

# Unveiling the Biological Role of Serglycin Proteoglycans

Studies on Serglycin Knock-Out Mice

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## Unveiling the Biological Role of Serglycin Proteoglycans - Studies on Serglycin Knock-Out Mice

### Abstract

Serglycin (SG) proteoglycans (PG) are predominantly located intracellularly in secretory vesicles of hematopoietic cells such as macrophages, cytotoxic T lymphocytes (CTLs), monocytes, basophils, neutrophils and mast cells (MCs). These PGs are characterized by their protease-resistant serine and glycine-rich core and also by their covalently attached negatively charged glycosaminoglycans (GAG) chains.

We showed that SG PGs are the dominant PGs species in CTLs where they contributed to proper sub-structural organization of the secretory vesicles. Furthermore, different granule-specific components exhibited distinct SG PG dependence for storage, where granzyme B levels were shown to be highly affected, while granzyme A or perforin levels were not.

MCs are important immune cells that release preformed mediators upon activation. We confirmed that SG PGs are the major PG species in mucosal-like bone marrow-derived MCs (BMMCs) and their absence leads to defects in the granule organization and inability to store specific granule components (mMCP-5, CPA). However, mMCP-1 and mMCP-7 are not dependent on SG for storage.

SG-dependent granule compounds exert many functions in homeostasis and interactions between immune cells. We noted that older SG<sup>-/-</sup> animals spontaneously developed enlarged peripheral lymphoid organs, namely the spleen and Peyer's patches. Additionally, investigations of the spleen cell population from SG<sup>-/-</sup> revealed an increased population of CD45RC expressing cells but a reduction in CD4 positive cells.

Sorting of SG PGs into secretory vesicles is a poorly understood process. We showed that in the presence of a reducing agent, granules in SG<sup>+/+</sup> BMMCs show a striking resemblance to those found in SG<sup>-/-</sup> cells lacking a defined granular organization. Moreover, CPA's storage was shown to be affected by the presence of the reducing agent, possibly due to its interaction with intra-granular SG PGs.

*Keywords:* serglycin, hematopoietic cells, secretory granules, mast cells, cytotoxic T lymphocytes, immune system, inflammation,

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## Dedication

To my family and friends...because you are the essence of my life

*Whatever you think, be sure it is what you think, whatever you want, be sure that is what you want, whatever you feel, be sure that is what you feel.*

T.S.Elliot

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Grujic M., Braga T., Lukinius A., Eloranta, ML., Knight, SD., Pejler, G., Abrink, M., (2005). Serglycin-deficient cytotoxic T lymphocytes display defective secretory granule maturation and granzyme B storage. *Journal of Biological Chemistry* 280(39), 33411-8.
- II Braga, T., Grujic, M., Lukinius, A., Hellman, L., Abrink, M., Pejler, G., (2007). Serglycin proteoglycan is required for secretory granule integrity in mucosal mast cells. *Biochemical Journal* 403(1), 49 -57.
- III Wernersson, S., Braga, T., Sawesi, O., Nilsson, K., Pejler, G., Abrink, M., (2008). Age-related enlargement of lymphoid tissue and altered leukocyte composition in serglycin-deficient mice. *Manuscript*.
- IV Braga T., Tveit, H., Abrink, M., Ringvall, M., Pejler, G., Reduction with Dithiothreitol Causes Serglycin-Specific Defects in Secretory Granule Integrity of Bone Marrow Derived Mast Cells. *Manuscript*.

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## Abbreviations

BMMC	Bone marrow-derived mast cell
CPA	Carboxypeptidase A
CS	Chondroitin sulfate
CD	Cluster of differentiation
CTMC	Connective tissue mast cell
CTL	Cytotoxic T-cell
DS	Dermatan sulfate
ECM	Extracellular matrix
ES	Embryonic stem
FGF	Fibroblast growth factor
Gal	Galactosamine
GlcA	Glucuronic Acid
GAG	Glycosaminoglycans
HS	Heparan sulfate
HexA	Hexuronic Acid
IdoA	Iduronic Acid
KS	Keratan sulphate
MC	Mast cell
MMP	Matrix metalloprotease
MMC	Mucosal mast cell
mMCP	Mouse mast cell protease
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
NDST	N-deacetylase/N-sulfotransferase
NK	Natural killer
PG	Proteoglycan
SG	Serglycin



# Introduction

## Genetically modified organisms

The discovery that molecules of foreign DNA can exchange parts of its sequence with chromosomal DNA of mammalian cells through a process called homologous recombination (Smithies *et al.*, 1985) opened new “doors” in the world of science, providing a new and useful tool to understand many biological processes. Taking advantage of this natural phenomenon, site-specific modifications could be introduced into the mouse genome and give rise to genetically modified animals (“knockout” if specific gene(s) are deleted; “knockin” if a gene is added).

Embryonic stem (ES) cells are characterized by their pluripotency which means that they can virtually differentiate into any kind of cell. When *in vitro* modified ES cells are placed together with a normal wild-type (WT or +/+) embryo, they will give rise to a “chimera”, which is an embryo containing both modified and normal ES cells. This embryo is then implanted into the uterus of a breeding female and gives rise to genetically modified animals that will carry the genetic modification through subsequent generations.

This technique has been used to address many important questions such as the characterization of regulatory sequences in gene expression, to study the function of specific genes *in vivo*, to characterize functional properties of gene products, and ultimately to produce animal models of human diseases.

In the studies presented in this thesis, the role and characterization of the serglycin (SG) protein was addressed by taking advantage of the previously established SG knock-out (KO or -/-) mouse strain where the SG gene had been deleted in all cells in the whole organism.

## The immune system

Everyday we find ourselves permanently in contact with a wide variety of microbes, ranging from viruses to bacteria and parasites. Our first line of defense comprises the skin and mucosal surfaces, which are efficient barriers in keeping these organisms outside the body. However, when microbes overcome these physical barriers, specialized cells are activated, triggering an immune response.

The immune system comprises two types of components, the innate and the acquired/adaptive response. Innate immunity is constituted of non-specific physical (skin, mucus) and chemical barriers (saliva, hydrochloric acid in the stomach), which block the passage of foreign organisms into our body. The adaptive immune system is specifically directed towards a particular pathogen and its specificity and efficiency increases upon recurrent exposures to the pathogen.

Leukocytes (*leuko* – white; *kytos* – cell) or white blood cells are key effector cells involved in the immune defense of the organism. All white blood cells derive from pluripotent hematopoietic cells in the bone marrow, and two main groups of leukocytes can be distinguished. One group is the lymphocytes, comprising T-cells, B-cells and natural killer (NK) cells. The T-cells are involved in the killing and elimination of degenerated or infected cells, whereas B-cells are mainly involved in the production of antigen-specific antibodies. In contrast to T- and B-cells, mediators of the adaptive immune response, NK cells mediate the killing and destruction of tumor and virus-infected cells as part of the innate immune system. The second group of leukocytes consists of phagocytic cells such as macrophages and monocytes, and the granulocytes (eosinophils, basophils, mast cells (MCs) and neutrophils). While the main function of the phagocytic cells is to ingest and eliminate pathogens, they also serve as antigen-presenting cells to T-cells, constituting a link between the innate and acquired immune response.

Granulocytes designate all the cells that are characterized by the presence of cytoplasmatic granules which are known to serve as reservoirs of important mediators involved in inflammatory processes. Importantly, these vesicles display different staining properties towards specific dyes, reflecting distinct intra-granular contents.

Eosinophils show preference for anionic (acidic) dyes indicating the presence of basic components in their granules, whereas MCs and basophils stain metachromatically with cationic (basic) dyes due to the presence of

intra-granular negatively charged molecules. Moreover, lymphocytes and macrophages are also known to possess such vesicles although these are often not regarded as granulocytes. The study of the underlying mechanisms of granule secretion and organization is therefore crucial in order to increase the knowledge of the immune response.

#### Mast cells and cytotoxic T cells

In general, mature T lymphocytes express either CD4 or CD8 molecules, hence allowing the identification of CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs). CTLs are pivotal cells of the immune system where they monitor all the cells in the body, acting readily against any that is considered a threat to the host; for example, CTLs kill virally infected cells. Moreover, CTLs are known to act against tumor cells due to their ability of recognizing antigenic differences between transformed cells and healthy cells. After maturation in the thymus, CTLs express a unique cell-surface antigen-binding molecule called T cell receptor (TCR), that will recognize antigens bound to the major histocompatibility complex (MHC) I-derived molecules (Moss *et al.*, 1992). It is this highly specific interaction that enables CTLs to recognize malignant cell alterations and target them to destruction (Castelli *et al.*, 2000).

Upon recognition of an MHC-I-peptide complex on a target cell by their TCR, CTLs are triggered to deliver secretory products stored in specialized cytoplasmic vesicles. These organelles store both lysosomal hydrolases which act at acidic pH, as well as other secretory compounds such as perforin, which functions at neutral pH (Peters *et al.*, 1991). CTLs may destroy target cells by one of three mechanisms. Two of these involve direct cell-cell contacts between effector and target cells. The third is mediated by cytokines such as interferon- $\gamma$  and tumor necrosis factor- $\alpha$ . Cytolytic activity involving cell-cell interactions can be mediated by two distinct mechanisms. In one case, CTLs release the lytic protein perforin and granzymes into the intercellular space. Perforin is able to integrate within the lipid bilayer of target cells where it polymerizes and creates pores in the membrane. The formation of such pores is regarded as essential for the granzymes to enter the cytosol of target cells. However, it has been shown that granzymes may be internalized independently of perforin possibly via receptor-mediated endocytosis (Froelich *et al.*, 1996; Shi *et al.*, 1997). The uptake of granular material by the target cell will cause cell death through a caspase-dependent or caspase-independent mechanism (Trapani & Smyth, 2002). Eleven granzymes have been identified in mice, and five in humans (Grossman *et al.*, 2003; Revell *et al.*, 2005), all with

different substrate specificities. The fact that granzymes may mediate apoptosis via alternative mechanisms targeting different substrates, contributes to the effectiveness of CTL-mediated killing.

The regulatory mechanism of these lytic proteins is well elaborated in that they only target and elicit their function in target cells. Firstly, they are released only upon contact of the CTLs with the target cell, and secondly, many of these components are only activated upon release, as they are kept in the secretory organelles in an inactive form. For example, perforin is only active at a neutral pH (Bashford *et al.*, 1988) and it is therefore kept inactive in the granules where the pH is acidic. Also, the presence of certain granule components that are able to entrap these molecules and release them upon activation has been documented. It has been demonstrated that after exocytosis, granzyme B was present as a complex with a proteoglycan (PG) and that this complex is able to induce apoptosis in target cells (Metkar *et al.*, 2002).

The other killing mechanism involves a protein called Fas ligand, which binds to its receptor Fas (or CD95) present in target cells, initiating a cascade of caspase cleaving events that quickly trigger apoptosis of the target cells.

MCs are highly granulated cells of the immune system and are usually located in the interface between the interior and the exterior environment, including the skin, airways and gastrointestinal tract. MCs progenitors arise from the hematopoietic stem cells in the bone-marrow (Kitamura *et al.*, 1981) and migrate to tissues where different types of mature MCs arise due to the influence of local microenvironment factors. Based on their tissue location, granule content and PG content, murine MCs can be subdivided into two major subclasses: connective tissue MCs (CTMCs) and mucosal MCs (MMC). CTMCs are located in connective tissues in the skin and in the peritoneal cavity and express tryptase, carboxypeptidase A (CPA), chymase, high levels of histamine and heparin PG. In contrast, MMCs are present in the lamina propria of the intestine and airways and are known to express chondroitin sulfate (CS) PGs, chymase, but lack tryptase and CPA. Human MCs can also be divided into two major subclasses according to their protease content. MC<sub>T,S</sub> contain only tryptase and they are situated in the lamina propria of the airways and gastrointestinal tract, while MC<sub>TC,S</sub> contain both tryptase and chymase and are found predominantly in the skin (Miller & Pemberton, 2002; Schwartz, 1994).

Table 1. Mast cell heterogeneity in human and mouse

	Mouse		Human	
	CTMC	MMC	MC <sub>T</sub>	MC <sub>TC</sub>
Tryptase	mMCP-6 mMCP-7		+	+
Chymase	mMCP-4 mMCP-5	mMCP-1 mMCP-2	-	+
Proteoglycan	Heparin	CS	Heparin/CS	Heparin/CS
CPA	+	-	-	+

Activation of MCs may occur by several mechanisms. The classical way of activating MCs is through their high affinity IgE receptor, FcεRI, present on the cell surface, leading to the release of a broad range of MC mediators stored in secretory granules. Furthermore, MCs can be activated by IgG receptors, complement proteins namely C3 and C5a (Johnson *et al.*, 1975) and toll-like receptors (Kulka *et al.*, 2004; Marshall *et al.*, 2003; Supajatura *et al.*, 2002). In addition, molecules such as substance P, neurotensin, compound 48/80, calcium ionophores can directly activate MCs (Metcalf *et al.*, 1997).

MC granules contain an abundance of mediators that elicit several functions upon release from the secretory vesicles (histamine, PGs, neutral proteases, lipid mediators and preformed cytokines).

Histamine is possibly the most well-known MC mediator. The physiological effects of histamine include vascular permeability, stimulation of smooth muscle contraction, bronchoconstriction and increased mucus secretion (Bachert, 2002). Moreover, studies using histidine decarboxylase (an enzyme involved in the formation of histamine)-deficient mice demonstrated the importance of histamine in other processes such as angiogenesis (Ghosh *et al.*, 2002) and gastric acid secretion (Tanaka *et al.*, 2002).

MCs also produce a multitude of cytokines. Preformed MC-derived cytokines include TNF-α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and TGF-β (Ishizuka *et al.*, 1999a; Ishizuka *et al.*, 1999b; Kobayashi *et al.*, 2000; Okayama *et al.*, 1995; Razin *et al.*, 1991). MCs cytokines may regulate several functions of other inflammatory cells, giving the MC a very

important role in interplay with other cells involved in the immune response.

The lipid mediators, leukotrienes and prostaglandins, are products derived from arachidonic acid. MCs express predominantly prostaglandin D<sub>2</sub> which can act as a vasodilator and bronchoconstrictor (Johnston *et al.*, 1995), and also promotes accumulation of inflammatory cells. Upon activation, MCs synthesize the cysteinyl leukotrienes LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>. These potent mediators are known to play important roles in several biological processes such as bronchoconstriction (Dahlen *et al.*, 1980), leukocyte recruitment (Medeiros *et al.*, 1999), vascular leakage (Beller *et al.*, 2004) and induction of cytokine production (Mellor *et al.*, 2002; Mellor *et al.*, 2003).

Up to 35% of the total protein content in mast cells corresponds to neutral proteases (Schwartz *et al.*, 1987). One of these proteases is CPA, which is a Zn<sup>2+</sup>-dependent exopeptidase that preferably cleaves substrates that possess C-terminal aromatic or aliphatic residues (Goldstein *et al.*, 1989). Due to its highly negative charge, CPA is found tightly bound to heparin PGs within the MC granule (Serafin *et al.*, 1987) and its activation has been shown to be highly dependent on these PGs species (Henningsson *et al.*, 2002). The biological effects of CPA are many, and include degradation of apolipoprotein B of low density lipoprotein (Kokkonen *et al.*, 1986), degradation of endothelin-1 (Metsarinne *et al.*, 2002) and formation and degradation of angiotensin II (Lundequist *et al.*, 2004).

Chymases are serine proteases with chymotrypsin-like substrate specificity, cleaving substrates at sites following aromatic residues (Powers *et al.*, 1985). Chymases are stored as active proteins in the MCs granules but they are synthesized as inactive precursors. The activation of pro-chymase occurs by the removal of a dipeptide by dipeptidyl peptidase I (Wolters *et al.*, 2001). Chymase has been implicated in a wide range of biological events that include attraction of neutrophils and eosinophils (He & Walls, 1998), extracellular coagulation and fibrinolysis (Tchougounova *et al.*, 2001), fibronectin, plasmin and thrombin degradation (Tchougounova *et al.*, 2003) and together with CPA, in the processing of angiotensin I to angiotensin II (Chandrasekharan *et al.*, 1996; Lundequist *et al.*, 2004).

Tryptases constitute a family of serine proteases characterized by their trypsin-like substrate specificity. Similarly to chymases, tryptases are stored in MC granules as active enzymes. Tryptases described in mice MCs include mouse MC protease (mMCP)-6, mMCP-7, mMCP-11 and mouse

transmembrane tryptase. There are two main types of human MCs tryptases:  $\alpha$ -tryptase and  $\beta$ -tryptases, where the latter is the main type expressed in human. Expression of tryptases differs between human and rodents in that all human MCs express tryptase, whereas in mice, tryptase is only found in CTMCs. Tryptase has been suggested to be involved in many biological processes. Bronchiodilating neuropeptides such as VIP (vasoactive intestinal peptide) and calcitonine gene-related peptide (Caughey *et al.*, 1988) have been shown to be degraded by tryptase leading to increase bronchial responsiveness and to other asthma-related events. Furthermore, tryptase has been suggested to play a role in atherosclerosis by degrading high-density lipoprotein (Lee *et al.*, 2002). Tryptase also cleaves fibronectin (Lohi *et al.*, 1992) and gelatin (Fajardo & Pejler, 2003), thus pointing to a role in tissue remodeling and angiogenesis.

## Glycobiology

Carbohydrates are the most abundant biomolecules occurring in nature. Due to their structural diversity, they have numerous roles in living organisms, such as the storage of energy and as fundamental building blocks of structural components. Furthermore, important biomolecules such as lipids, nucleic acids and proteins contain sugar residues as an integral part of their composition in many living organisms, reflecting the biological importance of carbohydrates. Proteins carrying a carbohydrate portion are the most common glycoconjugates, termed glycoproteins, where the carbohydrate portion is linked to the protein by means of glycosyl linkages.

## Proteoglycans

PGs comprise a wide range of macromolecules found distributed almost everywhere throughout the body. The structural diversity of PGs suggests that they are involved in a multitude of functions and several studies have demonstrated their importance in biological processes due to their ability to interact with a vast variety of biomolecules, namely proteins and components of the extracellular matrix (ECM) (Handel *et al.*, 2005; Iozzo, 2005; Kinsella *et al.*, 2004).

PGs are molecules containing long, unbranched polysaccharide chains (glycosaminoglycans or GAGs) covalently attached to a protein core. These GAG chains are composed of repeated disaccharide units, where each unit

is composed of one alternating amino sugar [N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc)] bound to either a hexuronic acid [HexA, either glucuronic acid (GlcA) or iduronic acid (IdoA)] or to galactosamine (Gal). Importantly, the sugar chains are found attached to the serine (Ser) or threonine residues of the core protein through an O-glycosidic bond (Bernfield *et al.*, 1999; Kjellen & Lindahl, 1991). There is an enormous diversity among PG species due to the variation of the core protein to which the GAG chains are attached, heterogeneity of GAG sulfation and length of the GAG chains. Furthermore, the number of GAG chains bound to the core protein may range from one to more than 100, varying between different types of PG species (Kjellen & Lindahl, 1991; Silbert & Sugumaran, 1995).

In addition to carrying single or multiple GAG chains, several PGs can accommodate GAG chains of more than one type (hybrid PGs), which further accounts for the extreme diversity of these molecules (Rapraeger *et al.*, 1985; Sugahara *et al.*, 1992). Although the underlying mechanism behind this feature is still poorly understood, previous studies have suggested that the amino-acid sequences of the core protein located in defined patches adjacent to the GAG binding site may play an important role (Chen & Lander, 2001; Esko & Zhang, 1996).

PGs can be defined by both their core protein as well as by the GAG side chains attached. According to the repeated disaccharide unit present, PGs can be divided into three main classes: heparan sulfate/heparin, keratan sulfate, and chondroitin sulfate/dermatan sulfate (fig.1).

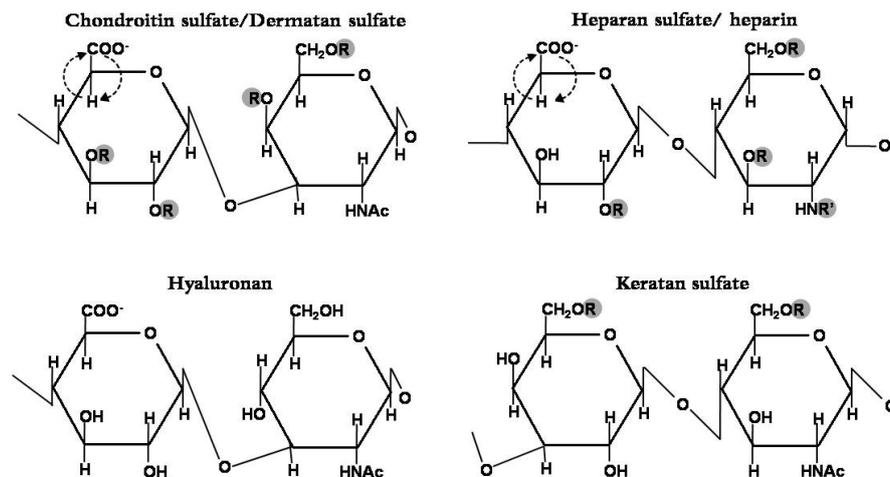


Figure 1. **Repeating disaccharide units of various GAGs.** Every unit is composed of uronic acid and an amino-sugar. Possible modifications of these basic units are represented by R (= H or SO<sub>3</sub>) and R' (= H, COCH<sub>3</sub> or SO<sub>3</sub>).

The GAG species CS and dermatan sulfate (DS) are termed galactosaminoglycans due to the presence of the N-acetyl-galactosamine residue in their composition. They are both derived from the same polymer  $-\text{HexA}\beta 1,3-\text{GalNAc}\beta 1,4-$ , where CS contains invariably GlcA while in DS some of the GlcA is epimerized to IdoA. They can accommodate sulfate groups in several positions of the disaccharide repeat unit, especially at positions 4 and 6 of GalNAc and position 2 of the hexuronic acid. Heparan sulfate (HS) and heparin both possess the same basic structure composed of repeating disaccharide units of  $\text{HexA}\alpha/\beta 1,4-\text{GlcNAc}\alpha 1,4$ . The uronic acid can be either GlcA or IdoA, and the GlcN is either free, N-acetylated (GlcNAc) or N-sulfated (GlcNS). As in CS/DS, both HexA and GlcN residues in HS/heparin can be modified with sulfate groups. The extent of epimerization of GlcA to IdoA and the degree of sulfation of the disaccharide units is used as the base to distinguish HS from heparin and CS from DS.

Keratan sulfate (KS) lacks the HexA unit, where the two repeating sugars are GlcNAc and Gal ( $-\text{Gal}\beta 1,4-\text{GlcNAc}\beta 1,4-$ ), having variable carbohydrate lengths and sulfation patterns. Two patterns of KS can be distinguished: KS I, which binds through an N-glycosyl linkage to the asparagine residues of the core protein, and KS II, which is found attached to a serine or threonine residue through an O-glycosyl linkage (Choi & Meyer, 1975). Hyaluronan is the only GAG that is synthesized in a free form, i.e, not covalently attached to a core protein. Its non-sulfated structure is made up of repeating disaccharide units of GlcA and GlcNAc ( $-\text{GlcA}\beta 1,3-\text{GlcNAc}\beta 1,4-$ ).

## Biosynthesis of proteoglycans

The biosynthesis of GAG chains takes place in the Golgi apparatus and consists of a multi-step mechanism involving the combined action of an extensive enzymatic machinery (Esko & Lindahl, 2001; Lindahl *et al.*, 1998; Prydz & Dalen, 2000). The first part of CS/DS and heparin/HS chain polymerization involves the formation of the linker tetrasaccharide (xylose-galactose-galactose-GlcA). The formation of this linkage oligosaccharide starts with the transfer of a xylose from UDP-xylose onto the hydroxyl

group of a serine residue in the core protein and occurs during the transfer of the latter from the rough endoplasmic reticulum to the cis-Golgi compartment (Lohmander *et al.*, 1989; Vertel *et al.*, 1993). Completion of the linkage tetrasaccharide involves the addition of two galactose units from UDP-galactose and the last member, GlcA is added by the enzyme glucuronosyltransferase I (Sugahara *et al.*, 2003). Once the linker tetrasaccharide is completed, the addition of the fifth saccharide unit determines whether the GAG chain becomes CS/DS or heparin/HS. This involves the action of either of the two distinct transferases, GlcNAc transferase or GalNAc transferase, resulting in the initiation of either CS/DS sulfate or heparin/HS sulfate, respectively (Lander & Selleck, 2000; Sugahara *et al.*, 2003), as shown in fig.2.

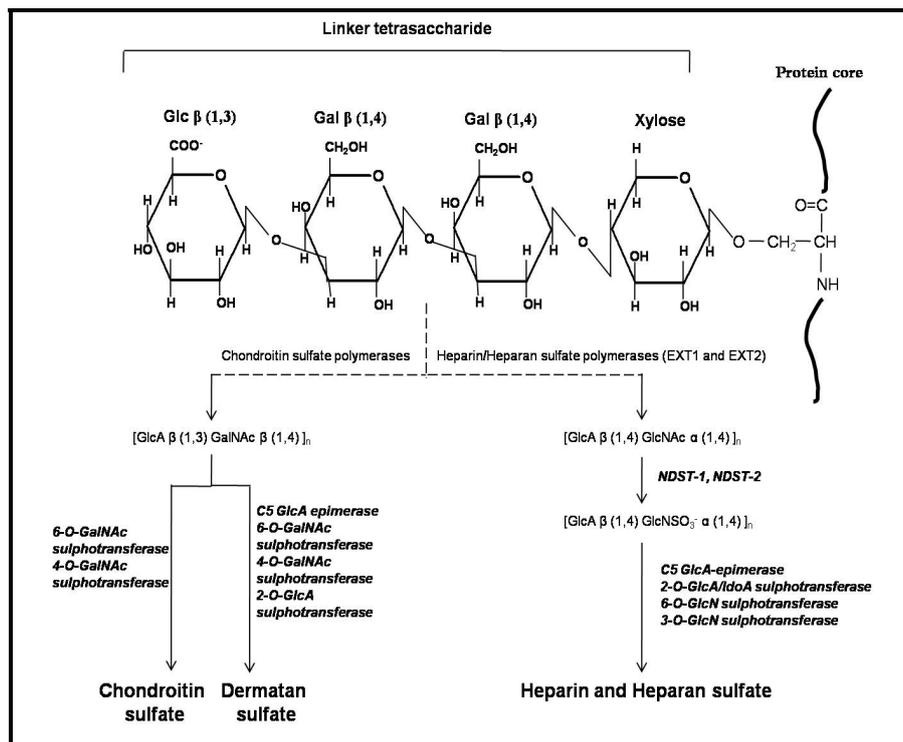


Figure 2. A general scheme representing the key steps in the synthesis of chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate GAGs.

In the case of heparin/HS, the chain is elongated through the action of GlcNAc and GlcA transferases (EXT1 and EXT2) and subsequently modified. The first modification reaction involves the enzyme N-deacetylase/N-sulfotransferase (NDST) and replaces N-acetyl groups on GlcNAc units with N-sulfated groups. This modification is extremely important since subsequent modifications can only take place in the proximity of N-sulfate groups. Four isoforms of the enzyme family of NDSTs have been identified in mice (Aikawa *et al.*, 2001; Eriksson *et al.*, 1994; Hashimoto *et al.*, 1992) : NDST-1 and NDST-2, expressed in embryonic and adult tissue; and NDST-3 and NDST-4 predominantly expressed during embryogenesis. Previous studies have shown that NDST-2 is involved in the generation of longer GAG chains with higher N-sulfate content when compared with NDST-1 (Pikas *et al.*, 2000). Heparin is mainly expressed in rodent CTMCs and CTMCs from mice lacking NDST-2 showed impairment in the storage of MC-specific proteases, namely CPA, mMCP-4, mMCP-5 and mMCP-6. Moreover, it was shown that some of the proteases require the presence of heparin to exert their biological role. For example, tryptase is dependent on heparin in the activation of biologically important tetramers, while the chymase-heparin complex was shown to play an important role in extravascular coagulation (Hallgren *et al.*, 2000; Tchougounova *et al.*, 2001; Tchougounova & Pejler, 2001). NDST-1 deficiency, on the other hand, was shown to lead to defects in lung and skull causing embryonic death, and also to a decrease in HS in basement membranes indicating that this enzyme is important in HS synthesis (Fan *et al.*, 2000; Ringvall *et al.*, 2000).

Once the modification reactions are initiated by an NDST, the other enzymes are able to catalyze the subsequent steps of the GAG chain synthesis. Further modifications steps include the C5-epimerization of GlcA to IdoA, 2-O-sulfation of IdoA and GlcA and 6-O-sulfation of GlcNS and GlcNAc residues. Occasionally, other modifications can occur, such as the 3-O-sulfation of GlcNSO<sub>3</sub>. Regions where the GlcNAc residues are not deacetylated remain almost unmodified while regions modified by NDSTs are extensively modified. The end result is that heparin is highly sulfated, whereas HS is sulfated to a less extent. However, HS can be highly sulfated in defined blocks.

Although both HS/heparin and CS/DS biosynthesis are initiated by the common linker tetrasaccharide, elongation and modification of CS/DS

involves different enzymes than those involved in HS/heparin synthesis. The addition of the initial GalNAc from UDP-GalNAc onto the linkage oligosaccharide is mediated by distinct enzymes (CS polymerases) (Rohrman *et al.*, 1985). Subsequent chain elongation then requires two glycosyltransferase enzymes to add the alternating residues of GlcA and GalNAc. In DS, like in HS, the generation of IdoA units occurs by epimerization at C5 of a portion of GlcA residues of the chain (Malmstrom, 1984; Malmstrom & Fransson, 1975). Additionally, distinct sulfotransferases (STs) add sulfate groups to the chain backbone residues usually of either the 4- and/or 6-hydroxyl of the GalNAc residues and at C2 of GlcA and IdoA. Eight CS/DS STs have been identified so far, one 2-O-ST, three 6-O-STs and four 4-O-ST (reviewed in (Kusche-Gullberg & Kjellen, 2003)).

## Cell surface proteoglycans

Cells need to build barriers that allow them to separate from other cells and also from the surrounding environment. At cell surfaces, two major families of membrane-bound HSPGs are found: the syndecans and the glypicans (fig.3).

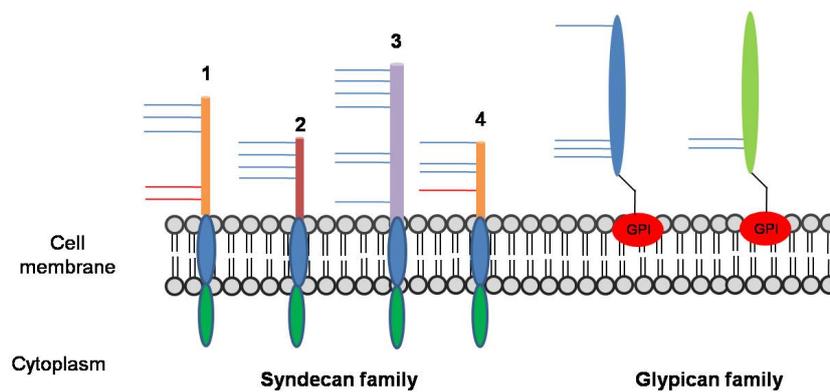


Figure 3. Representative scheme of cell-surface associated PGs. Heparan sulfate (light blue) and chondroitin sulfate (red) are the most common glysoaminoglycan chains found in these PGs.

Syndecans are type I transmembrane HSPGs forming a group of extracellular effector molecules harboring primarily, but not exclusively, HS

chains (Bernfield *et al.*, 1992). They possess an extracellular domain with GAG attachment sites, a hydrophobic transmembrane domain and a short cytoplasmic domain. So far, four mammalian syndecans (syndecan-1 to -4) have been identified and cloned (Mali *et al.*, 1990; Pierce *et al.*, 1992; Saunders *et al.*, 1989). The extracellular domain contains several Ser-Gly sequences where GAG chains bind and an N-terminal signal peptide that targets the extracellular domain outside the cell. Syndecan-1 and -3 exhibit two clusters bearing GAG attachment sites while syndecan-2 and -4 only possess one located near the plasma membrane. Syndecan-1 and -4 can, in addition to HS, bear CS chains (Carey, 1997). Sequences of transmembrane and cytoplasmic domains, as well as GAG attachment sites have been shown to be highly conserved between all the syndecan family members and species.

Syndecans have been shown to be involved in many biological functions such as cell-cell and cell-matrix adhesion and signaling. A vast range of extracellular proteins can bind to syndecan-1, via its HS chains, thus serving as a matrix receptor. Among the ECM components that can bind to syndecan, we can find collagen types I and III (Koda *et al.*, 1985), fibronectin (Saunders & Bernfield, 1988) and laminin (Elenius *et al.*, 1990). Furthermore, the cytoplasmic domain of syndecans are known to engage in interactions with intracellular components and are involved in processes such as cytoskeleton organization (Grootjans *et al.*, 1997) or in signaling pathways in response to antibody ligation (Carey *et al.*, 1996). Syndecans can also modulate the action of several growth factors. Growth factors such as fibroblast growth factor (FGF) family members, vascular endothelial growth factor (Gitay-Goren *et al.*, 1992), hepatocyte growth factor (Ashikari *et al.*, 1995) and others are known to bind to extracellular HS chains. Syndecan-1, -3 and -4 have been shown to bind specifically FGF-2 (Kiefer *et al.*, 1990) while syndecan-3 can bind to heparin-binding growth-associated molecule (Kinnunen *et al.*, 1996). Besides growth factors and cell adhesion molecules, syndecans are also found to bind other extracellular ligands which include low-density lipoprotein (Saxena *et al.*, 1990) acting as a lipoprotein receptor, or serine proteases and their inhibitors (serpins) (Kainulainen *et al.*, 1998). Moreover, tissue repair processes require regulated action of extracellular factors and proteases that can be modulated and activated by the presence of syndecans in the wound repair site. Virtually all tissues and cells express at least one syndecan, although some cells and tissues can express multiple family members (Kim *et al.*, 1994).

Glypicans constitute a family of HSPGs that are linked to the exocyttoplasmatic membrane through a glycosylphosphatidylinositol-anchor (David, 1993). Unlike syndecans, glypicans have their GAG attachment site located in close proximity to the cell surface. One striking feature of the glypican family is the presence of two to five consensus regions for insertion of GAG chains near the C-terminal and also the high degree of conservation of their primary polypeptide sequences. Six known glypicans have been identified (glypican-1 to -6) in mammals (David *et al.*, 1990; Saunders *et al.*, 1997; Stipp *et al.*, 1994). HS is the only GAG chain type known to be bound to glypicans *in vivo*, although *in vitro* experiments indicated that the insertion of CS chains is also possible (Saunders *et al.*, 1997). With regard to their expression, glypican-1 and -4 are known to be expressed in a large number of embryonic and adult tissues (Litwack *et al.*, 1998; Veugelers *et al.*, 1998), glypican-2 only in developing nervous system (Stipp *et al.*, 1994), glypican-3 is widely expressed during development (Li *et al.*, 1997) and glypican-5 expression is restricted to the nervous system, limb and kidney (Saunders *et al.*, 1997).

Glypicans, like syndecans, can also bind to FGF-2 and other heparin-binding growth factors (Brunner *et al.*, 1994; Steinfeld *et al.*, 1996). Furthermore, glypicans can also interact with molecules involved in cell adhesion and migration as, for example, the glypican-1 interaction with laminin (Carey *et al.*, 1993), and influence the extension process in Schwann cells. Although most of the reported interactions of glypicans with other components are associated with the GAG chains, it has been postulated that the core proteins could exert some GAG-independent functions (Stipp *et al.*, 1994). The reason for such an assumption resides in the fact that the protein sequence of the core protein of glypicans is highly conserved within all members of the glypican family (Karthikeyan *et al.*, 1992; Veugelers *et al.*, 1998) and that the insertion sites of GAG chains are only found near the C-terminus.

## Extracellular matrix proteoglycans

Perlecan is one of the most studied and best-characterized PG and it is found in all basement membranes and many extracellular matrices (Hassell *et al.*, 2002; Iozzo *et al.*, 1994).

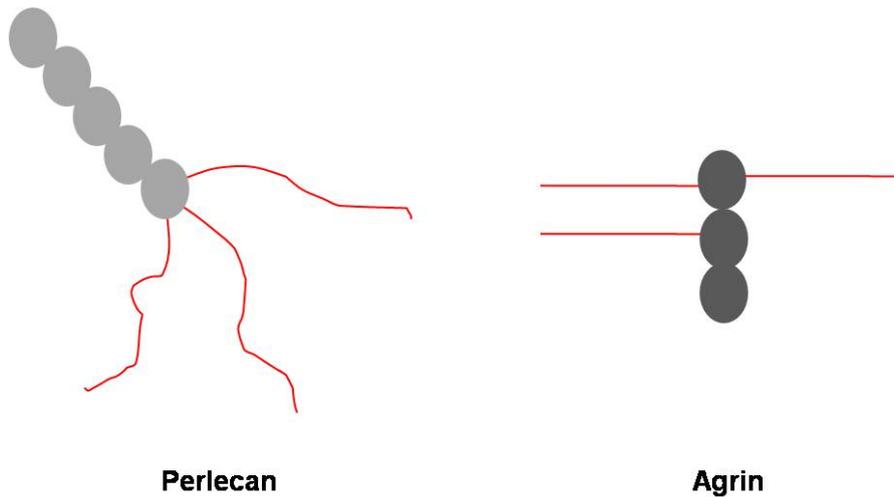


Figure 4. Schematic representation of perlecan and agrin, two well-characterized extracellular matrix-associated PGs.

The perlecan core protein (fig.4) contains five domains: one unique domain located at the N-terminal region and four other domains that are also found in other proteins. Studies using recombinant domain I expressed in several cell types demonstrated that this N-terminal domain contains three potential sites that can support both HS and CS chains.

Perlecan can be found in many extracellular matrices and it has been shown to interact with other ECM components. It has been reported that perlecan's core protein as well as its HS chains bind to fibronectin and laminin (Isemura *et al.*, 1987). Another ECM molecule shown to interact with perlecan is thrombospondin, which is localized at the cell surface of endothelial cells, and this interaction is dependent on perlecan's HS chains (Vischer *et al.*, 1997). Besides ECM components, perlecan is known to engage in interactions with several growth factors and cytokines. FGF family members were shown to require heparin or HS binding in order to be activated by their receptors. It appears that perlecan plays a very important role in the regulation of FGF stimulation not only through the binding of FGF directly to the HS chains, but also in being able to capture the growth factor in the basement membrane (Whitelock *et al.*, 1996). Moreover, platelet-derived growth factor (Gohring *et al.*, 1998),

transthyretin (Smeland *et al.*, 1997) and interferon- $\gamma$  (Lortat-Jacob *et al.*, 1991) display binding properties towards perlecan molecules. There has also been demonstrated a role for perlecan in cell-matrix adhesion interactions through its binding to integrin family members, particularly  $\beta 1$  and  $\beta 3$  integrins, which are critical for the proper formation of the basement membrane structure.

Agrin (fig.4) is a molecule that plays a key role in the aggregation of acetylcholine receptors during the development of the neuromuscular junction during embryogenesis (Nitkin *et al.*, 1987). Agrin consists of a 225 kD core protein containing two or possibly three HS side chains and the core protein possesses certain domains that are found in several other matrix proteins, such as laminin and perlecan (Bork & Patthy, 1995; Rupp *et al.*, 1991). Serine residues located at the N-terminal can serve as attachment sites for both HS and CS, although not all Ser-Gly sequences are substituted with GAG chains. One important feature in agrin is its splice variants and there are three alternate splicing sites, one located near the N-terminus, and the other two near the C-terminus of the core protein. Agrin is expressed in the basement membranes and extracellular matrices of a wide range of tissues which include neuronal, muscle and kidney (Bowe & Fallon, 1995). Agrin has been implicated in ionic filtration mechanisms in the kidney where it is the major HSPG found in the glomerular basement membrane (Groffen *et al.*, 1998; Raats *et al.*, 1998). Additionally, agrin has been shown to play an important role in cell-cell interactions and as a stabilizer of the basement membrane due to the ability of its HS chains to interact with components such as laminin and fibronectin. Furthermore, it has been implicated in the clustering of acetylcholine receptors in the synaptic basement membrane (Ma *et al.*, 1993).

Collagen XVIII is an ubiquitous basement membrane component, detected in all vascular and epithelial basement membranes. It belongs to the non-fibrillar group of collagens which are characterized by their heterogenic structure but share in common the presence of one or more non-collagenous sequences that interrupt the collagenous sequence. Collagen XVIII, like collagens IX and XII, contain Ser-Gly sequences that are potential attachment sites for GAG side chains. Nevertheless, unlike collagens IX and XII, which are sensitive to chondroitinase ABC indicating the presence of CS or DS side chains, collagen XVIII is resistant to chondroitinase ABC digestion (Oh *et al.*, 1994). Studies showed the presence of numerous acidic residues in the core protein flanking the Ser-Gly attachment sites, characteristic of HSPG.

Like agrin and perlecan, collagen XVIII is involved in the ionic filtration in the kidney, where it is found in the glomerular basement membrane. It has also been suggested to play a role in the regulation of signaling between mesenchyme and epithelium during organ morphogenesis (Lin *et al.*, 2001). Collagen XVIII is known to exist in three splice variant forms, which are expressed differently in different tissues. A short form is found mainly in basement membranes of blood vessels and in muscle while a long variant is primarily expressed in the liver sinusoids. Each variant contains a different signal peptide accounting for its tissue-specific expression (Rehn *et al.*, 1996). Endostatin, a 20 kD polypeptide known to inhibit endothelial cell proliferation, is a product of the proteolytic cleavage of the C-terminal of collagen XVIII, and it may act as a potential inhibitor of angiogenesis and cell growth (O'Reilly *et al.*, 1997; Zatterstrom *et al.*, 2000).

## Proteoglycans in other physiological contexts

Aggrecan is the large aggregating PG present in the cartilage and it is the first PG to have been identified and extensively characterized. Each aggrecan molecule possesses sulfated GAG chains and N- and O-linked oligosaccharides that account for up 90% of its mass. The large aggrecan core protein is commonly modified by the covalent attachment of approximately 100 CS chains, 30 KS chains and 8-10 shorter N- and O-linked oligosaccharides (Doege *et al.*, 1987). Aggrecan exerts a very important role in skeletal development and it is essential for the function of cartilage. Aggrecan molecules form large link protein-stabilized aggregates with hyaluronan in the ECM resulting in a unique structure able to entrap water molecules in this space. Due to its structural properties, aggrecan promotes the concentration of negative charges, maintaining cartilage hydration and endowing its characteristic resilience and ability to expand tissue volume.

Versican belongs to the family of large aggregating PGs named lecticans (Ruoslahti, 1996) and several versican core proteins have been identified due to the fact that it is expressed as several variants as a result of alternative splicing. The differences found in the isoforms reside in the middle portion of the core protein, where the GAG attachment sites are located. In versican V0, two CS-carrying portions are present, while the smaller V1 and V2 isoforms lack the GAG- $\alpha$  and GAG- $\beta$  respectively. However, none of the GAG-binding sites are included in versican V3, and this isoform may

therefore not be a PG (Zako *et al.*, 1995). As assessed by enzymatic digestion, the GAG chains present in versican family members were identified as being CS. Versican may interact with a number of different ligands either by specific core protein domains or by the GAG side chains. The most-well characterized interaction is between versican and hyaluronan, which involves the presence of link-protein, in analogy to the hyaluronan-aggrecan protein complex in cartilage (Morgelin *et al.*, 1989). Data retrieved from several studies point to a role of versican PGs in a wide range of biological events, such as cell adhesion, proliferation and migration. It has been shown that versicans, more exactly isoforms V0 and V1 affect the binding of various types of cells to collagen I, fibronectin and laminin (Yamagata *et al.*, 1989). This inhibition has been attributed to the interaction of the CS side chains with these ECM components, since chondroitinase ABC digestion abolished this effect. Furthermore, versican isoforms may also be involved in the control of cell proliferation specially in keratinocytes and dermal fibroblast proliferation (Zimmermann *et al.*, 1994). In addition to their involvement in such processes, it has been demonstrated that an increased versican expression pattern is correlated with smooth muscle cell proliferation during the formation of atherosclerotic lesions (Matsuura *et al.*, 1996).

Nervous tissues express many PGs. Although HSPGs can also be detected in detergent extracts of the membrane fraction of rat brain, 16 out of 25 putative PG core protein were identified as CSPGs (Herndon & Lander, 1990). Neurocan and brevican belong to the lectican family of CSPGs which include other members such as aggrecan and versican and they are both expressed in the nervous system in a highly specific manner (Rauch *et al.*, 1991; Seidenbecher *et al.*, 1995). Both neurocan and brevican share structural homologies with other members of the lectican family but the central domains show little homology with each other. Brevican is also called a “part-time” PG since it can be found in brain tissue without CS chains attached to the core protein (Yamaguchi, 1996). The interaction of these two CSPGs with other components has been established and neurocan is known to interact with N-CAM (Grumet *et al.*, 1993), Nr-CAM and amphoterin (Milev *et al.*, 1998) while brevican has been suggested to be a relevant ligand for tenascin-R (Hagihara *et al.*, 1999). NG2 is another transmembrane CSPG expressed in the nervous system although it can be found in skeletal myoblasts, developing cartilage and aortic smooth muscle cells (Levine & Nishiyama, 1996) and is known to

interact with various ECM and cell surface molecules that include type V and type VI collagens (Stallcup *et al.*, 1990).

Decorin and biglycan are two highly homologous core proteins belonging to the superfamily of small leucine-rich PGs and can be present in non-glycosylated forms (Roughley *et al.*, 1993). They have N-terminal domains which contain CS/DS GAG chains, but decorin can accommodate only one chain while biglycan has two GAG chains. Decorin is mainly found in many connective tissues and it has been suggested to be important in organogenesis (Scholzen *et al.*, 1994) and regulation of cell division and differentiation (Yamaguchi *et al.*, 1990). Biglycan is, in contrary to decorin, found on the surfaces of differentiating cells and it has been shown to interact with type I collagen and also to C1q, and can therefore play a role in inflammation events (Hocking *et al.*, 1996).

CD44 is a so-called “part-time” PG and it is a cell surface glycoprotein where it can act as an adhesion molecule and signal transducer in the immune system (Haynes *et al.*, 1989; Lesley *et al.*, 1993). The GAG chains, when present, can be either CS or HS. Ligands for CD44 include ECM components such as hyaluronan (Aruffo *et al.*, 1990), fibronectin (Jalkanen & Jalkanen, 1992) and collagen types I and VI (Wayner & Carter, 1987).

## Intracellular proteoglycans – Serglycin

PGs in intracellular cell compartments have been subject of increasing attention during the last years. The most important and the only known committed PG identified in intracellular locations so far is serglycin (SG). SG PGs are characterized by their unique protease-resistant, Ser and Gly rich protein cores, and by the covalently attached highly sulfated GAGs.

### The Serglycin gene

The SG PG was first discovered as a secretory and membrane-associated product isolated from the rat L2 yolk sac carcinoma cell line (Oldberg *et al.*, 1981), and it was the first PG gene to be cloned (Bourdon *et al.*, 1985). Using a gene-specific probe of this cDNA, related transcripts were found to be expressed in a wide range of cells of hematopoietic origin containing

secretory granules (Tantravahi *et al.*, 1986). The mouse SG gene, cloned and characterized from a mouse liver genomic library, consists of three exons separated by two introns (Avraham *et al.*, 1989). The first exon consists of 41 base pairs and codes for the first 25 amino-acid residues, which comprise the signal peptide of the core protein. A large (~8 kb) intronic sequence follows before the second exon, which codes for the N-terminal part of the protein (48 amino-acids). The third and last exon is preceded by an intronic sequence around 4 kb and encodes for a 79 amino-acid sequence that contains the GAG attachment site. The human SG gene consists of the same three exons as the mouse gene and both genes lack the classical TATA box. Also, the 5'-UTR of the human and mouse SG gene were found to share a high degree of similarity (96%) (Nicodemus *et al.*, 1990) implying that the sequence contains numerous cis-acting regulatory elements that are essential for the expression of the gene in different cell types. These elements may, depending on the cell type, either induce or suppress the expression of the gene (Avraham *et al.*, 1992). In fact, mouse MCs express at least three GATA factors (Zon *et al.*, 1991) where GATA-2 was shown to be essential for the development of MCs from bone marrow progenitors (Tsai & Orkin, 1997) and other factors may be important for the transcription of some MC specific genes such as CPA (Zon *et al.*, 1991) and the chymase gene (Caughey *et al.*, 1997; Liao *et al.*, 1997). Furthermore, the discovery that GATA-1, GATA-2 and/or GATA-3 are expressed in SG-containing cells (Martin *et al.*, 1990) together with proved the existence of a conserved GATA motif within the SG gene (Nicodemus *et al.*, 1990), suggests that DNA-binding proteins from the GATA family are involved in the transcription of the SG gene in hematopoietic cells. Interestingly, other cell types known not to express the SG gene do not express these transcription factors, but others such as GATA-4, GATA-5 and/or GATA-6.

#### Modifications of Serglycin

SG PGs examined to date show heterogeneity in molecular size due to variations in the number of chains per core and also the length and sulfation of each chain. The GAGs found attached to SG PGs are chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitin 4,6-sulfate, CS B, HS or heparin that display different negative sulfation patterns, where heparin is the most negatively charged GAG found in the body (Kolset & Gallagher, 1990).

Table 2. Main GAG chains found in serglycin in storage granule cells (adapted from (Kolset & Tveit, 2008))

Type of GAG chain	Cell type
Chondroitin 4-sulfate (GlcA-GalNAc 4-sulfate)	Platelets, monocytes, lymphocytes, NK cells
Chondroitin 6-sulfate (GlcA-GalNAc 6-sulfate)	Guinea pig platelets
Chondroitin sulfate E (GlcA-GalNAc 4,6-sulfate)	Mast cells, macrophages
Chondroitin sulfate B (IdoA-2-sulfate-GalNAc 4 sulfate)	Rat basophils
Heparin/Heparan sulfate (GlcA/IdoA $\pm$ 2-O-sulfate-glucosamine $\pm$ N-sulfate and/or 3 and 6-O-sulfate)	Mast cells, macrophages

Variations in the GAG composition may be due to a cytokine-controlled event that appears to be not entirely dependent on the protein core. In fact, c-kit ligand/SCF and other fibroblast-induced factors induce mouse MCs to incorporate heparin onto SG (Levi-Schaffer *et al.*, 1986), whilst IL-3 leads to the incorporation of chondroitin 4,6-sulfate onto the same core protein (Razin *et al.*, 1982). Other PGs are known to possess a specific amino-acid sequence that determines the type of chains that will be attached to the protein core (Esko & Zhang, 1996). Since SG has only one GAG-attachment region and any type of GAG can be attached to it (table 2), it is unlikely that the primary structure of SG determines which type of GAG to be polymerized onto the protein core. Consequently, posttranslational modifications of specific amino-acid residues in the SG protein core may dictate which type of GAG chain that will be synthesized. Depending on the cell type, different disaccharide units can be assembled onto the SG protein core. Basophils and MCs are known to contain highly sulfated GAG chains whereas NK cells and CTLs possess less sulfated GAGs attached to

the protein core. Interestingly, however, distinct subtypes of MCs present different GAG profiles, suggesting a role of the GAG structures as an indicator of cell differentiation. Despite these different MCs subtypes being involved in similar immunological events, CTMCs are known to express only heparin chains attached to the SG core and are the only cells able to do so, whilst MMCs contain GAGs of the CS type (Enerback *et al.*, 1985). In contrast, human MCs can display both GAG types in the same MC subclass (Stevens *et al.*, 1988). The use of gene-targeting approaches allowed a more thorough investigation regarding the mechanism and the enzymes involved in the GAG synthesis onto these PGs. Disruption of the NDST-2 gene led to severe defects in the storage of granule components in CTMCs, while CS-rich MMCs possessed normal granules. However, the levels of glycosyltransferases and sulfotransferases in MCs does not appear to be a determining factor when it comes to directing the synthesis of a particular GAG onto the protein core. Evidence for this fact came from studies where NDST-2 mRNA levels were found to be higher in MCs that predominantly express more CS on SG than heparin. Also, isolated rat peritoneal MCs were shown to possess all enzymes necessary for the synthesis of CS E even though they do not synthesize it onto SG protein cores (Stevens & Austen, 1982; Stevens *et al.*, 1983).

#### Serglycin as storage matrix of intragranular components

The role of SG PGs as storage matrices for several cell components has been elucidated in recent years due to the generation of SG KO mice (Abrink *et al.*, 2004) and MCs were the first cells to be used as a model to determine some of the functions of SG PGs. Because MC-derived SG PGs are the most negatively charged molecules in mammals, they are thought to be involved in the formation of macromolecular complexes with positively charged proteases in the granules. In this study, it was shown that CTMCs lacking SG had an abrogated ability to bind cationic dyes, indicating that SG PGs are the major PG species in the cytoplasmic granules of CTMCs. Although mRNA expression of MC specific proteases was not affected by the absence of SG, defects were seen in the storage of these proteases, namely mMCP-4, mMCP-5, mMCP-6 and CPA. This is in accordance with the studies performed in the NDST-2-deficient MCs where, besides altered granule morphology and staining properties, protease storage was abrogated due to the lack of highly sulfated heparin chains. Further studies using SG KO mice revealed that elastase, a protease present in the azurophil granules of neutrophils, is dependent on SG PGs existing in the

cytoplasmatic granules of these cells to be properly stored. Interestingly, when injected with gram-negative bacteria (*Klebsiella pneumoniae*), the virulence was increased in SG KO mice when compared with wild-type mice indicating that also the immune response is affected by the absence of SG (Niemann *et al.*, 2007).

#### Serglycin and granule formation

Dense-core secretory granules are specialized organelles of endocrine, neuroendocrine and other secretory cells that are responsible for the regulated secretion of several cargo molecules. More specifically, hematopoietic cells are known to store a wide range of specific mediators that upon activation are released to the site of action where they exert their biological functions. Granule components such as hormones, peptides and other enzymes are packaged into secretory granules at the trans-Golgi network through a complex and well regulated mechanism. The correct targeting and packaging of such mediators is of crucial importance for the proper assembly of a functional secretory vesicle. Dense-core secretory vesicles share a distinct characteristic in that their core that appears dark or dense in electron micrographs.

SG was seen to co-localize with the Golgi apparatus in immature human neutrophils (Niemann *et al.*, 2004) but was absent in cells with a higher degree of maturation, suggesting that SG may play a role in early events of granule formation. Importantly, the packaging and segregation of particular granule components may require the presence of SG for the correct accommodation of these mediators into secretory vesicles. The role of SG in granule formation was also studied in bone-marrow derived CTMCs where it was seen that lack of SG did not interfere with the formation of secretory vesicles (Henningsson *et al.*, 2006). Interesting, however, was the observation that SG mRNA levels could already be detected at day 0 of bone-marrow cultures suggesting that SG may play a role also in early events of hematopoietic cell development. Further studies on neutrophils revealed that lack of SG leads to defects only in one type of granules, the azurophil granules, whereas other secretory vesicles were not affected (Niemann *et al.*, 2007). This indicates that within a cell type there seems to exist heterogeneity within secretory vesicles, where SG may or not play an important role in the aggregation of specific granule components. Further support of this notion came from studies on MCs, where SG KO bone-marrow derived CTMCs displayed exclusively non-stainable empty-appearing vesicles lacking electron dense cores as assessed by electron

microscopy (Henningsson *et al.*, 2006), while these structures are present in lower numbers in SG wild-type MCs. Interestingly, previous reports have suggested that MC specific proteases may be located in different locations within the same granule pointing to a well-regulated and oriented granule organization of MCs mediators inside the secretory organelles (Whitaker-Menezes *et al.*, 1995).

#### Serglycin and activation of granule components

Many cell types exert their biological functions through the release of a wide range of mediators stored in secretory vesicles. Although being established as a storage matrix for such components in secretory vesicles, SG PGs are also thought to play an important role in the presentation and activation of some biologically active substances. As previously mentioned, the ability for bacteria clearance is severely affected by the absence of SG, which may indicate that stored components such as elastase cannot act against such pathogens, possibly because they require SG for storage or activity (Niemann *et al.*, 2007). A recent study in macrophages revealed that SG plays a role in the regulation of macrophage tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) where the secretion of this mediator in response to lipopolisaccharide stimulation is increased in SG KO macrophages (Zernichow *et al.*, 2006). Furthermore, lysozyme is released and remains bound to PGs when exocytosed by macrophages (Lemansky & Hasilik, 2001). Another well-characterized example of the importance of SG PGs in modulating biological functions of granule components involves certain MC specific proteases. Previous studies have demonstrated that MC proteases are released in macromolecular complexes with PGs during MC degranulation (Schwartz *et al.*, 1981). Interestingly, MC-derived heparin PGs were shown to promote chymase cleavage of certain substrates by simultaneously interacting with both chymase and its positively charged substrates, facilitating the contact between both components and reducing electrostatic repulsions (Pejler & Sadler, 1999). Moreover, the presence of heparin PGs after release from MCs is thought to have a protective role against some protease inhibitors, preventing their binding to the proteases (Lindstedt *et al.*, 2001; Pejler & Berg, 1995). Matrix metalloproteases (MMPs) are important mediators in tissue remodeling and inflammation processes. It has been shown that certain MCs proteases are involved in the processing and activation of these mediators, namely that the activation of proMMP-9 and proMMP-2 was abrogated in mice lacking the chymase

mMCP-4 (Tchougounova *et al.*, 2005) which is known to be SG-dependent, and that MC-dependent processing of proMMP-2 was shown to be affected in SG-deficient mice (Lundequist *et al.*, 2006).

SG has also been suggested to participate in cytotoxic events mediated by CTLs. CTL-mediated cytotoxicity of infected or tumor cells involves the action of perforin and granzyme B and it has been shown that both are exocytosed in a macromolecular complex with SG PGs (Metkar *et al.*, 2002; Raja *et al.*, 2002). Importantly, granzyme B was shown to enter target cells independently of perforin (Froelich *et al.*, 1996) and upon release, granzyme B was still present as a complex with a PG (Metkar *et al.*, 2002). Additionally, a complex formed by granzyme B and free chondroitin 4-sulfate was shown to induce apoptosis in Jurkat cells (Galvin *et al.*, 1999). Moreover, a protease-resistant PG thought to be SG was shown to be exocytosed by NK cells when incubated with target cells, suggesting that SG may also be involved in effector mechanisms of NK-induced killing (MacDermott *et al.*, 1985; Schmidt *et al.*, 1985).

Furthermore, SG mRNA was shown to be present in endothelial cells and SG was seen to co-localize with tissue type plasminogen activator (Schick *et al.*, 2001). Given that in endothelial cells, SG is constitutively secreted (Schick *et al.*, 2001), it has been proposed that, similarly to MC MMPs, SG may play an important role in tissue remodeling and cell migration events involving this protease. Another cell migration molecule known to interact with SG is CD44, which is a cell surface adhesion molecule expressed in a wide range of cell types. Previous studies have demonstrated that SG interacts with CD44 present on the cell surface of cells, leading to degranulation events on CD44 positive CTL clones (Toyama-Sorimachi *et al.*, 1995). These findings further suggest that extracellular SG may be involved in cell-cell interaction and activation of lymphoid cells.

#### Turnover of exocytosed Serglycin

Little is still known about the fate of SG after exocytosis from MCs, macrophages and other cell types. It has been shown that when SG is injected into the blood stream of rats, liver sinusoidal cells are able to capture it and degrade it, contributing to the reduced levels that are found in plasma (Oynebraten *et al.*, 2000). 90% of the injected radiolabelled SG PGs were found in the liver, 5% in the blood and 5% in the kidney, spleen and urine, pointing for a major uptake by liver cells. This study also showed that it is mainly the hyaluronan receptor present on the surface of these cells

that is responsible for the elimination of SG from the bloodstream. Interestingly, a subsequent study showed that the core protein is degraded more rapidly than the GAG chains and that the final products of GAG degradation are lactate and sulfate, indicating that this process is anaerobic (Falkowska-Hansen *et al.*, 2006). So far, this is the most-well documented mechanism for elimination of SG. However, due to the interaction of partner cells in processes such as inflammation, it does not rule out other mechanisms through which SG is eliminated. In fact, MCs and macrophages are known to co-exist in inflammation sites and it has been suggested that macrophages may be involved in the uptake of SG PGs released in complex with MCs mediators (Lindahl *et al.*, 1979).

## Present Investigations

### Aim

The aim of the current studies was to investigate and elucidate the functional aspects of SG PGs as components of secretory granules, and to provide further insights into their interactions with other intra-granular mediators, using a genetic SG-deficient mouse strain.

### Results and discussion

#### *Paper I: Serglycin-Deficient Cytotoxic T Lymphocytes Display Defective Secretory Granule Maturation And Granzyme B Storage*

CTLs play a pivotal role in the defense of the organism by attacking and eliminating degenerated harmful cells (e.g. tumor cells, virus infected cells). Fas-ligand and the granzyme B/perforin complex have long been recognized as the main components of the lymphocyte-mediated killing and have received particular attention in several studies. Nevertheless, the full understanding of the molecular mechanism behind their action still holds some questions that remain to be answered, namely the presence and role of “helper” molecules such as SG PGs. We thus investigated the importance of SG PGs in the granule organization as well as their interactions with other granular mediators in CTLs

Deletion of the SG gene did not result in an inability of granule formation in concanavalin A -stimulated CTLs from either spleen or blood. However, striking differences were seen sub-structurally where SG<sup>-/-</sup> CTLs possessed exclusively spherical-shaped electron translucent granules filled with an

amorphous matrix. In contrast, SG<sup>+/-</sup> CTLs contained mostly heteromorphous cytotoxic granules with an electron-dense irregular core, but also spherical electron translucent granules filled with an amorphous matrix. Furthermore, SG<sup>-/-</sup> CTLs incorporated low amounts of radiolabeled sulfate both in secreted and intracellular GAGs in contrast to GAGs recovered from SG<sup>+/-</sup> cells, indicating that in fact SG PGs are the dominating PGs species both intra and extracellularly. However, as assessed by RT-PCR, CTLs were shown to express other PGs species, namely glypican-1, glypican-4 and syndecan-4, which may account for the remaining cell associated sulfated GAG chains.

Previous reports have shown that SG PGs interacted with granzymes and perforin (Masson *et al.*, 1990; Metkar *et al.*, 2002) and that granzyme B engages in tight interactions with SG PGs as well as with free CS chains (Galvin *et al.*, 1999; Raja *et al.*, 2002) forming a complex that withholds the capacity to induce apoptosis (Metkar *et al.*, 2002). We therefore addressed the possible dependence of CTLs granule mediators on SG PGs. As demonstrated by means of Western-blotting, granzyme A, perforin and Fas-L are not dependent on SG PGs for storage. However, granzyme B levels were shown to be dramatically reduced in SG<sup>-/-</sup> CTLs indicating that SG is essential for its storage, which is consistent with other previous findings (Galvin *et al.*, 1999; Raja *et al.*, 2002). Although both are basic proteins, granzyme A and B exhibit different affinities towards SG PGs possibly due to a distinct protein folding and/or special arrangement.

In conclusion, this study shows for the first time that SG PGs are the dominating PG species synthesized by CTLs and are important for the storage of granzyme B, but not granzyme A or perforin.

#### *Paper II: Serglycin Proteoglycan is required for Secretory Granule Integrity in Mucosal Mast Cells*

MCs are specialized cells involved in the host defense against a variety of pathogens such as bacteria (Echtenacher *et al.*, 1996; Malaviya *et al.*, 1996) and parasites (Ha *et al.*, 1983; Nawa *et al.*, 1985). Upon activation, MCs degranulate and release a broad range of mediators found enclosed in cytoplasmic granules which include histamine, cytokines, proteases and proteoglycans. Depending on their tissue localization and micro-environmental stimuli, MCs may exhibit distinct phenotypical features and two major subtypes can be distinguished: CTMCs present in the skin and peritoneal cavity, and MMCs which are found in mucous membranes and

in the intestinal lamina propria (Galli, 1990; Metcalfe *et al.*, 1997). The protease expression pattern of these MC subclasses is remarkably different where CTMCs are known to express MC CPA, mMCP-4, mMCP-5, mMCP-6 and mMCP-7 whereas MMCs contain mainly mMCP-1 and mMCP-2. Also, CTMCs and MMCs express different PGs species with heparin PGs being the most prominent PG species found in CTMCs (Yurt *et al.*, 1977) whilst MMCs express predominantly CS PGs (Enerback *et al.*, 1985). Insights into the role of heparin PGs in CTMCs came from studies where bone marrow-derived MCs (BMMCs) from NDST-2 (an enzyme involved in the epimerization and sulfation of heparin) knock-out mice displayed severe defects in the storage of MC secretory granule proteases (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). Furthermore, SG<sup>-/-</sup> BMMCs showed dramatic defects in granule morphology and also a compromised ability to store MC proteases, namely mMCP-4, -5, -6 and CPA demonstrating the importance of the highly sulfated heparin PGs as storage scaffolds in MC secretory granules (Abrink *et al.*, 2004).

In this study we assessed the role of SG PGs in mucosal-like BMMCs. BMMCs from both SG<sup>+/+</sup> and SG<sup>-/-</sup> were differentiated into a MMC-like phenotype in the presence of IL-3, IL-9, SCF and TGF- $\beta$ , specifically regarding the expression of MMC proteases mMCP-1 and -2. (Pemberton *et al.*, 2003).

Mature MCs are typically stained with cationic dyes due to the presence of negatively charged molecules in their secretory granules. SG<sup>+/+</sup> in vitro-derived MMC-like cells exhibited a strong metachromatic granular staining with May-Grunwald/Giemsa whereas SG<sup>-/-</sup> cells displayed “empty”-appearing vesicles, indicating that lack of SG leads to dramatic defects in the staining properties of MC with cationic dyes. Moreover, transmission electron micrographs revealed striking differences regarding granule organization between both phenotypes. Granules in SG<sup>+/+</sup> cells showed a well defined sub-structural organization mainly divided into distinct compartments of low electron density (electron translucent) and electron dense cores. SG<sup>-/-</sup> granules are completely filled with material of intermediate electron density displaying an amorphous-like arrangement lacking the subdivision into electro dense and translucent regions, clearly showing that SG is crucial in the intra-granular organization process.

Analysis of the cell-associated PGs upon biosynthetic labeling revealed an ~80% reduction of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> incorporation into PGs recovered from SG<sup>-/-</sup> cells when compared to SG<sup>+/+</sup> counterparts, which indicated that SG accounts for most of the secretory granule PGs. In addition, enzymatic digestion by

chondroitinase ABC resulted in the depolymerization of most of the GAGs from both SG<sup>+/+</sup> and SG<sup>-/-</sup> cells confirming that both SG- and non-SG species accommodate GAGs of CS type. As assessed by anion exchange chromatography, these GAGs in both phenotypes display a similar anionic charge density corresponding to a low degree of sulfation.

We next analyzed how the MC specific proteases were affected by the lack of SG. Although no differences were seen in terms of expression of MC proteases at the mRNA level, at protein level SG<sup>-/-</sup> cells were devoid of mMCP-4, mMCP-6 and CPA while mMCP-5 levels were dramatically reduced, suggesting that these proteases are strongly dependent on SG PGs for storage. However, mMCP-1 and mMCP-7 levels were present in similar amounts in both SG<sup>+/+</sup> and SG<sup>-/-</sup> cells indicating that these are independent on SG for storage whereas mMCP-2 is only partially dependent.

Taken together, these results clearly establish that SG is the major cell-associated PG in *in vitro*-derived MMC-like cells and plays an important role in the granule maturation process where SG PGs act as a preferential storage matrix for selected mediators. However, the differential degree of dependence on SG PGs varies between individual granule compounds. A likely explanation may reside in the fact that MC proteases possess different cationic properties which will mediate their stronger or weaker binding to the negatively charged GAG chains.

*Paper III: Age-Related Enlargement of Lymphoid Tissue and Altered Leukocyte Composition in Serglycin-Deficient Mice*

In paper III, we noted that older SG-deficient mice commonly showed enlarged spleens and anomalies in other lymphoid organs, namely Peyer's patches and bronchus-associated lymphoid tissue (BALT).

In spleen, SG deficiency resulted in a reduction of CD4<sup>+</sup> cells as compared with SG<sup>+/+</sup> controls whereas the percentage of CD45RC expressing cells was higher in SG<sup>-/-</sup> animals. Nevertheless, these results occurred independently of spleen enlargement which indicates that SG is possibly involved in the maturation process of immune cells in the spleen. In agreement with previous studies (Abrink *et al.*, 2004; Braga *et al.*, 2007), the lack of SG affects the metachromatic staining properties of MCs. However, and as judged by FACS analysis of peritoneal MCs, CD117 (c-kit) intensities were much lower in SG<sup>-/-</sup> exudates indicating a role for SG in the

regulation of this surface marker. Moreover, the number of peritoneal macrophages was shown to be markedly decreased in older  $SG^{-/-}$  animals.

The results in this paper indicate that SG deficiency leads to multiple age-related abnormalities in several lymphoid organs. This provides an indication that SG may be involved in the homeostasis of the leukocyte population, most likely by affecting growth and differentiation factors that are important for cell differentiation.

*Paper IV: Reduction with Dithiothreitol Causes Serglycin-Specific Defects in Secretory Granule Integrity of Bone Marrow Derived Mast Cells.*

Secretory granules are specialized cytoplasmatic organelles whose main function is to serve as storage pools for specific cell mediators such as hormones, proteases and signaling molecules that are generally released in response to stimulation. These structures are present in many cell types, including endocrine and neuroendocrine cells as well as in a variety of hematopoietic cell types such as macrophages, neutrophils, basophils, CTLs and MCs. Previous reports using  $SG^{-/-}$  mice provided important insights into the role of SG PGs in the granule maturation process in MCs (Abrink *et al.*, 2004; Braga *et al.*, 2007), where  $SG^{-/-}$  MCs displayed granules lacking a normal sub-structural organization accompanied by an absence of several MC-specific proteases. Also, a recent study showed that SG-dependent MC proteases were correctly targeted into the secretory vesicles in both  $SG^{+/+}$  and  $SG^{-/-}$  MCs but, in the latter, these proteases were either targeted for degradation or secreted (Henningsson *et al.*, 2006). However, the underlying mechanisms behind the sorting of SG PGs into secretory vesicles are yet to be disclosed.

In the present study we addressed the possibility that a defined N-terminal motif involving a disulfide bridge may be involved in the correct sorting of SG PGs into secretory vesicles. It has been described that certain secretory proteins in neuroendocrine cells are known to possess an N-terminal bonded loop proven to be essential for their correct sorting into secretory granules (Benedum *et al.*, 1987; Chanat *et al.*, 1993; Tooze *et al.*, 2001). Interestingly, alignment of SG sequences from several species showed a striking conservation of an N-terminal motif comprising two cysteine residues likely to form a disulfide bond similar to the one found in other secretory proteins. In order to investigate whether the disruption of this disulfide bond would lead to defects in the secretory granule organization, both  $SG^{+/+}$  and  $SG^{-/-}$  BMDCs were cultured in the presence of

dithiothreitol (DTT). In fact, DTT caused to defects in the staining properties of SG<sup>+/+</sup> cells with May-Grunwald/Giemsa, which was an indication that the intra-granular PGs were affected. Furthermore, ultra-structural investigations using transmission electron microscopy revealed that the sub-structural organization of these granules was strikingly similar to that found in SG<sup>-/-</sup> BMMCs. Interestingly, however, SG<sup>-/-</sup> BMMC granule morphology was shown not to be affected by the presence of the reducing agent, suggesting that DTT interacts specifically with SG PGs within the granules.

Biosynthetic radiolabelling experiments showed that in the presence of DTT, SG<sup>+/+</sup> BMMCs incorporate low amounts of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> into the GAG chains when compared with non-treated cultures, and these levels are similar to the ones previously observed in PGs recovered from SG<sup>-/-</sup> BMMCs. Also, DTT did not lead to differences in incorporation of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> into GAG chains recovered from DTT-treated SG<sup>-/-</sup> BMMCs when compared with non-treated cultures. Together, these data clearly suggests that DTT interacts specifically with SG resulting in similar defects as seen in the SG<sup>-/-</sup> BMMCs.

Since lack of SG leads to a compromised ability to store specific MC-proteases (Abrink *et al.*, 2004; Braga *et al.*, 2007), we analyzed whether the effect of DTT would result in loss of function of SG as storage matrix of these proteases. However, immunoblot analysis of cell extracts showed that DTT did not affect the level of mMCP-6 protein. In contrast, incubation with 5mM DTT led to a considerable decrease in expression of the active form of CPA. Moreover, quantitative real-time PCR analysis revealed that the levels of both SG and CPA mRNA were not affected by DTT, indicating that these effects occur at the protein level.

The results in this study suggest that this N-terminal motif comprising a disulfide bonded-loop present in the SG protein may be involved in the correct sorting of SG PGs into secretory granules and that the reductive cleavage of this bond leads to an impairment of function of SG PGs in BMMC secretory vesicles.

## Concluding remarks

PGs comprise a collection of biomolecules that are involved in many different biological processes such as cell adhesion, cell-cell and protein-cell interactions, and many others. Importantly, many immune cells of hematopoietic origin are known to possess cytoplasmic vesicles where effector mediators are stored, which are released upon activation and stimulation. SG PGs have been shown to be the only known committed intracellular PG in hematopoietic cells. Although considerable progress has been made in the SG PG field of research especially in the last five years due to the generation of a SG-deficient mouse strain, many aspects regarding SG biology and function still remain to be answered. More knowledge of SG interactions with other biological ligands, their dependence on SG for storage and activation would be of fundamental importance to understand more about the biology of many immune-related reactions and other physiological mechanisms.

The work presented in this thesis focused on the role of SG PGs in secretory granule organization, their interaction with other granule components as well as their importance in other immune-related events. Moreover, the importance of the highly conserved amino-acid sequence present in the N-terminus of the SG protein has been addressed. We have demonstrated that SG PGs play a very important role in granule maturation and organization in CTLs and MMCs and further showed that specific granule components in these cells are SG-dependent for their correct storage. Our finding that the lack of SG may lead to defects in immune-related organs in older animals, and that SG may be involved in the maturation and regulation of immune cells in lymphoid organs suggests that SG may not be only confined to intracellular compartments, but also play important roles extracellularly.

Even though the studies presented in this thesis have provided more insights about the importance of SG PGs, many questions still remain unanswered. It has yet to be determined how the expression of the SG gene is regulated and what are the trans-activating factors involved in cells where the expression of SG is undesired. From the studies presented here it is clear that SG PGs play a very important role in secretory granule maturation and organization but it remains to be elucidated why and by which mechanisms SG PGs are mainly secreted and released from other cell types such as macrophages. It seems likely that SG expression could be modulated through alternative splicing processes according to the stage of maturation and differentiation of the cell, but this aspect of SG biology remains to be addressed. Furthermore, it would be of fundamental interest to gain further insights into the role of the GAG chains and the determinants that drive the synthesis of either CS or heparin/HS into the SG core protein. Due to its role and interactions with many different biological ligands, it is therefore of interest to study in more detail the ligand interactions before and upon release and their biological effects as well as to learn more about the structural requirements involved in such interactions. Moreover studies should be conducted to address whether or not abnormal expression of SG or its absence leads to pathological conditions, especially in humans and if SG could be used as a biological marker of such conditions.

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