose we labeled CTLs with [35S]SO4 for 3 days with ConA, we observed a marked general increase in the total incorporation of [35S]SO4 into all pools of GAGs, except into the extracellular pools derived from SG−/− cells (TABLE TWO). Strikingly, after stimulation for 3 days with ConA, we observed a marked general increase in the total incorporation of [35S]SO4 into all pools of GAGs, except into the extracellular pools derived from SG−/− cells (TABLE TWO). Moreover, ConA stimulation resulted in a markedly lower incorporation of [35S]radioactivity into cell-associated PGs from SG−/− cells as compared with wt counterparts (TABLE TWO). This indicates that most of the cell-associated GAGs in CTLs indeed are attached to the SG core protein. An even more dramatic effect was observed for the secreted GAGs, where the SG knock-out resulted in a ~10-fold reduction in [35S]SO4 incorporation (TABLE TWO). These results thus indicate that SG is the dominating, both intracellular and secreted, PG species in CTLs. However, we cannot rule out other explanations for the strong reduction of GAG synthesis in SG−/− cells. For example, the knock-out of SG may, by unknown mechanisms, alter the expression of GAG modifying enzymes.
TABLE TWO
Incorporation of $^{35}$S$^{2-}$ into GAGs of non-stimulated and ConA-stimulated spleen cells

| Source of $^{35}$S-labeled GAGs | $SG^{+/+}$ Non-stimulated | $SG^{+/+}$ Stimulated | $SG^{-/-}$ Non-stimulated | $SG^{-/-}$ Stimulated |
|-------------------------------|---------------------------|-----------------------|---------------------------|-----------------------|
| Cell fraction                 | 120                       | 2,150                 | 80                        | 700                   |
| Medium fraction               | 1600                      | 6600                  | 700                       | 650                   |

*p* CS, chondroitin sulfate.

FIGURE 2. Identification of PG core protein mRNAs in CTLs. Total RNA was isolated from $SG^{+/+}$ and $SG^{-/-}$ spleen-derived CTLs. The RNA was used for RT-PCR analysis utilizing primers (see TABLE ONE) specific for glypicans 1–6 (G1–G6) and syndecans 1–4 (S1–S4). HPRT expression was used as the housekeeping control.

Importantly, because the SG knock-out did not completely abolish $^{35}$S incorporation, other PG species than SG are apparently expressed by CTLs. To identify other PG species than SG we therefore performed RT-PCR analysis utilizing primers specific for two major classes of PGs, the glypicans (glypican-1–6) and the syndecans (syndecan-1–4). As a positive control of the primer pairs described in TABLE ONE total RNA from day 14–16 embryos was isolated and used for RT-PCR. All of the expected products, i.e. syndecan-1–4 and glypicans 1–6, were amplified from this source, thus validating the PCR primers used (data not shown). As shown in Fig. 2, CTLs were found to express glypican-1, glypican-4, and syndecan-4, but not the other known isoforms of glypicans or syndecans. The identification of glypican-1, -4, and syndecan-4 was verified by DNA sequencing. Hence, it is possible that the GAG chains that are synthesized in $SG^{-/-}$ cells are attached to any of these identified additional PG core proteins.

The isolated sulfated macromolecules were further analyzed by anion exchange chromatography. All of the isolated samples, both from $SG^{+/+}$- and $SG^{-/-}$-cells and both from cell (Fig. 3A) and medium (Fig. 3B) fractions, showed co-elution with standard chondroitin sulfate A. This indicates that the overall degree of sulfation of all CTL GAGs is relatively low as compared with, e.g. heparin (see elution position of standard heparin in Fig. 3A and B). To analyze the nature of the isolated GAGs, samples were digested by chondroitinase ABC followed by Sephadex G-50 chromatography to separate undigested and digested material. These experiments showed that most of the GAGs synthesized by both $SG^{+/+}$ and $SG^{-/-}$ cells, both before and after ConA stimulation was composed of chondroitin sulfate (TABLE TWO and Fig. 3C). These results are thus in agreement with the previous identification of chondroitin sulfate A as the major GAG species present in NK cells (17).

**Effects of the SG Knock-out on the Capacity of CTLs to Lyse Target Cells**—Next we asked whether the lack of SG affected the ability of CTLs to induce apoptosis in target cells. To answer this important question we used the JAM test (28). $[^{3}H]$thymidine-labeled target cells were mixed with effector cells in a ratio of 1:60 or 1:30. The samples were harvested onto glass fiber filters after 4, 7 (not shown), and 21 h, and the filter-bound radioactivity, corresponding to non-degraded DNA, was harvested onto glass fiber filters after 4, 7 (not shown), and 21 h, and the filter-bound radioactivity, corresponding to non-degraded DNA, was measured. As a negative control we used $[^{3}H]$thymidine-labeled syngeneic EL4 cells (H-2b haplotype). As expected, we determined low cytotoxicity of CTLs toward the syngeneic EL4 cell line (Fig. 5). In contrast, allogegenic tumor cells, YAC1 (H-2a), J558 (H-2d), and P815 (H-2d), were highly susceptible to apoptosis by the spleen-derived CTLs (Fig. 5). Importantly, there was no significant difference between $SG^{+/+}$, $SG^{-/-}$, and $SG^{-/-}$ CTLs in their capability to induce apoptosis, indicating that the late apoptosis-inducing mechanism of spleen-derived CTLs is independent on SG.
This is the first work in which gene targeting methodology is used to assess the role of SG in CTLs. SGPGs are unique in that they are primarily destined for storage in secretory granule and subsequent exocytosis, whereas other PG species are mainly associated with the cell surfaces (e.g. glypicans and syndecans) or deposited in extracellular matrixes (e.g. aggrecan and perlecan). SG expression has been reported for a variety of cell lineages, in particular of hematopoietic origin (reviewed in Ref. 34). However, the identification of the biological function of this particular PG has previously been hampered by the lack of a genetically SG-deficient mouse strain, whereas the targeting of numerous other PG genes has been reported (reviewed in Ref. 35). An important consideration when studying the biology of SGPGs is that the nature of GAG chains attached to the SG core protein varies tremendously between SGPGs expressed by different cell types. Thus, connective tissue type mast cells present in, e.g. the peritoneum synthesize...
predominantly highly sulfated GAGs of heparin type ([IdoUA-GlcNAc]$_n$ carbohydrate backbone) (36), whereas bone marrow-derived mast cells synthesize GAG chains of equally high charge density albeit of chondroitin sulfate type ([GlcUA-GalNAc]$_n$ carbohydrate backbone) (37). In contrast, SG produced by macrophages (38) and NK cells (17) contain chondroitin sulfate chains of much lower charge density. In a recent study we reported for the first time the successful targeting of the SG gene and showed that the SG knock-out had a profound impact on granule maturation in connective tissue type mast cells, including a complete absence of mature secretory granule accompanied by an absence of all of the proteases that are normally present in mast cell secretory granule (25). In the present study we extend the knowledge of the biology of SG by examining the role of SG in CTLs. We show here that the SG inactivation, similar to the effect on connective tissue type mast cells, affects the granule morphology also in CTLs. However, the effect on CTL granule is not as dramatic as that observed in connective tissue type mast cells. Whereas the lack of SG completely abrogated the assembly of any metachromatically staining secretory granule in MCs, lack of SG did not prevent granule formation in CTLs. Instead, the SG knock-out resulted in an inability to assemble mature granule of high electron density, whereas immature granule of lower electron density were present both in SG-deficient and -competent CTLs. These findings suggest that SGPGs is mainly present in the highly electron dense granule structures but is absent from other types of granule. Possibly, the granule of lower electron density may thus be devoid of PGs or may contain PGs of other type than SG (see below). A further implication of the present findings is the possibility of secretory granule segregation similar to the situation in neutrophil granulocytes, i.e. the existence of subtypes of granule with distinct composition (reviewed in Ref. 39).

This study indicates that SG is the dominating PG species in CTLs, thus in agreement with the widely accepted but previously not proven view (see the introduction and Ref. 40). However, our results clearly show that other PG species than SG are also present in CTLs and that these other PG species account for ~15–30% of the cell-associated sulfated GAG chains. The identity of the non-SG PGs is not certain. However, we report here for the first time the expression of glypican-1 and -4 and of syndecan-4 in CTLs, and we may thus hypothesize that the remaining GAGs that are observed in the absence of SG may be attached to either or several of these identified additional core proteins. Syndecan-4 expression has previously been observed in lymphocytes (41), but, to our knowledge, expression of glypican-1 and -4 by lymphocytes has not been reported previously.

Froelich and coworkers have in a series of important studies introduced the concept that granzyme B forms a functional entity with SGPGs. This notion is based on the findings that purified SGPGs as well as free chondroitin sulfate chains interact tightly with granzyme B (19, 23) and is further supported by the finding that granzyme B is exoyctosed in complex with a PG, although the identity of the granzyme B-binding PG was not revealed (15). The present study shows clearly that PGs of the SG species are essential for storage of granzyme B in CTL granule (39), thus in strong agreement with the previous findings of Froelich and coworkers. Considering that the SG knock-out specifically affected the electron dense granule structures without affecting granule compartments of lower electron density, and that granzyme B storage was essentially completely abrogated in SG$^{-/-}$ CTL, it is reasonable to assume that granzyme B is specifically located in the mature electron dense structures but is absent in other types of cytolytic granule. Interestingly however, SG did not appear to be necessary for storage of granzyme A. This might be unexpected considering that granzyme A is a basic protein (granzyme A has a net charge of +15.8 at pH 7.0; mouse granzyme B has a net charge of +20.5) and has previously been suggested to interact with GAGs (18). One possibility would therefore be that granzyme A specifically interacts with PG species other than SG. Another possibility is that the GAG-binding properties of granzyme A differ in some way from those of granzyme B. To address this second possibility we constructed three-dimensional models of mouse gran-
zyme A and B, based on the known structures of the human or rat counterparts (30, 31), and used these models to search for potential GAG binding regions on the molecular surfaces. As shown in Fig. 6A (right panel, marked by a white oval), there is a quite extensive patch surrounded by positively charged side chains on the rear side (opposite side from the side containing the active site) of granzyme B, that might potentially interact with anionic GAGs. Interestingly, the corresponding region of the granzyme A dimer (white oval in Fig. 6B) contains significantly fewer positive charges and an additional number of compensating negative charges. GAGs may thus be less prone to interact with this site in granzyme A than in granzyme B. On the other hand, granzyme A contains contiguous positively charged areas both at the top and bottom of the dimer (Fig. 6B, marked by yellow ovals), and these have a clear potential to mediate interactions with GAGs. It is thus apparent that both granzymes A and B have clearly defined regions of positive charge that may interact with GAGs. However, these areas are located in different regions of the respective proteases, and it cannot be excluded that the different arrangements of the potential GAG-binding surfaces may be related to the differences in dependence on SG for storage.

Based on the high impact of the SG knock-out on granzyme B storage and on cytolytic granule morphology, it may have been expected that SG-deficient CTLs would have an impaired ability to induce apoptosis of target cells. Furthermore, because granzyme B and possibly also perforin is released in complex with a PG (15), it could be speculated that SG acts as a common vehicle for the delivery of these cytolytic compounds. However, we did not see any reduction in cytotoxic activity toward a panel of allogeneic cell lines due to the SG deficiency, thus arguing against a major role for SG in inducing apoptosis. However, these findings do not necessarily exclude a role of SG in inducing apoptosis in vivo. For example, it may be speculated that SG protects granzymes, and possibly other SG-associated compounds, from initiators that are present in vivo but absent in the cell culture medium used in our in vitro cytotoxicity assays. This would be in accordance with the reported protective effect of SGPGs (of heparin type) on mast cell chymase (42). Furthermore, we may speculate that SG in vivo may have a role in anchoring the cytolytic machinery to the vicinity of the proper target cells; this notion is also in accordance with the effect of SGPGs on the localization of mast cell proteases (43).

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