REVIEW ARTICLE

Intracellular proteoglycans

Svein Olav KOLSET^{*1}, Kristian PRYDZ[†] and Gunnar PEJLER[‡]

^{*}Institute for Nutrition Research, University of Oslo, Box 1046 Blindern, 0316 Oslo, Norway, [†]Department of Molecular Biosciences, University of Oslo, Box 1041 Blindern, 0316 Oslo, Norway, and [‡]Department of Molecular Biosciences, Swedish University of Agricultural Sciences, BMC, Box 575, S-75123 Uppsala, Sweden

Proteoglycans (PGs) are proteins with glycosaminoglycan chains, are ubiquitously expressed and have a wide range of functions. PGs in the extracellular matrix and on the cell surface have been the subject of extensive structural and functional studies. Less attention has so far been given to PGs located in intracellular compartments, although several reports suggest that these have biological functions in storage granules, the nucleus and other intracellular organelles. The purpose of this review is, therefore,

to present some of these studies and to discuss possible functions linked to PGs located in different intracellular compartments. Reference will be made to publications relevant for the topics we present. It is beyond the scope of this review to cover all publications on PGs in intracellular locations.

Key words: glycosaminoglycan (GAG), protease, proteoglycan (PG), serglycin.

INTRODUCTION

The proteoglycan (PG) structures we know today have been elucidated mainly from classical studies with material isolated from tissues like tendon and cartilage, and cell types such as fibroblasts and chondrocytes (reviewed in [1–4]). With the exception of studies on PG biosynthesis and degradation, including studies of mucopolysaccharidosis, the experimental approaches adopted so far have focused on the functions of PGs in the extracellular matrix and on cell surfaces, and their effects on cell function and behaviour, often in concert with other molecules. The biology of PGs residing in different intracellular organelles and their effects on cell behaviour has traditionally received less attention. There are numerous obvious reasons for the focus of PG research in relation to the extracellular matrix and the interplay between matrix and cells. Such studies have provided us with insight on cell surface and matrix PGs in relation to proliferation, development, pathology etc. [4–6]. However, PGs are also expressed in cell types that do not typically take part in extracellular-matrix or tissue construction. There are several cell types that are inherently mobile, particularly those in the immune system and those found in metastasizing tumour cells. These cells do not engage in matrix formation to a large extent, but they synthesize PGs, many of which will probably have as wide a functional repertoire as their relatives residing in matrices and on cell surfaces. But, many of these cells will also express PGs with defined intracellular functions.

The interplay between cells and their matrices and cell neighbours is highly dynamic and depends on signals secreted and received by cells, and on signalling through adhesion processes. An important aspect here is the role played by cell surface PGs as part of cellular signal-transductions systems [7]. Another aspect is the endocytosis of signalling molecules or molecules important for metabolic regulation. PGs are known to participate in endocytic processes and can be found in endosomes, and other organelles in degradation pathways. What role these PGs play is largely unknown. Thirdly, the possibility that PGs, or their corresponding glycosaminoglycan (GAG) chains, may reach the

nucleus is a fascinating aspect that deserves more attention. What, if any, are the functions of such molecules and what regulates their transport to this destination? Finally, the site of hyaluronic acid (HA; also known as hyaluronan) biosynthesis is not in the Golgi apparatus, but is assumed to take place at the cell surface. This is in contrast with the biosynthesis of other GAGs. The mechanisms for such biosynthesis, and the co-ordinate regulation of the biosynthesis of partner molecules such as aggrecan and versican, are challenging and fascinating topics.

Biosynthesis

The biosynthesis of PGs has been extensively studied, and for detailed presentations there are several excellent and extensive reviews on different aspects of these processes [1,3–6,8,9]. In this review we will only briefly outline parts of the biosynthetic process.

A wide range of protein cores serve as acceptors for the initiation and polymerization of GAG chains. Serine is the anchor site in the protein for xylose, the first monosaccharide in GAG chains, to be attached through the action of a xylosyltransferase. The exceptions are keratan sulphate and HA. Keratan sulphate chains are linked to proteins either through GlcNAc–asparagine or GalNAc–serine/threonine linkages. HA is synthesized as a GAG without covalent binding to a protein core. The serine attachment site for chondroitin and heparan polymerization is typically located in Ser–Gly dipeptide or repeating sequences with flanking acidic amino acids.

The first part of chondroitin/dermatan and heparin/heparan polymerization is the completion of what is referred to as the tetrasaccharide linker (Xyl–Gal–Gal–GlcA). The galactose units are transferred from UDP–galactose to the initiated chain by galactosyltransferase I and II, respectively. The last member of the linker unit is glucuronic acid (GlcA), transferred from UDP–GlcA by the enzyme glucuronosyltransferase I [9,10].

At this point the biosynthesis machinery is faced with the decision to transfer either GalNAc or GlcNAc to the terminal GlcA,

Abbreviations used: ChSy, chondroitin sulphate polymerase; GAG, glycosaminoglycan; FGF, fibroblast growth factor; GPI, glycosyl-phosphatidylinositol; HA, hyaluronic acid (or hyaluronan); HAS, HA synthase; HSPG, heparan sulphate proteoglycan; IdoA, iduronic acid; MDCK, Madin–Darby canine kidney; mMCP, mouse mast-cell protease; NDST, N-deacetylase, N-sulphotransferase; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; PG, proteoglycan.

¹ To whom correspondence should be addressed (e-mail s.o.kolset@basalmed.uio.no).

through the actions of either of the two distinct transferases, GlcNAc transferase or GalNAc transferase, resulting in the initiation of either chondroitin/dermatan sulphate or heparin/heparan sulphate, respectively [1,9]. These enzymes are different from those responsible for further chain elongation. The ensuing polymerization is achieved through the activities of chondroitin sulphate polymerase (ChSy; probably in complex with other factors [11]) or heparan sulphate polymerase (also called the EXT1-family), adding disaccharide blocks comprising either GalNAc/GlcA or GlcNAc/GlcA, respectively. Both enzyme complexes contain two independent catalytic domains, one transferring the hexosamine unit, the other the hexuronic part. The growing GAG chains are thereafter subject to various degrees of epimerization of GlcA residues to iduronic acid (IdoA) through C-5-uronosyl epimerase activity. This structure is present in dermatan sulphate and heparin/heparan sulphate, but not in chondroitin sulphate. So far, only the epimerase acting on heparin/heparan sulphate chains has been identified at the molecular level.

Finally, and most importantly, the GAG chains become sulphated at defined locations both in the hexosamine units and the IdoA units, and occasionally in the GlcA units, through the actions of defined sulphotransferases. There are several specific sulphotransferases that have been identified, responsible for the transfer of sulphate units from the activated sulphate donor adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to defined positions in the different types of GAG chains [1].

The biosynthesis of heparan sulphate differs from that of chondroitin sulphate in one important aspect, deacetylation and N-sulphation of hexosamine units. A key enzyme in heparin/heparan sulphate biosynthesis is the N-deacetylase, N-sulphotransferase (NDST), of which four isoforms have been identified so far. Regions where the GlcNAc units are not deacetylated remain almost unmodified with regard to epimerization of the GlcA moieties and sulphation of the disaccharide units. In contrast, regions where the GlcNAc residues have been modified by NDST are extensively modified through the ensuing epimerization of D-GlcA to L-IdoA, 2-O-sulphation of IdoA and 6- and/or 3-O-sulphation of the N-sulphated GlcN entities. The end results, as shown by a series of structure analyses of both heparin and heparan sulphate, is that heparin is highly sulphated, whereas heparan sulphate is highly sulphated in defined blocks [12,13]. Approx. 40–50 % of the GlcNAc units generally become N-sulphated in heparan sulphate.

There are now several studies where genes coding for different enzymes in GAG biosynthesis have been deleted, providing us with new insight into the biological importance of defined structures in the different types of GAG chains in mammalian systems, *Caenorhabditis elegans* and *Drosophila melanogaster* [2,9,14]. It is interesting to note that much of the current knowledge on heparin/heparan sulphate biosynthesis has been obtained from studies in mast cells and subcellular fractions thereof. In mast cells, the dominating PG is heparin, which is found in storage granules. Intracellular PGs have, consequently, been instrumental for our present understanding of GAG biosynthesis.

HA is not a sulphated GAG, nor is it covalently attached to protein. Biosynthesis of HA in mammalian cells has been demonstrated in subcellular fractions enriched in plasma membrane markers [15]. The synthesis machinery is not inactivated, but rather stimulated by protease treatment of cells that synthesize HA. The interpretation of these and other findings has been that HA is synthesized at the cytoplasmic face of the plasma membrane and extruded through the plasma membrane and shedded [16] from the cell surface. A substantial amount of HA, however, is also found intracellularly in many cell types [17–20] in granules,

Table 1 GAG structures of intracellular PGs

Examples of disaccharide structures in GAG chains of PGs also found in intracellular compartments. Heparin/heparan sulphate is shown with three different examples, two with different degrees of sulphation, and one unmodified disaccharide unit. The chondroitin sulphate (CS) family contains other disaccharide units, but these have not been seen in intracellular proteoglycans. NK, natural killer.

GAG	Disaccharide units	Cell type
Heparin/heparan sulphate	GlcNSO ₃ (3,6-OSO ₃) α 1-4GlcA/IdoA(2-OSO ₃) GlcNSO ₃ (6-OSO ₃) α 1-4GlcA/IdoA(2-OSO ₃) GlcNAc α 1-4GlcA	Mast cells
CS-A(CS-4)	GalNAc(4-OSO ₃) β 1-4GlcA	Granulocytes, NK cells
CS-B	GalNAc(4-OSO ₃) β 1-4IdoA or GalNAc(4-OSO ₃) β 1-4IdoA(2-OSO ₃) (diB)	Lymphocytes, platelets Basophilic leukocytes
CS-C(CS-6)	GalNAc(6-OSO ₃) β 1-4GlcA	Mucosal mast cells Platelets
CS-E	GalNAc β 1-4GlcA(4,6-OSO ₃)	Macrophages, mucosal mast cells
Keratan sulphate	GlcNAc(6-OSO ₃) β 1-3Gal	Eosinophils
HA	GlcNAc β 1-4GlcA	Mast cells

the cytoplasm and the nucleus [18,21–24], and in endocytic organelles, although not in mature lysosomes [19].

A cytoplasmic localization of HA synthesis would require supply of UDP-GlcA and UDP-GlcNAc at concentrations compatible with the respective K_m values for these substrates. Most reports on HA synthase (HAS) K_m values, however, conclude with values similar to those found for glycosyltransferases in the Golgi lumen, ranging from 29 μ M to 60 μ M for UDP-GlcA and 82 μ M to 500 μ M for UDP-GlcNAc [25–27]. These K_m values are also significantly higher than those reported for UDP-sugar transporters in Golgi membranes [28–31].

A multi-subunit complex involved in HA synthesis has been reported, with K_m values for UDP-GlcNAc (1.6 μ M) and UDP-GlcA (12.2 μ M) that are much lower [32,33] than those described above, but still higher than that reported for a UDP-GlcNAc transferase (0.545 μ M) acting on cytoplasmic and nuclear proteins [34]. The multi-subunit complex involved in synthesis of HA [33] has also been postulated to manage degradation of HA and has been termed the 'hyaluronasome' [20]. The hyaluronasome model makes rapid co-ordination of synthesis and degradation possible. Most of the intracellular HA detected in mammalian cells is located in endosome-like structures [19], but cannot always be quantitatively accounted for by the HA internalized by invagination of the plasma membrane [18]. In such cases, the HA located to endosomes could either be inserted into endosomes after cytoplasmic synthesis, or some synthesis of HA could take place at the luminal side of a membrane-limited compartment.

GAG structures

Heparin is found only inside storage granules in connective-tissue mast cells, whereas the closely related heparan sulphate PGs are found at the plasma membrane, either intercalated or glycosylphosphatidylinositol (GPI)-anchored, and in the extracellular matrix. The repeating disaccharide unit of heparin is GlcA/IdoA attached to GlcN that may be either N-acetylated, deacetylated with a free amino group in position 2, or N-sulphated in the same position (Table 1). In addition, the GlcN units may be O-sulphated in position 3 and/or 6, and IdoA in position 2.

Heparin contains the antithrombin-binding pentasaccharide with the unique 3-O-sulphate group [35] and N-sulphated GlcN unit in the middle unit of the five saccharides. Such high-affinity structures are found on approximately one-third of the chains of

unfractionated heparin. The basis for the anticoagulant activity of heparin is the high-affinity binding to the serpin protease inhibitor antithrombin, mediated by the pentasaccharide sequence. When heparin binds to antithrombin, the protease inhibitor undergoes a conformational change that dramatically increases its activity towards coagulation proteases such as thrombin and Factor Xa [36]. However, this effect of heparin is the result of exogenous administration. The biological ligands for heparin in the granules of mast cells are histamine, chymases, carboxypeptidases and other granule components [37]. The 3-O-sulphated residues are not necessary for any of these interactions. At present the actual biological significance of the antithrombin binding sequences in heparin in storage granules in mast cells is still not clarified. A minor fraction of endothelial-cell-surface heparan sulphate carries antithrombin binding sequences, which may be sufficient for proper regulation of blood coagulation. Mice carrying a deleted version of one of the genes for 3-O-sulphotransferase do not exhibit a thrombotic phenotype, suggesting the presence of compensatory system(s) in the endothelium [38,39]. However, it is also important to note that humans possessing an antithrombin variant with reduced heparin-binding properties show marked tendencies for thrombosis [40], indeed providing support for a physiological role of the antithrombin-binding pentasaccharide in regulation of blood clotting. Finally, it is also important to note that 3-O-sulphated GlcN units have been found in heparan sulphate of clam, an animal without blood circulation, further supporting the notion that inhibition of blood coagulation is not the prime function of heparin/heparan sulphate with antithrombin-binding sequences [41].

The biological ligands and physiological role for 3-O-sulphated heparin sequences in mast-cell granules remain to be defined. This may seem surprising, given the vast number of studies devoted to this highly sulphated polysaccharide. Interestingly cellular entry of herpes simplex virus has been shown to depend on cell surface heparan sulphate with 3-O-sulphated residues [42]. Further research, making further use of gene deletion technology, should have the potential to provide us with answers about the biological functions of such well defined and unique saccharide sequences.

In haematopoietic cells other than connective tissue mast cells, chondroitin sulphate is the major GAG, and in most cell types chondroitin 4-sulphate (CS-A) is the dominant form [43]. But there are also cell types that synthesize chondroitin 6-sulphate (CS-C) [43]. The repeating disaccharide unit in these GAGs is GlcA-GalNAc (Table 1). GAGs with IdoA and 4-O-sulphated GalNAc units are also found in some haematopoietic cells. This structure is conventionally named CS-B (or dermatan sulphate). One unique feature of chondroitin sulphate in e.g. macrophages, basophils and mucosal mast cells is the presence of highly sulphated chondroitin sulphate units. In macrophages and mucosal mast cells GalNAc with O-sulphate units in both position 4 and 6 have been identified [44,45], generally referred to as CS-E. In addition, basophils and mucosal mast cells [46] have been shown to contain the disaccharide 2-O-sulphated IdoA-4-O-sulphated GalNAc (CS-diB), as shown in Table 1. The presence of both intracellular keratan sulphate [47] and hyaluronan [18,21] has also been documented.

The presence of highly sulphated chondroitin sulphate in several haematopoietic cells is an interesting feature of intracellular PGs. In rodent mucosal mast cells, oversulphated chondroitin is the dominant GAG, and these cells perform their biological functions, e.g. in connection with helminth infections [45]. By comparison it is evident that mucosal mast cells are not as electron dense and contain less PGs and histamine than their corresponding connective tissue relatives [48]. Still, the functions of the mucosal mast cells are not in any way compromised.

Human mast cells have also been shown to contain both oversulphated chondroitin sulphate and heparin [49,50], as have mouse connective-tissue mast cells [51]. The regulation of the biosynthesis of such hybrid molecules is not understood in any great detail. Serglycin core protein can possibly serve as a model substrate for detailed analyses of factors regulating the glycosyltransferases at the decision point where the biosynthesis machinery initiate heparan or chondroitin chains.

From the studies of heparin and heparan sulphate biosynthesis, it has been shown that heparan sulphate in particular contain segments with several consecutive N-acetylated and unsulphated GlcN residues, whereas other regions are more heparin-like and highly sulphated. The different regions and specific sequences in the sulphated regions contain binding sites for different growth factors and other ligands [1,12]. In the highly sulphated chondroitin sulphates found in several haematopoietic cells, which in most of these cells are probably attached to the serglycin core protein, it is possible that a similar operational segmentation might take place. In essence, it is possible that the oversulphated units are arranged in blocks. There is some evidence for this [52], and the idea is intriguing. It is conceivable that blocks of oversulphated disaccharide units can promote the binding of specific ligands [53], either in storage granules, in secretory vesicles or in the extracellular environment. Highly sulphated chondroitin sulphate D [GlcA(2-OSO₃)-GalNAc(4-SO₃) disaccharides] in brain has been shown to bind with high affinity to pleiotrophin [54] and chondroitin sulphate E and heparin bind midkine, fibroblast growth factor-16 and epidermal-like growth factor with comparable affinities [55]. The partners of highly sulphated chondroitin sulphates in granules of several immune cells await further characterization. Such studies could potentially both broaden our understanding of the biological role of different chondroitin sulphates in general and increase our general knowledge of the role of PGs in immune reactions. The generation of mice with a deleted version of the chondroitin sulphate 6-O-sulphotransferase gene revealed decreased numbers of naïve T-lymphocytes in the spleen, suggesting a biological role of chondroitin 6-sulphate, possibly in concert with chemokines or growth factors [56].

Intracellular sorting

Several reports indicate that glycans might act as sorting mediators in the anterograde direction of the secretory pathway [8]. Both N-linked and O-linked glycans have been shown to mediate apical sorting in the Madin–Darby canine kidney (MDCK) cell line. For instance, addition of sites for attachment of N-glycan chains to rat growth hormone, a normally unglycosylated protein, resulted in secretion to the apical side of polarized MDCK epithelial cells [57]. The role of GAG chains in sorting of protein moieties has been studied to a lesser extent than is the case for other glycans. However, it has been shown that chondroitin sulphate PGs, and free chondroitin sulphate chains initiated on exogenously added xylosides are secreted selectively into the apical medium of MDCK cells [58]. Furthermore, the heparan sulphate PG (HSPG) glypican was transported predominantly to the basolateral membrane domain in MDCK and CaCo-2 epithelial cells, whereas glypican lacking heparan sulphate attachment sites was sorted to the apical surface in MDCK cells [59], presumably due to N-glycans [60]. Interestingly, sorting of serglycin to secretory vesicles in acinar pancreatic cells was shown to depend on GAG chains. Core protein without GAGs did not reach these vesicles [61]. In addition, cells expressing serglycin without GAG chains contained few granules with their typical enzyme content (Table 2).

Table 2 Storage granule cells expressing serglycin

Cell type	Type of GAG	Other granule constituents
Platelets	Chondroitin sulphate	Platelet factor 4, von Willebrand factor
Natural killer cells	Chondroitin sulphate	Granzymes
Cytolytic T cells	Chondroitin sulphate	Granzymes
Polymorphonuclear leucocytes	Chondroitin sulphate	Gelatinase, lactoferrin, myeloperoxidase
Connective tissue mast cells	Heparin	Histamine, chymases, (mMCP-4,5), tryptases
Mucosal mast cells	Chondroitin sulphate	Histamine, chymases, (mMCP-1,2)
Endothelial cells	Chondroitin sulphate	Plasminogen activator (tissue type)
Pancreatic acinar cells	Chondroitin sulphate	Procarboxypeptidase, amylase

An interesting discovery has been the PG leprecan, which possesses a C-terminal KDEL motif, a signal known to function in retrieval to the endoplasmic reticulum from more distal regions of the secretory pathway [62]. Despite this KDEL motif, leprecan is not only found inside L-2 cells, but also in the extracellular basement membrane. A KDEL-modified variant of the extracellular matrix PG decorin is also secreted, but to a lesser extent than the corresponding wild-type molecule [63]. The KDEL-based retrieval system is not absolutely stringent, but allows delayed export of KDEL-containing proteins [64]. Thus, leprecan might have important functions both in the extracellular matrix and in the early secretory route or even in other intracellular locations. Leprecan has been predicted to contain 2-oxoglutarate- and iron-dependent dioxygenase activity [65], also found in the DNA-repair enzyme AlkB [66].

Endocytosis

Newly synthesized PGs that are constitutively transported to the cell surface may be released to the body fluids and/or the extracellular matrix, or may attach cells to matrix components due to a membrane spanning protein core and extracellular GAGs. Some PGs, however, may be rapidly internalized [67]. The GPI-anchored HSPG glypican-1 arrives at the surface of ECV 304 cells with full-length heparan sulphate chains, but undergoes rapid endocytosis [68]. Most of the intracellular glypican-1 carries truncated heparan sulphate chains with more N-unsubstituted GlcNs than the cell-surface variant. The truncation occurs by an autocleavage mechanism dependent on nitric oxide and divalent copper ions [69]. The NO-dependent cleavage takes place in caveolin-1-containing endosomes [70] after an initial degradation step catalysed by an endoheparanase [71]. The heparan sulphate stubs may be extended to full size before transport of glypican-1 back to the cell surface. Where inside the cell this heparan sulphate re-polymerisation takes place is not clear. Enzymes involved in heparan sulphate polymerisation usually re-localize to the endoplasmic reticulum in brefeldin A-treated cells [8], but in ECV 304 cells, the extension of heparan sulphate chains takes place in the presence of brefeldin A [68,71]. This could happen either because the polymerizing enzymes are present in the *trans*-Golgi network of ECV 304 cells, or that polymerization takes place in a compartment previously not known to be engaged in heparan sulphate biosynthesis. GPI-linked proteins may move more freely in membrane bilayers than transmembrane proteins and might thus define additional recycling pathways.

Endocytosis of PGs has been demonstrated to be of importance for signalling and/or internalization of several kinds of growth factors [72] and for cellular uptake of polyamines, a function of the glypican-1 cycle as described above [73]. Polyamines, on the other

hand, may protect heparan sulphate against degradation [74]. Both growth factors and GAGs have been observed in the nucleus or nuclear fractions of mammalian cells [75–77], and nuclear localization signals have even been demonstrated in the protein cores of glypican and biglycan [78]. In total these observations indicate that PGs and/or GAGs might have important functions in the guidance of growth factors and other important factors in the direction of the nucleus.

Nuclear PGs

Several reports have indicated previously the presence of GAGs or PGs in the nucleus ([76,79], see also [78] but the purity of nuclear fractions obtained was a concern [80]). However, with the use of antibodies and confocal laser microscopy, more recent studies confirm what has previously been suggested with regard to PGs in the nucleus. In addition, nuclear localization signals have been identified in the core proteins of glypican-1 and biglycan, and their presence in the nucleus of neurons and glioma cells has been demonstrated with confocal laser microscopy [78]. By the same technique syndecan-1 has been shown to be associated with the nucleus in epithelial mesothelioma cells 2–3 days after seeding [81], as has HA in the nuclei of smooth-muscle cells and fibroblasts [18].

Further, nuclear PGs have also been detected in fibroblasts and CHO cells, where fibroblast growth factor (FGF)-2 is transported to the nucleus after teaming up with heparan sulphate [77,82]. The nuclear localization of heparan sulphate raises questions concerning regulation of intracellular trafficking of such molecules [83]; how do PGs or GAGs end up in the nucleus and what are their molecular partners along the way?

Cell-surface PGs are typically degraded in the classical endocytic pathway involving lysosomal enzymes. While most of the internalized heparan sulphate is completely degraded in lysosomes, a fraction may diverge from the main pathway and end up in the nucleus [77]. From what compartment(s) in the endocytic pathway PGs or partial degradation products may cross the membrane to be able to interact with the nuclear import machinery is not clear, but the whole process might be influenced by protein partners such as FGF-2 [77].

PGs or GAGs in the nucleus might have different functions other than being transport vehicles from the cell surface for growth factors such as FGF-2. Some studies have indicated that nuclear GAGs might influence gene expression [84,85]. Cell-surface heparan sulphate may contain attachment sites for viruses and bacteria, and, if these GAG entities are routed to the nucleus, they may provide a free ride for infectious micro-organisms. Such GAG-dependent delivery of molecules to the nucleus is relevant to medical and pharmaceutical research and might be used in gene or drug delivery [86].

PGs in secretory granules

PGs are present in cytoplasmic granules in a wide range of haematopoietic cells, including mast cells, neutrophils, cytotoxic T lymphocytes and eosinophils, as well as in endocrine and neuroendocrine cells and secretory cells in epithelia. GAG chains of intracellular PGs in these cells are typically attached to the serglycin core protein [43]. The serglycin core protein, which was actually the first PG core protein to be cloned, was initially cloned from a rat yolk-sac carcinoma [87], and from a mouse mastocytoma [88] and has subsequently been identified also in human mast cells [89] as well as in several haematopoietic cells [90], endothelial cells [91,92] and even in embryonic stem [93] cells (Table 2).

Most studies have identified serglycin mRNA, but some studies have also identified serglycin at the protein level [90,92,94,95]. Although it is likely that serglycin is the dominating PG present in the secretory granule of most haematopoietic cell types, this notion remains to be proven by gene targeting experiments. While the core protein may be the same in the PGs in many secretory granules, the type of GAG attached may differ between cell types, as presented above. From Table 2 it is evident that serglycin-expressing cells are involved in a wide range of biological processes, such as regulation of blood coagulation, tumour cell killing, and bacterial defence and wound repair. In several of these processes the release of storage granule content is fundamental to the function of the cell in question. Clearly, PGs in granules could have several different functions, as discussed below and also indicated in Figure 1.

Storage

An obvious candidate function for PGs in secretory granules is to mediate storage of various other granule components. According to this concept, PGs may be relatively inert as regards their own biological activities and are merely acting as a scaffold for other granule constituents. Indeed the high negative charge density of, in particular, the heparin PG in mast cells, makes the PGs well suited for binding positively charged compounds, thereby neutralizing their charge and facilitating their packaging. Early proof for this notion was the demonstration that mast cells, after degranulation, secreted PGs to which basically charged proteases were attached [96]. Much later, unequivocal evidence for a central role of PGs in the storage of compounds in mast-cell granules was obtained at the same time by two laboratories. Targeting of the gene for NDST-2 resulted in a drastic reduction in the various proteases that are normally found in the secretory granules of connective-tissue mast cells, accompanied by greatly distorted morphology of the secretory granule [37,97]. Since the mRNA levels for the proteases were not affected, it was concluded that the defect occurs at the level of storage rather than expression. In addition, the storage of histamine was markedly reduced [37,97].

It may seem surprising that the targeting of NDST-2 gives such a striking mast-cell phenotype, since NDST-1 may have the potential to provide back up for the lack of NDST-2. Indeed, the lack of obvious phenotypic changes in tissues in the NDST-2^{-/-} mice, other than the connective-tissue mast cells, indicates that NDST-1 compensates for the lack of NDST-2. However, in contrast with many other cell types, connective-tissue mast cells do not appear to express NDST-1 [98], emphasizing the large impact of NDST-2 deficiency in this cell type.

Another intriguing issue is why mast cells have 'selected' NDST-2 in favour of NDST-1. An attractive hypothesis, which has been given some support [99], is that NDST-2 is specifically designed for the biosynthesis of highly sulphated GAGs, (heparin), whereas NDST-1 predominantly gives lower sulphated variants (heparan sulphate). Although serglycin PGs undoubtedly have an established role in the storage of granule components in haematopoietic cell types, recent data have shown that serglycin [61] and, possibly, other PGs [100] are present in granules also of pancreatic acinar cells, indicating that PGs may have a role in the storage of the pancreatic digestive enzymes. Furthermore, there is evidence suggesting that PGs are important for the storage granule in platelets: the human gray platelet syndrome shows resemblance to hereditary macrothrombocytopenia in Wistar Furth rats, a disorder that is characterized by a defect in platelet formation leading to large platelet mean volume and α -granule defects [101]. The PG content in the affected platelets is only one-third of what is seen in normal cases and the PGs expressed are smaller in size

with shorter chondroitin sulphate chains, and it may therefore be speculated that the disorder is closely linked to the observed defects in the platelet PGs.

It is evident from the NDST-2^{-/-} mast cells that the lack of functional secretory-granule PG causes severe defects in the storage of basic granule components (see above), but what about the opposite situation, i.e. how does the lack of the corresponding basic compounds affect PG storage? This important question received some attention in a study where the gene for histidine decarboxylase, the enzyme that converts histidine into histamine, was targeted [102]. It was shown that the lack of histamine resulted in a marked reduction in the ability of the mast cells to bind cationic dyes, indicating a reduction of stored PG. Moreover, it was observed that the level of stored mast-cell proteases was considerably lower than in wild-type cells. The most likely explanation for these findings is that the granule PGs are dependent on the positively charged histamine for neutralization of their negative charge and, thereby, proper storage. The reduction in PGs caused by the histamine deficiency will then, in turn, cause impaired storage of the mast cell proteases as discussed above. It may therefore be speculated that the exact composition of the secretory granule is a result of a careful electrostatic balance between the various charged granule components, where the lack (or reduction) of one compound will lead to a disturbed electrostatic balance and alterations in the capacity to store partner molecules. It has also been demonstrated that alterations of the electrostatic balance may affect the biosynthesis of charged molecules. Transfection of pituitary cells with a construct coding for a highly basic protein, basic proline-rich protein, caused an increase in the level of sulphation of the corresponding secretory granule PGs [103]. This indicates that the cells have the ability to respond to an increased level of basic charge by increasing the charge density of the balancing negatively charged PG. Thus, the exact composition of a secretory granule may be subject to regulation at many levels.

Delivery from secretory granules

It is clear (see above) that intracellular PGs are crucial for the actual storage of various secretory granule compounds. It may also be speculated that PGs are important for the delivery of secretory granule compounds during exocytosis. After secretion, the particular secretory granule compounds may be either rapidly dissociated from the PG or they may remain associated. A striking example of the former mode is histamine, which is present in the secretory granule of mast cells and basophils. Inside the secretory granule the low pH prevailing will cause complete protonation of histamine, allowing electrostatic interaction with the PGs present. However, when secreted, the higher pH in the extracellular milieu will cause deprotonation of histamine and release from the PG, indeed an ingenious mechanism for delivering this potent inflammatory mediator into the tissue. Interestingly, a similar mechanism appears to be operating for one of the mast-cell proteases, mouse mast-cell protease (mMCP)-7 [104]. mMCP-7 interacts with heparin via specific histidine residues, which are assembled in a cluster. At the acidic pH in the mast cell granule these histidine residues are protonated and can interact with heparin but, similarly to histamine, the increase in pH after secretion will abolish the interaction with heparin, allowing diffusion of mMCP-7 away from the mast-cell surface [105].

A similar mechanism for release has been reported for perforin [106], although a later study has indicated that perforin may remain associated with serglycin after extracellular release [107]. In the latter study it was suggested that serglycin PGs actually function as vehicles for the delivery of perforin/granzymes into the

Figure 1 Possible functions of intracellular PGs

(A) Intracellular PGs (red core protein, blue GAG chains) interact with other proteins to organize and concentrate their contents in granules, and also to protect these from inactivation or destruction. (B) After release of granule content from activated cells, granule PGs can assist bound molecules (black) with transport to defined destination (see C–F), either for presentation (alternative 1) or release (alternative 2). (C) After release, proteins bound to GAG chains can be activated and perform functions while still bound to GAG chains (blue; alternative 1) or can be activated and released (red; alternative 2). This can occur on the cell surface or in the extracellular environment. (D) PGs in granules or vesicles interact with other proteins (pale blue), either through GAG chains or protein core, to mediate sorting to defined locations, such as the basolateral cell surface. (E) After release of granule content, PGs and/or molecules bound to PGs bind to new partner molecules in the extracellular matrix. (F) Secreted PGs may interact with the surface of collaborating cells through PG/cell surface protein interactions (alternative 1), or with target cells in, e.g., immune reactions (alternative 2). (G) Cell-surface or extracellular PGs can be endocytosed, partially degraded and transported to the nucleus (blue). (H) Cell surface or extracellular PGs can be endocytosed and completely degraded (alternative 1) or partially degraded PGs can be recycled or subject to re-synthesis (alternative 2).

target cells destined for granzyme-mediated apoptosis. Strikingly, it appeared as if this mechanism of granzyme/perforin delivery did not require pore formation in the plasma membrane [107]. In many other cases it is apparent that the secretory granule compounds remain associated with its intracellular PG partner also after secretion. For example, the interaction of granzyme B, a serine protease expressed by cytotoxic T lymphocytes, with serglycin PG is sufficiently strong to resist extracellular conditions [108]. Furthermore, it has been shown recently that the neutrophil compound, myeloperoxidase, is associated with serglycin during cellular transport and it was suggested that serglycin constitutes a general transport vehicle for cationic granule components of neutrophils [109]. It is also clear that certain mast-cell chymases remain tightly associated with heparin PG after mast cell degranulation [110] and that lysozyme exocytosed by macrophages remains complex-bound to PGs [111].

An interesting finding that may be of relevance in this context is provided by the NDST-1 null mouse strain. Pups carrying the NDST-1 defect die neonatally from alveolar collapse [112]. It was noted that the lung epithelium lacked surfactant proteins on their cell surface and that, instead, these proteins were trapped inside the cell. It is thus possible, although this remains to be proven, that intracellular PGs may have a role in delivering the surfactant proteins to the cell surface. In addition, it has been demonstrated that a significant part of the matrix metalloproteinase 9 that is secreted by monocytes is covalently attached to a PG [113]. This is in contrast with the above examples in which the interactions between secretory granule PGs and other granule components are of a non-covalent nature, and suggests that the mode of interaction between PGs and secretory products may be much more complex than hitherto recognized. An important implication of these combined findings is that the physiological form of many secretory granule compounds is the complex with their partner PG. This association may indeed have profound effects on various properties of the specific compound and this should be taken into careful consideration during studies of their respective biological activities.

Modulation of activities

In many cases the secretory granule PGs may be primarily involved in storage of granule compounds and in acting as vehicles for their delivery to the extracellular space. However, it is becoming evident that PGs may also influence the biological activities of secretory granule constituents. A well-characterized example is certain mast-cell chymases, serine proteases that are tightly bound to heparin PG *in vivo*. A number of studies have shown that the PG moiety of the chymase/PG complex will bind to various heparin-binding proteins that are present in the medium, thus 'presenting' them to the protease and enhancing the rate of proteolysis markedly [110,114]. It is important to stress that the PG does not influence the actual enzymic activity of chymase. This mechanism is thus analogous to the co-receptor function of cell-surface HSPGs ([12] for review), which facilitates interaction of, e.g., FGFs with their receptor.

The consequence of this mechanistic model is that the PG part of the chymase-PG complex will favour the selection of heparin-binding substrates over proteins with low affinities for heparin. Indeed, many of the known substrates for mast-cell chymase have heparin-binding properties. Although this mechanism has so far only been described in detail for mast-cell chymase, it is not unlikely that similar mechanisms apply to other haematopoietic proteases. It is important to note that many haematopoietic serine proteases, e.g. cathepsin G and granzymes, are basic proteins, and that their physiological form is most likely in a complex

with serglycin PGs. Several studies have shown that elastase, a serine protease present in secretory granules of neutrophils, is inhibited by heparin and to a lesser extent by its physiological ligand chondroitin sulphate [115,116]. In contrast with the effect of heparin on mast-cell chymase, the effect of GAGs on elastase appears to be a direct effect on the catalytic activity.

Another mode by which PGs may influence the activity of a secretory granule compound is exemplified by tryptase, a tetrameric mast-cell serine protease that is stored in the secretory granule as a complex with heparin PG. It has been known for a long time that heparin is needed for stabilization of the tryptase tetramer [117] and more recent studies have provided evidence that heparin is also required in the initial assembly of the tryptase tetramer with concomitant enzymic activation [118–120]. In the latter case it was shown that heparin induces tetramerization by cross-linking the individual tryptase monomers [120], yet another example where PGs facilitate interaction between proteins. Moreover, it has been suggested that heparin is involved in the processing of the N-terminal activation peptide in tryptase [121]. Similarly, it has been shown that heparin may be important for the removal of the activation peptide in mast-cell carboxypeptidase A [122] and in chymase [123], although the latter findings have been questioned [124].

Finally, it is interesting to note that optimal inhibition of thrombin requires that heparin binds both to antithrombin and to the coagulation protease in a ternary complex [125], another example where a GAG acts by bringing two proteins in contact (see above also).

Protection

It may be hypothesized that the association of intracellular PGs with secretory granule compounds could serve a protective role. For example, many haematopoietic cell types contain fully active proteases in their granules and it may be important, therefore, to have mechanisms that protect the cells from damage caused by their endogenous proteases. Possibly, the intracellular PG partner, in combination with the low pH in the secretory granules, may fulfil such a function. It is also possible that PGs may have a protective function for secretory granule compounds after secretion into the extracellular space. Indeed, it has been shown that heparin PG protects mast-cell chymase [126], elastase [127] and cathepsin G [128] from inhibition by plasma protease inhibitors such as α_1 -antitrypsin, α_1 -antichymotrypsin and α_2 -macroglobulin. In contrast, it has been shown that the inhibition of mast-cell chymase by secretory leucocyte proteinase inhibitor [129] and the inhibition of cathepsin G by mucus proteinase inhibitor [130] are in fact accelerated when the respective protease is bound to heparin. These results may at first glance seem contradictory; however, it should be noted that both secretory leucocyte proteinase inhibitor and mucus proteinase inhibitor are heparin-binding proteins whereas the plasma protease inhibitors are not. Hence, the accelerated inhibition of heparin-bound proteases by heparin-binding protease inhibitors clearly conforms to the concept that heparin enhances the rate of molecular encounter between heparin-binding proteins (see above; see also [131,132] for a further discussion of this mechanism), whereas proteins that are not heparin binding are subject to electrostatic repulsion when its potential binding partner is attached to heparin.

Nature of interactions

The interaction between secretory granule PGs and partner compounds appears to be predominantly electrostatic in the cases studied. Moreover, the strength of the interaction seems to be

largely dependent on anionic charge density rather than on specific structural elements of the GAGs. This is in contrast with the interaction of heparin with the blood plasma protease inhibitor antithrombin, which is strictly dependent of a specific pentasaccharide sequence [132]. Detailed studies of the interaction between secretory granule proteins and their cognate PGs have only been carried out in a few cases, e.g. for lactoferrin [133], granzyme B [108], elastase [116], myeloperoxidase [134], mast cell tryptase [120] and mast cell chymase [135,136]. Out of these, the GAG binding characteristics have been particularly well studied for the mast-cell proteases. Mast-cell chymase has been shown to bind tightly to heparin, its physiological ligand, and it has been shown that this interaction is highly dependent on the anionic charge density of the GAG [135,136]. Importantly, structurally unrelated but highly sulphated polysaccharide species could substitute for heparin in binding to the chymase, indicating 'non-specific' electrostatic interactions. It was found that the smallest oligosaccharide capable of interacting with chymase was a tetradecasaccharide [136], a size that is considerably larger than the size required for optimal interaction with e.g. lactoferrin [133], platelet factor 4 [137] and antithrombin [132]. The reason behind this unexpectedly large size of the minimal saccharide required for binding is not clear, but could be related to the molecular architecture of mast-cell chymase, having two potential GAG-binding clusters of positively charged amino acid residues on the molecular surface [138,139]. Possibly, optimal interaction of heparin with chymase might require simultaneous binding to both of these sites. Similarly, characterization of the binding of GAGs to tryptase revealed a strong dependence on anionic charge density for binding, without any obvious stringent requirements as regards specific structural elements of the GAG. Octasaccharides were found to be the smallest oligosaccharides capable of binding to the tryptase monomers. However, approximately twice that size (20 saccharide units) was required for cross-linking the tryptase monomers into tetramers and subsequent acquirement of enzymic activity [140]. Although the interaction of most granule proteins with their respective partner PGs appears to be of relatively non-specific nature, a more defined binding mode has been suggested for the interaction between histamine and heparin. Studies by NMR showed that the diprotonated form of histamine interacts preferentially with the carboxy and, possibly, the sulphamido group of the GAG, while no interaction was observed with the 6-O- and 2-O-sulphate groups [141]. Interestingly, histamine and heparin were identified and were also shown to co-localize in the tunicate *Styela plicata* [142], indicating that the interaction of heparin with histamine shows evolutionary conservation.

Fate of exocytosed serglycin

An interesting but scarcely studied question is what happens to granule PGs after release to the extracellular environment. In one study it was shown that serglycin injected into the bloodstream was rapidly taken up by sinusoidal scavenger endothelial cells in the liver [143]. Other reports indicate that secreted serglycin might interact with CD44 [90] and thereby participate in cell adhesion processes. It is evident that there are several questions concerning the fate of secreted granule PGs that await exploration, such as whether these PGs are degraded by endoglycosidases, incorporated into the extracellular matrix, or engage in new partnerships with molecules destined for presentation to cell-surface receptors and/or endocytosis.

Concluding remarks

Studies on PGs are constantly advancing into new territories. The intracellular environment has so far been surprisingly unchal-

lenged as a venue for acquiring novel information about these complex molecules. There are, however, aspects receiving increasing attention, such as the importance of PGs for intracellular granule formation, and the fate of heparan sulphate taken up by endocytosis from the extracellular environment or the cell surface (Figure 1).

The presence and functions of PGs in intracellular granules may be viewed as a reflection of previously characterized functions of extracellular PGs and their contribution to the construction of a functioning matrix. However, with our increasing understanding of the complex dynamics of PGs in the extracellular matrix in relation to cell attachment and migration, binding and regulation of growth factors, binding to proteolytic enzymes and protease inhibitors, it would not be surprising if additional functions and activities of intracellular and granule-associated PGs will eventually be demonstrated. Proper organization of the intragranular environment is in itself not a trivial function. The generation of granules, granule homeostasis, protection and regulation of granular proteins, and regulation of secretion may depend on granule PGs. In future studies of secretion it will be important to study the differences between constitutive and regulated pathways of secretion and the role(s) played by the distinct PGs in the different cell types with PG-containing granules.

Complete degradation in the lysosomal compartment might not be the only destiny awaiting endocytosed PGs. A minor part of endosomal cargo containing GAGs or PGs could be targeted to other intracellular destinations to perform vital functions, with or without partners, in the communication between the extracellular environment and intracellular regulatory systems. In several aspects, the GAG chain is the 'business end' of PG molecules, and accumulating data show that heparan sulphate may contain several domains with different structural features and functional potentials [1]. Early studies on heparan sulphate in the nucleus suggested that specific structures could be recovered [75]. The possible functional basis for such a finding has not been defined, and still represents a fascinating challenge. However, several PGs have large protein cores with several distinct protein motifs with different features, as is the case for agrin, perlecan and versican. Intracellular processing and sorting of such entities has not been studied in any great detail, except in the context of biosynthetic regulation. Although speculative, it may be that some of the protein domains are destined for functions intracellularly. The fact that newly synthesized glypican, with and without heparan sulphate chains, receives different intracellular travelling instructions in epithelial MDCK cells shows that regulation of intracellular trafficking is a relevant theme for PG research.

The intracellular routing of PGs has not been studied in great detail. What are the functions of PGs with nuclear sorting signals? Is there a way by which PGs could reach the nucleus without first appearing on the cell surface? Furthermore, granule PGs may have importance for the functions of a rainbow alliance of molecules in the same organelles. Several of these molecules await structural and functional characterization. Intracellular PGs may turn out to have a functional repertoire as complex and fascinating as their family members travelling to more distant destinations.

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