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Heparan sulfate proteoglycan as a cell-surface endocytosis receptor

Helena C. Christianson [a], Mattias Belting [a,b,*]

[a] Department of Clinical Sciences, Section of Oncology, Lund University, Lund, Sweden
[b] Skåne University Hospital & Oncology Clinic, Lund, Sweden

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ABSTRACT

How various macromolecules are exchanged between cells and how they gain entry into recipient cells are fundamental questions in cell biology with important implications *e.g.* non-viral drug delivery, infectious disease, metabolic disorders, and cancer. The role of heparan sulfate proteoglycan (HSPG) as a cell-surface receptor of diverse macromolecular cargo has recently been manifested. Exosomes, cell penetrating peptides, polycation–nucleic acid complexes, viruses, lipoproteins, growth factors and morphogens among other ligands enter cells through HSPG-mediated endocytosis. Key questions that partially have been unraveled over recent years include the respective roles of HSPG core protein and HS chain structure specificity for macromolecular cargo endocytosis, the down-stream intracellular signaling events involved in HSPG-dependent membrane invagination and vesicle formation, and the biological significance of the HSPG transport pathway. Here, we discuss the intriguing role of HSPGs as a major entry pathway of macromolecules in mammalian cells with emphasis on recent *in vitro* and *in vivo* data that provide compelling evidence of HSPG as an autonomous endocytosis receptor.

1. Introduction

1.1. HSPG diversity

Heparan sulfate proteoglycans (HSPGs) encompass a diverse class of proteins defined by the substitution with HS glycosaminoglycan (GAG) polysaccharide chains. HS is a polymer of repeating N-acetyl glucosamine (GlcNAc)-D-glucuronic acid (GlcA) disaccharide units. General modifications occurring in the synthesis of an HS chain are: N-deacetylation/N-sulfation of GlcNAc residues; epimerization of GlcA to L-iduronic acid (IdoA); sulfation at the 2-O position of IdoA; and sulfation at 6-O and 3-O positions of GlcNAc. The abundant carboxyl and sulfate groups of HS significantly contribute to the polyanionic nature presented by mammalian cells to their neighboring cells and extracellular environment. Accordingly, several nucleic acid binding molecules interact with HS that exert their functions *via* association with nucleic acids once internalized through an HSPG-dependent mechanism (Belting, 2003; Sarrazin et al., 2011).

HS can be conjugated onto a variety of proteins with different spatial distributions, *e.g.* perlecan in the extracellular matrix, and cell-surface associated syndecans (SDCs) and glypicans (GPCs), which further determines the biological outcome of HS ligand binding. Notably, whereas SDCs are transmembrane proteins, GPCs are associated with the plasma membrane *via* a glycosyl–phosphatidyl–inositol (GPI) anchor. Depending on the repertoire of other membrane proteins, perhaps most importantly integrin and lipoprotein receptors, cell-surface HSPGs may be located to various plasma membrane domains. Notably, the topographical localization of HS chains on core proteins differs; GPC HS chains are attached to the core protein close to the plasma membrane, whereas SDC HS chains are attached at more peripheral sites. Moreover, the status of the HS biosynthesis, modification and degradation machineries set the stage for how cells bind to a wide variety of extracellular, polybasic ligands. Thus, an interesting and challenging study subject is the biological relevance of HS-ligand interactions.

1.2. HSPG endocytosis diversity

The exact role of HSPGs in the uptake of macromolecular cargo has been controversial. For long, the prevailing model was that cell-surface ligand binding by HSPG resulted in ligand conformational alteration followed by presentation to "specific", high-affinity endocytosis receptors. Endocytosis can broadly be divided into clathrin dependent and independent internalization, of which the latter can be further classified into caveolin-mediated endocytosis, macropinocytosis and caveolin-independent pathways involving lipid rafts (Howes et al., 2010). Differential modes of membrane attachment of SDCs and GPCs (see above) may preferentially sort them to clathrin-coated and lipid raft plasma membrane domains, respectively. Numerous HS-binding ligands, including FGF2, DNA-peptide polyplexes, anti-HS antibody-conjugated nanoparticles and VLDL (Tkachenko et al., 2004; Wilsie et al., 2006; Paris et al., 2008; Wittrup et al., 2010) appear to utilize lipid raft-mediated pathways. Uptake of GPC-bound ligands may proceed primarily through caveolin-dependent endocytosis (Cheng

* Corresponding author at: Lund University, Department of Clinical Sciences, Section of Oncology, Barngatan 2B, SE-221 85, Lund, Sweden.
E-mail address: Mattias.Belting@med.lu.se (M. Belting).

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et al., 2002). The small Rho GTPase dynamin was proposed to have a role in HSPG-mediated uptake of cationic polymers and polypeptides (Payne et al., 2007), whereas FGF2 and anti-HS antibody uptake were dynamin-independent (Tkachenko et al., 2004; Wittrup et al., 2010). Other small GTPases, *e.g.* Cdc42, Rac1, RhoA, RhoG and ARF6 as well as actin microfilament interactions may also regulate HSPG internalization (Zimmermann et al., 2005; Bass et al., 2007), *e.g.* SDC-4 was found to orchestrate the activation of all four GTPases during endocytosis and recycling of $\alpha_5\beta_1$-integrin (Brooks et al., 2012). HSPG endocytosis is thus not restricted to one particular pathway, and varies depending on the cellular context and type of extracellular ligand. A key remaining question is how the endocytic route used in conjunction with ligand characteristics dictates the intracellular sorting and fate of internalized cargo, *e.g.* lysosomal degradation, recycling, endosomal escape into the cytosol, or fusion into multivesicular bodies and exosomes for extracellular secretion. In addition, how HSPG expression and localization are modulated according to the cell's need to internalize extracellular ligands still remains ill-defined. However, it was demonstrated that cells made dependent on the uptake of exogenous polyamines display cell-surface HSPGs with increased polyamine affinity (Belting et al., 1999). Also, abnormal insulin and fatty acid exposure of hepatocytes triggered the exchange of CS for HS on SDC, which resulted in decreased affinity for lipoprotein particles possibly contributing to insulin resistance-associated dyslipidemia (Olsson et al., 2001). Later *in vivo* studies, however, indicated that HS structure remained unaltered in hepatocytes of diabetic as compared with non-diabetic mice (Bishop et al., 2010). Future studies will have to clarify how various environmental factors regulate HS structure and HSPG-dependent endocytosis.

1.3. Regulatory role of HSPG shedding

Shedding of HSPGs from the cell-surface represents another level of regulation of HSPG-mediated endocytosis. GPCs can be shed by GPI-specific lipases, whereas SDCs are enzymatically released by a variety of matrix proteinases (Fransson et al., 2004; Nam and Park, 2012). Several pathogens have been shown to release proteases that induce shedding of SDC-1 from host cells, which may represent a general defense mechanism against microbial internalization and HS-binding antimicrobial peptides. Moreover, injection of lipopolysaccharide into mice induced shedding of hepatic SDC-1, resulting in impaired VLDL catabolism and hypertriglyceridemia (Deng et al., 2012). HSPG shedding can thus down regulate HSPG-dependent functions by sequestration of HS binding ligands, making them unavailable for cellular uptake. However, it remains to be investigated how shed HSPG–ligand complexes are further metabolized; in analogy with serglycin (Raja et al., 2005), shed HSPG may actually act as an extracellular chaperone that transfers ligands in a paracrine manner to cell-surface HSPGs on neighboring cells for internalization.

2. Regulation of HSPG-mediated endocytosis

2.1. HS structural specificity in macromolecular uptake

Almost half a century ago, it was shown that polybasic peptides greatly enhance protein uptake by cultured cells (Ryser and Hancock, 1965); however, the mechanism of this phenomenon remained unknown until 1996, when Mislick and Baldeschwieler suggested that poly-lysines can mediate DNA uptake through a route strictly dependent on intact HSPG biosynthesis in target cells, and an optimum peptide-DNA complex charge ratio of +1.5 (Mislick and Baldeschwieler, 1996). Our group could demonstrate that uptake of HIV-Tat peptide (GRKKRRQRRRPPQ)–DNA complexes with a charge ratio of >1 was strictly dependent on HSPGs, whereas HSPG deficiency in recipient cells favored the uptake of complexes with a net negative charge (Sandgren et al., 2002). It soon became established that HSPG has a general role in the uptake of polyarginines, polylysines, and arginine/lysine hybrid peptides (Belting, 2003). More recently, it was shown that tryptophan interspersed between basic amino acids could trigger HS-dependent endocytosis through the formation of beta-sheet stable aggregates. Interestingly, peptide internalization was more efficient in cells deficient in sialic acid, *i.e.* another class of cell-surface polyanion that may sequester and prevent peptides from interacting with internalizing HSPG (Bechara et al., 2013). The abundance of such domains and peptide stretches in naturally occurring proteins is consistent with HSPG ligand promiscuity, as mentioned above (Belting, 2003; Sarrazin et al., 2011; Raff et al., 2013). Further, therapeutic antibody cationization has been associated with increased target tissue retention and increased clearance from the circulation, including transport over the blood brain barrier, potentially owing to enhanced HSPG-mediated transcytosis (Boswell et al., 2010).

Key questions are whether HSPG is a true internalizing receptor, part of a multi-receptor complex, or merely an initial attachment site during macromolecular delivery, and whether HSPGs of both the SDC and GPC type are involved. To address this, the internalizing activity of a library of phage display derived anti-HS antibodies (αHS) was explored. Among several αHS clones, only one was shown to efficiently enter cells, through a process dependent on 2-O-sulfated but not N-sulfated HS. Using metabolic labeling of HSPGs and internalization of αHS-conjugated superparamagnetic nanoparticles, it was concluded that HSPG acts as an independent, internalizing receptor of macromolecular cargo (Wittrup et al., 2009). Notably, the strict dependence on HSPG for αHS internalization as opposed to other HS-binding ligands, *e.g.* growth factors and lipoproteins that may utilize alternative receptors, allowed specific isolation of HSPG-derived endosomes. Further, HSPGs of both the SDC and GPC type were able to mediate nanoparticle uptake and resided in the same vesicular compartment during early steps of endocytosis. Plasma membrane situated proteins with key regulatory functions during uptake of HS-binding cargo remain to be defined. One such component, Glucose Regulated Protein 75 (GRP75), was identified using the magnetic nanoparticle approach (Fig. 1) to isolate HSPG-associated endocytic vesicles (Wittrup et al., 2010). Together, the sorting of HSPG to endocytic vesicles may be determined by 2-O-sulfated iduronic acid-containing HS domains that can be carried by both SDC and GPC proteins.

In line with the studies above, conditional knockout mice deficient in 2-O-sulfotransferase, but not in 6-O-sulfotransferase, showed delayed clearance of lipoproteins in the liver (Stanford et al., 2010). On the other hand, type 2 diabetic mice exhibited increased sulfatase-2 activity, which is known to remove 6-O-sulfate groups, resulting in decreased lipoprotein clearance (Chen et al., 2010). Inhibition of sulfatase-2 expression in the diabetic mouse model restored VLDL-binding capacity in hepatocytes and normalized triglyceride levels (Hassing et al., 2012). These and other studies point at the importance of HSPG as an independent receptor during hepatic lipoprotein clearance *in vivo*, and suggest that the functional role of specific HS sulfation modifications, *e.g.* 2-O and 6-O-sulfation, may depend on whether they occur during initial synthesis or once HSPGs have arrived at the plasma membrane for post-synthetic processing.

2.2. HSPG core protein specificity in macromolecular uptake

Specific roles of individual HSPG core proteins have been proposed, *e.g.* by *in vivo* studies providing evidence of a specific and independent role of SDC-1 in lipoprotein uptake by hepatocytes (Stanford et al., 2009). Mouse hepatocytes expressed a variety of SDCs and GPCs, and it remains unclear why only SDC-1 depletion was associated with perturbed lipoprotein uptake. The SDC transmembrane domains have a tendency to form dimers and/or oligomers due to a conserved GxxxG dimerization motif. Although SDCs have a conserved transmembrane domain sequence, the homodimerization was shown to be weak for SDC-1, strong for SDC-3 and SDC-4, and very strong for SDC-

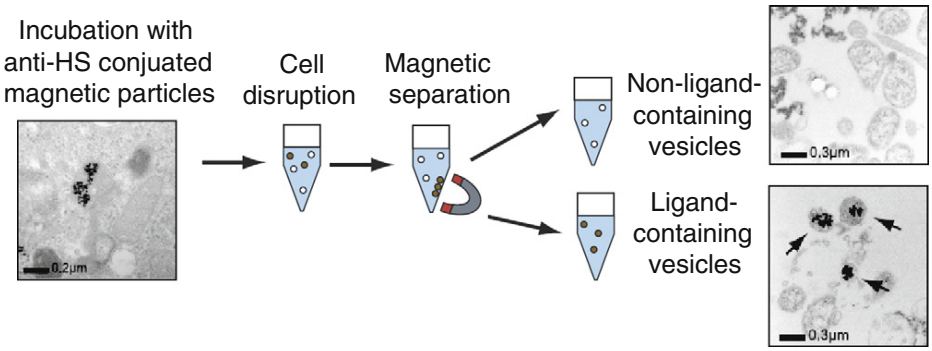

Fig. 1. Schematic presentation of magnetic nanoparticle-based isolation of HSPG endocytic vesicles. Cells are incubated with anti-HS antibody conjugated superparamagnetic beads that accumulate in endocytic vesicles. Intact cells are disrupted leaving intracellular vesicles intact, followed by several rounds of magnetic separation into non-ligand containing vesicles and ligand-containing vesicles, as shown by electron microscopy (right panels).

2 transmembrane domains (Dews and Mackenzie, 2007). Different levels of self-association suggest that dimerization of specific SDC core proteins may play different roles in directing membrane clustering following ligand binding. The cytoplasmic domain of SDCs contains two highly conserved regions (C1 and C2) and a variable region (V), situated between C1 and C2, that is unique to each SDC. The C2 region of all four SDCs terminates in an EFYA sequence, which can interact with proteins containing PDZ domains, *e.g.* syntenin (Grembecka et al., 2006). Syntenin can bind both SDC-4 and PIP2, and its lipid binding function has been shown to be essential for recycling of SDC/syntenin from endosomal compartments to the cell surface (Zimmermann et al., 2005). Two additional PDZ domain partners for SDCs are GIPC/synectin and synbindin. GIPC/synectin was suggested to be involved in removal of SDC-4 from the cell surface to endocytic vesicles (Naccache et al., 2006), and synbidin–SDC-2 interactions seem to have a role in the recruitment of intracellular vesicles to postsynaptic sites (Ethell et al., 2000). More recently, it was reported that SDC-1 follows a raft-mediated endocytic pathway dependent on a conserved motif, MKKK, in its cytoplasmic domain (Chen and Williams, 2013). It was further suggested that upon ligand binding, rapid, MKKK-dependent ERK activation triggers the dissociation of SDC-1 from tubulin. Down-stream signaling events may involve Src family kinases that trigger SDC-1 phosphorylation, recruitment of cortactin, and actin-dependent endocytosis. Whether MKKK-dependent ERK/Src signaling activation is a general mechanism of SDC-1 mediated macromolecular uptake remains to be determined.

It is less obvious how ligand interaction with GPI-linked GPCs can trigger endocytic signaling. An established view has been that GPI-linked proteins have preference for and assemble in cholesterol-rich raft membrane domains, as exemplified by co-internalization of GPC-1 and the GPI-anchored prion protein (Cheng et al., 2006). However, GPC-4 was shown to regulate Wnt signaling when localized both within and outside of lipid raft domains (Sakane et al., 2012). GPI-linked proteins may indeed be sorted to coated pits for clathrin-mediated endocytosis *via* co-association with low density lipoprotein receptor related protein-1 (LRP-1). LRP-1 represents a promiscuous receptor of clathrin-mediated endocytosis of macromolecular ligands. A recent study proposed a model in which sonic Hedgehog uptake through GPC-3 operates *via* a mechanism where the role of the HS chain is to co-associate GPC-3 with LRP-1 in coated pits (Capurro et al., 2012). Thus, the spatial distribution of specific HS epitopes, *e.g.* close to or distant from the core protein linkage region, may provide a favorable steric situation to trigger HSPG/co-receptor clustering, down-stream signaling activation and membrane invagination. In this way, the balance between SDC and GPC isoforms and HS structure together may determine the intracellular fate of endocytosed cargo molecules.

3. Functional regulation of HSPG-mediated ligand internalization

Studies of morphogen gradients in *Drosophila* and mice highlight the importance of GPC endocytosis in the regulation of Hedgehog (Hh) signaling. Interestingly, the outcome of GPC-mediated endocytosis was opposite in the two species (Beckett et al., 2008). The GPC analog Dally like protein (Dlp) was required for efficient binding of Hh to its receptor Patched (Ptc) in *Drosophila* (Gallet et al., 2008). Hh–Ptc binding was followed by cellular internalization aided by Dlp that, in turn, alleviated the inhibition of Smoothened and activated downstream signaling. In mice, GPC-3-mediated endocytosis of Sonic Hh (Shh) negatively regulated signaling activation by continuous removal of Shh from the cell surface (Capurro et al., 2008). As another example, intracellular trafficking and endosomal processing of GPC-1 were suggested to have a role in the turn-over of scrapie prion protein (PrP^{Sc}). PrP^{Sc} may trigger GPC-1 co-internalization followed by autoprocessing and subsequent HS degradation and prion clearance, whereas GPC-1 silencing may elevate the levels of intracellular prion aggregates (Lofgren et al., 2008). Moreover, constitutive endocytosis and lysosomal accumulation of SDC-1 were demonstrated in poorly differentiated cancer cell lines, and expression of intracellular SDC-1 correlated with an aggressive tumor phenotype (Burbach et al., 2003). Rapid SDC-1 turnover may serve as a means for constitutive delivery of extracellular cargo, adhesion and/or signaling molecules. Surface availability of cell adhesion and signaling molecules can also be regulated by syntenin-mediated SDC recycling, which has been suggested to control intracellular integrin turnover and cell spreading (Zimmermann et al., 2005).

The mode of internalization and the biological response to endocytosed growth factors may be influenced by HSPG interactions. This notion was supported by a report on the nuclear co-localization of HSPG and FGF-2 where HSPG was proposed to function as a shuttle for the nuclear transport of the growth factor (Hsia et al., 2003). Interestingly, even though nuclear targeting of FGF-2 required initial binding to FGFR-1, the high affinity receptor and HSPG did not seem to share the same transport route, as FGFR-1 instead accumulated in perinuclear vesicles. More recently, the distinct contributions of FGFR-1 and SDC-4 in the endothelial response to FGF-2 were investigated (Elfenbein et al., 2012). The data suggested that SDC-4-mediated macropinocytosis of an FGFR-1–SDC-4–FGF-2 signaling complex determined the kinetics and magnitude of down-stream MAPK activation. Interestingly, disruption of SDC-4 resulted in increased endocytosis and MAPK activation, suggesting that the signaling output to FGF-2 stimulation is regulated by differential endocytosis dependent on the regulatory role of SDC-4. Work by others (Uhrig et al., 2012) demonstrated that non-HSPG binding adeno-associated virus vectors enter recipient cells through clathrin-dependent endocytosis more

efficiently resulting in higher transgene expression as compared to clathrin-independent uptake of HSPG-binding vectors. It is thus clear that all levels of HSPG regulation, including HS structure, mode of internalization and compartmentalization, contribute to the biological effects of internalized ligands (Fig. 2).

4. Therapeutic implications of HSPG-mediated internalization

Several therapeutic approaches to capitalize on the functional role of HSPG as a cell-surface endocytosis receptor have been explored. For example, conjugating β-glucuronidase with the cationic HIV-TAT peptide, or guanidinylated neomycin utilized the high capacity of HSPG-mediated uptake to decrease storage defects in experimental models of lysosomal storage disease (Grubb et al., 2010). HSPG-mediated transport of both viral and non-viral vectors of nucleic acid delivery has long been the subject of intense research for gene therapy purposes. A more detailed picture of the mechanism of HSPG-mediated, nucleic acid delivery has emerged; lipoplexes and nucleic acid polyplexes were captured by SDC clustered on actin-rich plasma membrane extensions prior to cell entry, followed by actin cytoskeleton retrograde flow, moving the polyplexes towards the cell body (Ur-Rehman et al., 2012). Notably, contrasting roles of individual HSPGs in gene delivery were suggested using polyethyleneimines (PEI)–DNA complexes; whereas SDC-1 efficiently mediated PEI–DNA endocytosis and promoted gene expression, and SDC-2 had an inhibitory effect (Paris et al., 2008). A recent study suggested that baculoviruses in analogy with lipoproteins specifically rely on SDC-1, but not on other SDCs or GPCs for binding and entry in vertebrate cells with implications for the further development of these vectors in gene delivery (Makkonen et al., 2013). Polyamines are essential for normal cell growth and development and elevated levels have been associated with various malignant conditions. They are synthesized *de novo* or enter cells through HSPG-mediated uptake (Belting et al., 1999). Accordingly, pharmacological inhibition of polyamine synthesis in combination with perturbed HSPG synthesis had anti-tumor effects *in vivo* (Belting et al., 2002). Further studies with HIV-TAT peptide (Mani et al., 2007) and a phage display anti-HS antibody (Welch et al., 2008) revealed that competitive inhibition of HSPG-dependent polyamine uptake can inhibit tumor growth.

Finally, in other cases it may be desirable to achieve HSPG-detargeting modifications to reduce off-target sequestration and toxicity by gene delivery vectors. Strategies to retarget adenoviruses include genetic modifications to incorporate peptide ligands directed against alternative target cell surface antigens simultaneously with genetic or chemical ablation of native, HSPG-binding determinants (Coughlan et al., 2010).

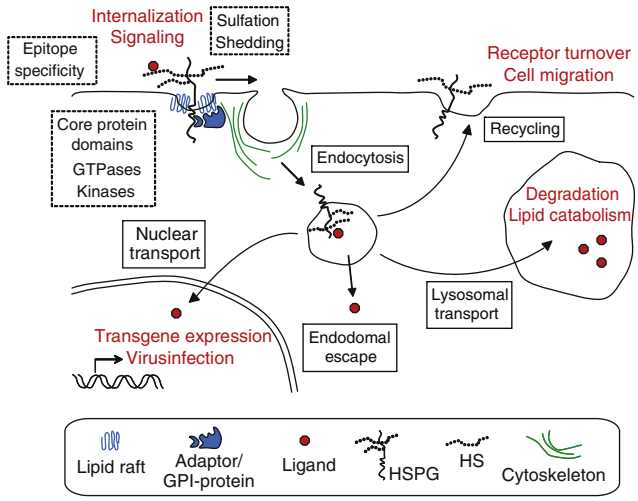

Fig. 2. Schematic presentation of HSPG ligand internalization and sorting. Cell-surface HSPGs of both the SDC and GPC type cluster and assemble in membrane lipid rafts upon ligand binding that may be HS epitope specific. Post-synthetic modification of HS structure by sulfatases and heparanase, and core protein shedding represent extracellular processes that modulate HSPG internalizing activity. Membrane invagination, and endosome formation involve the activity of several small GTPases, intracellular kinases, and remodeling of the cytoskeleton. Internalized cargo can be sorted for lysosomal degradation ("off" mechanism), escape into the cytosol *e.g.* nuclear translocation, or recycle back to the plasma membrane. Notably, both SDCs and GPCs can also follow a clathrin-dependent uptake route, as exemplified by GPC-mediated uptake of Wnt5a and Hedgehog (Capurro et al., 2012; Sakane et al., 2012).

5. Conclusions

HSPG clearly represents a highly versatile receptor for the uptake of macromolecular cargo. It may be anticipated that future discoveries will reveal new and exciting functions of HSPG-mediated endocytosis. The emerging field of extracellular vesicles, including exosomes, microvesicles and microparticles as "endogenous viruses" with the capacity to transfer genetic material between cells represents one interesting area where HSPGs may prove to have an important role (Christianson et al., 2013). Although recent studies have identified specific biological functions of HSPGs in virus infection, lipid metabolism and cancer, therapeutic strategies directed at HSPG-mediated endocytosis are still far from clinical implementation. The development of more specific and efficient HS-mimicking drugs and HSPG inhibitors should pave the way for such interventions.

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