Specificities of heparan sulphate proteoglycans in developmental processes

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Heparan sulphate proteoglycans are abundant cell-surface molecules that consist of a protein core to which heparan sulphate glycosaminoglycan chains are attached. The functions of these molecules have remained mostly underappreciated by developmental biologists; however, the actions of important signalling molecules, for example Wnt and Hedgehog, depend on them. To understand both the mechanisms by which ligands involved in development interact with their receptors and how morphogens pattern tissues, biologists need to consider the functions of heparan sulphate proteoglycans in signalling and developmental patterning.

Biochemical studies and cell-culture assays have implicated heparan sulphate proteoglycans (HSPGs) as co-receptors[1] in processes ranging from mechanical support to functions in adhesion, motility, proliferation, differentiation and morphogenesis (see ref. 2 for a review). Enormous structural heterogeneity can be generated through specific heparin sulphate (HS) chain modifications during their biosynthesis, as well as from the diverse nature of their core proteins. Furthermore, biochemical studies have shown how interactions of cell-surface HSPGs can be specific at various levels. Tissue-specific isoforms of the polymerizing and chain-modifying enzymes can produce HS chains with distinct sequences and macroscopic organizations[3,4]. Various ligands and their receptors can show selectivity in their binding affinities to distinct HS chain structures[5,6]. These chains can be attached to one or more specific core proteins, each with a distinctive tissue-specific expression pattern and cellular localization. Finally, HSPGs can be selectively shed from the cell surface to yield soluble effectors.

Despite extensive biochemical evidence that HSPGs have specific functions *in vivo*, the roles of these molecules have not been appreciated, especially by developmental biologists. The prevalent view is that these highly abundant, strongly anionic molecules interact non-specifically with secreted growth factors and extracellular matrix (ECM) components, and have a general function in protecting these ligands from proteolysis or sequestering them in the ECM. Indeed, these ligands are frequently purified by chromatography on columns of heparin, the heavily modified HS-related glycosaminoglycan that is pharmaceutically manufactured from mast-cell-rich tissues[7]. This has contributed to the notion that HSPGs lack specificity and simply act as 'extracellular fly paper' that binds and concentrates ligands at the cell surface.

This idea is rapidly changing, however, because of insights from genetic studies in *Drosophila* and mice. Indeed, genetic studies have begun to reveal dedicated functions for HSPGs in specific signalling pathways involved in cell differentiation and morphogenesis. Here we discuss the developments in the field and illustrate the complex and specific roles of HSPGs *in vivo*. From the analysis of mutant phenotypes that are associated with mutations in either the enzymes that synthesize the HS chains linked to the proteoglycan core proteins or the core proteins themselves, it is now apparent that HSPGs are critical in the interactions between specific extracellular ligands and their signal-transducing receptors.

Enzymes involved in HSPG biosynthesis

Precursor HS chains are initially synthesized in the Golgi as non-sulphated copolymers attached to HSPG core proteins. The chain is initiated at specific Ser-Gly residues within defined amino-acid sequences on the core protein. The number of such chain attachment sites varies with the core protein, but is typically only between two and four. The first step in HS chain biosynthesis is the enzymatic transfer of xylose from the sugar nucleotide to the hydroxyl group of these serine residues (for details, see ref. 5). Each sugar in a common tetrasaccharide linkage region (-GlcA-Gal-Gal-Xyl) is then transferred from the sugar nucleotide by individual Golgi enzymes. A single b-linked GlcNAc residue is added to the tetrasaccharide linker followed by alternating GlcA and GlcNAc residues catalysed by HS polymerase.

Once this chain is assembled, the individual saccharide units undergo a number of sequential modifications by various Golgi enzymes: *N*-deacetylase/*N*-sulphotransferase (NDST), uronosyl C5-epimerase, 2-*O*-sulphotransferase (2-OST), 6-*O*-sulphotransferase (6-OST) and 3-*O*-sulphotransferase (3-OST) (Fig. 1). The enzymes do not modify all the available sugars in the chain, which

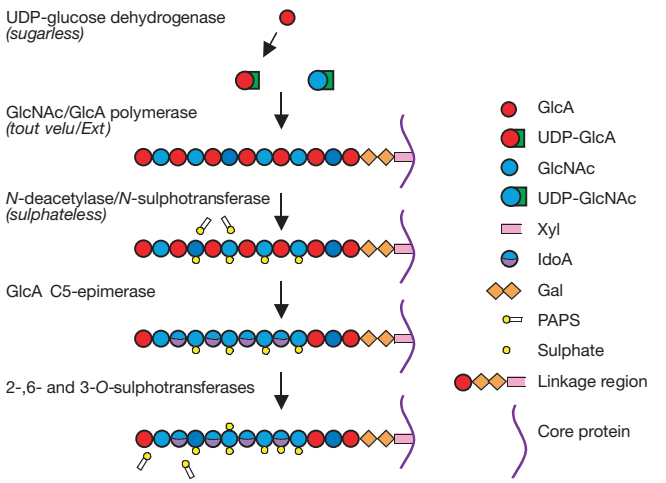

Figure 1 Scheme of HS chain biosynthesis. The chains are synthesized on the core protein by the sequential action of individual glycosyltransferases. A common tetrasaccharide linkage region is formed, followed by the addition of alternating GlcA and GlcNAc residues, producing in turn the precursor chain. This chain is then enzymatically modified by deacetylation and *N*-sulphation, epimerization and *O*-sulphation, yielding individual chains whose sequence is distinct from all other chains.

results in extensive sequence diversity in the final chain. This structural heterogeneity includes a macroscopic organization in which regions of 10–16 highly modified disaccharides alternate with longer regions of relatively unmodified disaccharides. The overall size of the HS chain can vary from 20 to 150 disaccharides, which adds another level of complexity to HS chains.

In the past few years, many of the enzymes that participate in HS biosynthesis have been identified on a molecular level in vertebrates and invertebrates[3]. In mammals, multiple isoforms of HS biosynthetic enzymes have been identified[4]. Similarly, many of the same genes have been characterized in *Drosophila*, and, rather surprisingly gene redundancy is minimal in this organism, multiple polymerases and sulphotransferases have now been identified (Table 1).

Core proteins involved in HSPG biosynthesis

A variable proportion of some proteins can become glycanated with HS chain(s) (CD-44, betaglycan) but we will restrict our discussion to core proteins that are consistently and fully glycanated with HS chains. These HSPGs are diverse and can be transmembrane (syndecans), bound by a glycosyl phosphatidylinositol (GPI) linkage to plasma membrane lipid (glypicans), or secreted into basement membranes (perlecan, agrin)[8] (Fig. 2). Glypicans and syndecans represent the two main cell-surface HSPGs, and in mammals four syndecan and six glypican separate genes have been identified. In mice, these generally show distinct developmental and tissue-expression patterns. In *Drosophila*, only a single syndecan gene is known, but two glypican genes, *dally*[9] and *dally-like* (G. Baeg, X. Lin and N.P, unpublished data) have been characterized (Table 1). The *unc-52* gene is the perlecan ortholog in *Caenorhabditis elegans*, and although basement membrane HSPGs have not been described in *Drosophila*, the fly genome does contain a sequence corresponding to a perlecan-like core protein.

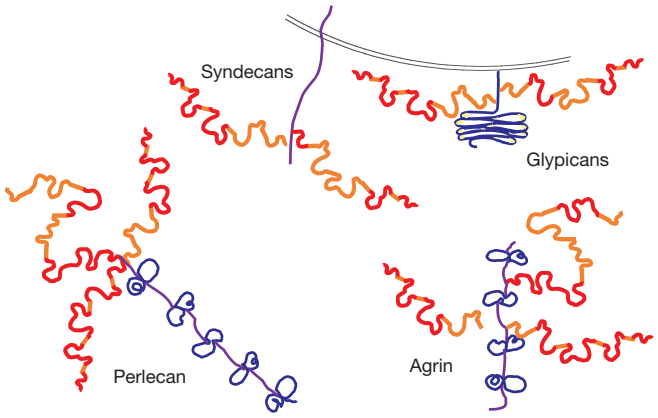

Figure 2 Depiction of HSPGs associated with cell surfaces. The syndecan and glypican family members are integral membrane proteins. The syndecan core proteins are apparently highly extended transmembrane proteins that contain a short (34–38-residue) carboxy-terminal cytoplasmic domain. The HS chains on the syndecans are linked to serine residues that are distal from the plasma membrane. The glypican core proteins are apparently disulphide-stabilized globular core proteins linked to the plasma membrane by an apparently extended C-terminal region that culminates in a GPI linkage. The HS chains on the glypicans are linked to serine residues adjacent to the plasma membrane in the apparently extended region. Basement membranes in mammalian tissues contain perlecan and agrin. Perlecan is in nearly all basement membranes as well as in cartilage, whereas agrin is a component of basement membranes in muscle, myoneural junctions, the central nervous system, lung and kidney. Both are large multidomain proteins bearing HS chains near their amino termini. Although both proteins exist as multiple isoforms that arise by alternative splicing and contain globular domains that are homologous to laminin G and epidermal growth factor domains, on the basis of their genomic organization, they are quite distinct proteins.

Although syndecans and glypicans are associated with the plasma membrane, it is important to note that under certain conditions the syndecan core proteins can be proteolytically cleaved near the cell surface, releasing the intact ectodomain replete with all the HS chains of the parental molecules (see ref. 2). Glypicans may also be shed, as they are reported to be soluble components of conditioned cell-culture media and follicular fluid.

Although there is no evidence for regulated shedding or shedding *in vivo* (see ref. 10), this shedding has been suggested to be mediated by a phosphoinositide-specific phospholipase C. In mammalian cells, syndecan ectodomain shedding is mediated by cell-surface-associated metalloproteinase(s), is regulated by intracellular signalling pathways, and results in soluble effectors[11]. Soluble syndecan ectodomains are found in wound fluids[12] where they can inhibit growth factor[13] and enhance proteinase activities[14]. Interestingly, enhanced shedding in the skin wounds of transgenic mice overexpressing syndecan-1 results in inhibition of cell proliferation and altered proteolyic activity in the wound, causing defective repair (V. Kainulainen, O. Reizes, J. Madri and M.B., unpublished data). Thus, the soluble ectodomains can act as dominant-negative inhibitors of the cell-surface HSPGs. In principle, the cleavage of the extracellular domain of HSPGs could be associated with other functions; for example, the solubilized ectodomain could regulate the movement of growth factors within extracellular spaces.

Developmental roles of HS enzymes

The critical roles of HSPGs in developmental processes and specific signalling pathways have been illustrated by the identification of mutations in enzymes involved in HS chain biosynthesis in *Drosophila* and mice.

In *Drosophila*, mutations in the homologue of UDP-D-glucose dehydrogenase, which produces UDPGlcA (*sugarless* (*sgl*))[15–17], and in the NDST homologue (*sulfateless* (*sfl*))[18] have been identified. In either *sgl* or *sfl* mutant embryos, Wingless (Wg), a member of the Wnt family, fibroblast growth factor (FGF) and Hedgehog (Hh) signalling pathways are impaired. Interestingly, the phenotype associated with these mutations is very severe and corresponds to a complete loss of activity of these pathways, indicating that the HSPGs are absolutely required for signalling of these growth factors and morphogens.

The *Drosophila* gene *tout velu* (*ttv*) has been identified as an enzyme involved in the production of HS chains. Surprisingly, the *ttv* mutant only affects a specific signalling pathway, suggesting an unexpected level of specificity of the HSPG involved. Mutations in the *ttv* gene were first isolated based on the inability of Hh molecules to move through a field of cells[19]. Further studies suggested that the membrane-targeted cholesterol-modified Hh molecule requires HSPGs either to be trapped by receiving cells or to move from cells to cells[20]. Molecular characterization of *ttv* revealed that it is

Table 1 Core proteins and enzymes

	Drosophila	Mice
Core proteins		
Glypican	Dally, Dally-like*	Glypican-1, -2, -3, -4, -5, -6*
Syndecan	Dsyndecan	Syndecan-1, -2, -3, -4
Enzymes		
UDP-D-glucose dehydrogenase	Sugarless	
N-deacetylase/*N*-sulphotransferase	Sulfateless	NDST-1, -2, -3, 4
GlcNAc/GlcA polymerase	Tout velu, DExt2?	Ext 1, Ext2, EXTL1?†
α-GlcNAc transferase?		EXTL2
Uronosyl C5 epimerase		Ep-1
2-*O*-Sulphotransferase	Pipe, HS2-OST	2-OST-1
3-*O*-Sulphotransferase		3-OST-1, -2, -3
6-*O*-Sulphotransferase		6-OST-1, -2, -3, -4, -5, -6, -7

* Although *Drosophila* Dally and Syndecan are known to contain HS chains, it is important to note that in most instances the enzymatic activities of the proteins listed have not been shown and are inferred from their amino-acid sequence homologies.
† Proteins of the EXT/EXTL families encode either HS-copolymerases (Fig. 1) or α-GlcNAc transferases (the enzyme that begins the HS chain after linker biosynthesis[38]). In particular, there is no direct evidence that Sgl and Sfl have the enzymatic activities listed, and the biochemical activities of DExt2 or mouse EXTL1 have not yet been reported.

homologous to a member of the *Ext* gene family, which has been implicated in the human multiple exostoses (Ext) syndrome[21]. This dominantly inherited disease is characterized by short stature, inequalities in limb length, bone deformities and the presence of bone outgrowths, called exostoses, at the ends of long bones. The function of Ext proteins as HS polymerases has recently been elucidated. First, the inability of herpes simplex virus, which binds to cell-surface HS, to infect the mouse cell line sog9 correlates with a defect in HS biosynthesis[22]. This defect, however, can be rescued by introducing an *Ext1* complementary DNA into the mutant cell line. Second, an *Ext2* homologue from bovine serum has been isolated[23] and shown to have HS polymerase activity.

Because *ttv* encodes a putative HS polymerase, it is surprising that in the absence of *ttv* activity only Hh signalling is affected, and not Wg and FGF signalling as observed in *sgl* and *sfl* mutants. This, together with the observation that the overall HS concentration is reduced in *ttv* mutants[20,24], suggests that other *Drosophila Ext* genes exist. Consistent with this finding, at least one other *Ext* gene is present in the fly genome[20]. A possible explanation of the specificity of Ttv for Hh action is that Hh signalling may be more sensitive to a reduction in HSPG concentration than Wg and FGF signalling. Alternatively, Hh-specific HSPG(s) may exist and Ttv may be responsible for its production.

In addition to Ttv/Ext, *Drosophila* mutants in the *pipe* gene, which encodes a putative 2-*O*-sulphotransferase[25], show defects in the establishment of dorsal–ventral (D–V) polarity in the embryo. *pipe* is expressed in the ventral part of the egg chamber and has been proposed to activate the serine protease cascade that leads to production of the active Toll ligand Spätzle. The effect of *pipe* on Toll signalling is probably indirect. The role of the proteoglycan involved might be either to activate or to assemble a protease complex which processes Spätzle or concentrates Spätzle to the ventral side of the egg chamber. Finally, although Pipe encodes a putative 2-OST, it is not yet known whether it encodes a sulphotransferase for a HSPG or another class of proteoglycan.

Similar examples of specific effects associated with enzymes involved in HS chain biosynthesis have also been found in mice. Compelling evidence for tissue-type selectivity of HSPG function comes from studies of embryos that are homozygous for a mutation in the gene encoding HS 2-OST[26]. These mice show multiple developmental abnormalities including lack of kidney induction. The abnormalities probably arise from compromised activities of the specific HS-dependent growth factors responsible for this organogenesis. In addition, targeted disruption of a single gene encoding one of four known NDST genes prevents mast-cell biosynthesis of heparin chains but does not affect the biosynthesis of other HS chains[27,28]. Thus, the HS biosynthetic enzymes probably have activities that are specific to cell type. Differences in the activities or regulation of these enzyme isoforms may account for the reproducible structural distinctions of HS chains from different cell and tissue types[29,30] and for reproducible differences in growth factor binding of HS chains from distinct developmental stages[31].

The HSPG core proteins have specific developmental roles

In contrast to the analysis of the HS biosynthetic enzymes, much less is known about the phenotypes associated with mutations in the core proteins. In *Drosophila*, mutations in both *syndecan* and one of the *glypican* genes (*dally*) have been identified[9]; however, detailed phenotypic analysis are only available for *dally*.

Genetic analyses of *dally* have implicated this glypican in both Wg and Decapentaplegic (Dpp, a member of the transformin growth factor-β family) signalling. Loss of *dally* activity, in both the embryo and imaginal discs, generates phenotypes reminiscent of loss of Wg activity. Genetic experiments are consistent with a model in which Dally acts as a co-receptor for the Wg-transducing receptor encoded by the seven-transmembrane protein Frizzled 2 (refs 18, 32). Interestingly, *dally* expression is developmentally regulated and is co-expressed with Frizzled 2 in the embryo, suggesting that, as previously observed for Frizzled 2 (ref. 33), the level of the HSPG co-receptor has to be tightly regulated for proper Wg signalling.

Interestingly, Dally has been found to act in Dpp signalling but only in imaginal discs[34]. Dpp is also expressed during embryonic stages; however, there is no evidence from the studies of *sfl*, *sgl* or *dally* mutants that HSPGs are involved in the early function of Dpp in the establishment of D–V embryonic polarity. A reduction in *dpp* levels enhances the defects associated with *dally* mutations in the eye, antenna and genitalia. Furthermore, additional copies of *dpp* rescue the defects in these tissues. These genetic interactions have led to the hypothesis that Dally regulates Dpp activity[32]. Analysis of Dally provides a striking example of an HSPG that is developmentally regulated and that interacts with only a subset of signalling pathways.

In mice, targeted disruptions of glypican genes yield either no apparent abnormalities (glypican-2; S. Saunders and A. Lander, personal communication) or a phenotype similar to the Simpson–Golabi–Behmel syndrome[35], a congenital overgrowth syndrome of unknown pathogenesis in humans associated with mutations in the *glypican-3* gene[36]. Interestingly, glypican-3 may function to regulate bone morphogenetic protein (BMP)-4 signalling in as much as the progeny of crosses between the glypican-3 knockout with haploinsufficient mutants of BMP-4 in mice show skeletal abnormalities that are not seen in either parental strain (S. Paine-Saunders, W. C. Skarnes and S. Saunders, personal communication). Targeted disruption of the *syndecan-1* gene, the main syndecan gene expressed by epithelia and plasma cells, yields apparently normal fertile mice despite the early and intense expression of syndecan-1 during embryogenesis[1]. The absence of abnormalities presumably results from compensatory expression of other syndecan family members. However, epidermal or corneal epithelial wounds in these mice undergo markedly delayed repair (H. Gibson, M. Hinkes and M.B., unpublished data; M. A. Stepp *et al.*, unpublished data). Furthermore, mammary epithelia in these mice resist the tumorigenic action of the transgenically expressed Wnt-1 oncogene[37] and also resist lung infection when certain bacteria are inoculated intranasally, but are normally susceptible when inoculated via the peritoneal cavity (P. W. Park, G. Pier and M.B., unpublished data). Thus, abnormalities are detected in these syndecan-1-deficient mice only by perturbing their epithelia. These data suggest that compensation by other HSPGs is sufficient to maintain normal development and reproduction, but cannot replace the specific functions of syndecan-1.

Perspectives

We have discussed the contribution of the genetic approach to the understanding of HSPG functions in developmental processes. It is now apparent that analysis of the mutant phenotypes associated with specific gene disruptions in either the various enzymes involved in HS chain biosynthesis or the core proteins provide a wealth of information about the complex roles of HSPGs *in vivo*. To gain further insights into these roles, it is now critical to analyse the mutant phenotypes associated with the remaining enzymes and core proteins. The availability of a repertoire of mutants in each of the components involved in HS chain biosynthesis will allow many other issues to be critically addressed. In particular, detailed analysis of HS chain structure in various mutants will define the physiological roles of HS chains expressing different binding specificities. Furthermore, issues of functional redundancy between HSPG core proteins should be directly testable by analysing the phenotypes of double mutants, or by expressing one specific HSPG core protein in the mutant background of another.

The availability of HSPG mutants will allow one to characterize the function of HSPGs in signalling. In most models the HSPG is viewed as a co-receptor for the signal-transducing receptor; however, in some instances the extracellular domain can be cleaved,

released from the cell surface and potentially used as a carrier of extracellular signals. Using genetic mosaics, the cellular autonomy of HSPG function could be readily determined. In the case of Hh signalling, the HSPG appears to be critical to the cell-to-cell movement of Hh molecules. Although the precise roles of the HSPGs are not yet understood, it is clear that these complex molecules are fundamental to the distribution and processing of extracellular signals that pattern fields of cells. Finally, additional roles of HSPGs in developmental, physiological and behavioural processes are likely to be uncovered by the analysis of mutants. □

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