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Fine-tuning of cell signaling by glypicans

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Abstract Signaling peptides of the extracellular environment regulate cell biological processes underlying embryonic development, tissue homeostasis, and pathophysiology. The heparan sulphate proteoglycans, glypicans, have evolved as essential modulators of key regulatory proteins such as Wnt, Bmp, Fgf, and Shh. By acting on signal spreading and receptor activation, glypicans can control signal read-out and fate in targeted cells. Genetic and embryological studies have highlighted that glypicans act in a temporal and spatially regulated manner to modulate distinct cellular events. However, alterations of glypican function underlie human congenital malformations and cancer. Recent reports are starting to reveal their mechanism of action and how they can ensure tight modulation of cell signaling.

Keywords Glypican · HSPG · Cell signaling · Signaling cell-based therapy modulator · Morphogen · Human disorder · Cancer · Stem cells

Introduction

Glypicans are cell-bound heparan sulphate proteoglycans (HSPGs) that are evolutionarily conserved in organisms as distinct as nematodes, fruit flies, and mammals [1, 2]. Due to their high negative charge, the heparan sulphate (HS) chains of glypicans interact with a multitude of extracellular matrix proteins, including chemokines, growth factors/morphogens, and their receptors [1–3]. Disruption of glypican functions in *Drosophila*, Zebrafish, *Xenopus laevis* and mouse results in phenotypes reminiscent of defects in cellular responses to regulatory signaling molecules [1, 4]. Yet, genetic and embryological studies link glypicans to the regulation of cell signaling events during morphogenesis and adult physiology [1, 4, 5]. Here we discuss recent findings concerning the function of glypicans in regulating the activity and distribution of these extracellular signals and their implication in human pathologies.

Glypican assembly

Glypicans are attached to the exocytoplasmic surface of the cell membrane by a glycosylphosphatidylinositol (GPI) linkage [2]. Vertebrates typically contain six glypican genes (*gpc1* to *gpc6* [6]) whereas one glypican has been identified in zebrafish (knypek [7]), two in *Drosophila* (*dally* and *dally-like* [8, 9]) and two in *Caenorhabditis elegans* (*gpn*-1 and *lon*-2 [10, 11]). All glypican core proteins are ~60–70 kDa in size and share a pattern of 14 conserved cysteine residues, which might confer a conserved globular tertiary structure on all glypicans (Fig. 1 [2]). In their C-terminal regions, glypicans also share attachment sites for the heparan sulphate glycosaminoglycan (HSGAG) polysaccharide side chain in addition to the signal sequence for the GPI anchor (Fig. 1 [2]).

The HSGAG of proteoglycans can undergo complex patterns of modification consisting of sulphations of hydroxyl groups in individual sugar molecules, epimerizations of specific carbon atoms and changes in length of the individual sugar residues [2, 3]. Such modifications are

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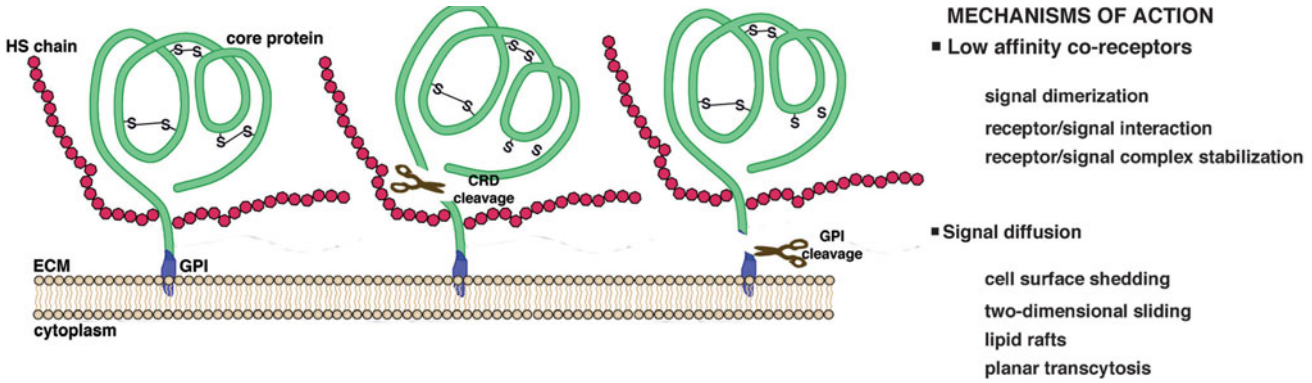

Fig. 1 Schematic representation of glypican structure and potential mechanism of action. *Left* the glypican core proteins, located in the extracellular matrix, consist of a cysteine-rich domain (CRD) possibly forming a globular structure stabilized by disulphide bridges. Glypicans are bound to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage. HS chains are linked to serine residues adjacent to the plasma membrane (adapted from De Cat et al. [6]). Proteolytic cleavages of glypicans either downstream of the CRD or at the level of the GPI would give rise to additional glypican forms. *Right* glypicans can function as classical co-receptors by favoring signal/ligand dimerization, ligand binding to the cell membrane high-affinity receptor, and in stabilizing the ligand-receptor complex. Alternatively, glypicans may control signal diffusion. Potential involved mechanisms include (1) shedding of glypicans from the plasma membrane, (2) binding of extracellular signals to favor two-dimensional 'sliding' along the HS chains, (3) lipid rafts, and (4) vesicular transport

thought to generate a large structural diversity that might encode information for the selective binding of protein ligands [2, 3]. In support of this possibility, analysis of HSPGs from different mammalian tissues has revealed tissue-specific modification of the HS chains [12, 13]. Moreover, biochemical, genetic, and embryological studies of enzymes involved in the polymerization and modification process of HSPG biosynthesis have illustrated the involvement of distinct modifications in specific biological events [14–16]. It is also important to note that structural modifications of HSGAG appear to be a cell-specific signature, which varies between proteoglycans from different cellular or tissue sources, rather than between different proteoglycans (e.g., syndecans and glypicans) from the same source [17, 18]. Several reports have already described the extent to which sugar structures can dictate specificity in glypican–protein interactions [2, 3, 19].

The GPI-anchorage is yet another feature that could make glypicans susceptible to additional post-translational modifications. In particular, the GPI anchor appears to provide a system of regulated release of glypicans to the extracellular environment. The lipid anchor can be removed by proteases or lipases, leading to shedding of glypicans from the plasma membrane to generate either soluble or glypican forms associated with low-density particles. It is now emerging that while cell-autonomous functions of glypicans are presumably exerted by membrane-linked forms, released forms can be transported across tissues and elicit their functions in a broader field of cells [1, 4]. The functional relevance of glypican shedding and binding to lipoprotein particles will be discussed in detail below.

Proteolytic cleavages of the core proteins can also contribute to generate distinct glypicans forms. As shown for several vertebrate and invertebrate proteins, the N-terminal cysteine-rich domain (CRD) of glypicans can be separated from the HS-modified and GPI-anchored C-terminal domain following endoproteolytic processing (Fig. 1 [20]). To what extent this event occurs in physiological conditions is under investigation, also because the ratio between un-cleaved and cleaved glypicans varies according to the glypican family member and the tissue-specific context. It is important to note that the N-terminal glypican fragment is not membrane-associated and, once generated, it can remain attached to its C-terminal half through one or more disulphide bridges [21, 22]. Thus, proteolytic processing can provide a mechanism to make rapidly available a secreted glypican product, if needed, as such a form could be released from producing cells simply following redox changes of the extracellular environment.

Glypicans as modulators of regulatory extra-cellular signals

The functional relevance of glypican-encoding genes in modulating activity and distribution of key regulatory extracellular signals has come from the genetic analysis and embryological manipulation of glypicans in different species (Table 1). Mice lacking gpc3 are affected by overgrowth, renal cystic dysplasia, and limb defects. These phenotypes are consistent with defects in Wnt and bone morphogenetic protein (Bmp) signaling pathways [23–25]. The *C. elegans* glypican lon-2 also controls body

Table 1 Glypican function in model organisms

Core protein	Species	Major defect	Signal disrupted	Reference
Lon-2	*C. elegans*	Body length	Bmp	[11]
Knypek	Zebrafish (mutant)	Gastrulation	Wnt	[7]
gpc4	*Xenopus* (morpholino)	Gastrulation	Wnt	[25]
		Dorsal forebrain	Fgf	[27]
gpc3	Mouse (null allele)	Body size	Wnt	[25]
		Limb mesenchyme	Bmp	[23]
		Ureteric mesenchyme	Bmp, Fgf	[24]
Dally	Drosophila (mutant)	Embryogenic epidermis	Hg, Wg	[3, 28]
		Wing imaginal disc	Wg, Dpp, Hh	[3, 28]
		Eye-antennal discs	Dpp	[1, 4]
Dally-like	Drosophila (mutant)	Wing imaginal disc	Wg	[3, 19, 30]

length [11]. It has been proposed that Lon-2 negatively regulates Bmp signaling as *lon*-2 mutants recapitulate phenotypes caused by Bmp overexpression [11]. Another example is the zebrafish *knypek*, which encodes the glypican homologue to mammalian gpc4/gpc6 [7]. *knypek* controls convergent-extension movements during zebrafish gastrulation by modulating Wnt11 activity [7]. Modulation of extracellular signals by glypicans has also been reported in *Xenopus*. In particular, reducing gpc4 (xgly4) protein levels disrupts cell movements during gastrulation [26] and dorsal forebrain patterning from early neural plate stages on [27]. Xgly4 also physically interacts with Wnt11 and might function in the Wnt/PCP pathway during gastrulation [26]. In addition to Wnt11, Xgly4 also binds fibroblast growth factor 2 (Fgf2). Inhibition of Fgf signaling results in dorsal forebrain phenotypes similar to those of Xgly4-depleted embryos, indicating that establishment and patterning of the dorsal forebrain territory may require modulation of Fgf signaling by Xgly4 [27]. In *Drosophila*, the glypican dally modulates Hedgehog (Hh) signaling during embryogenesis, whereas both glypicans dally and dally-like are required and redundant in Hh movement in developing wing imaginal disc [28]. Additional studies on wing disc patterning have also demonstrated that in dally and dally-like mutants, the distribution and signaling of Wnt and Bmp family members, wingless (Wg) and decapentaplegic (Dpp) respectively, are altered [3]. Furthermore, dally and dally-like also act on Wg during segment polarity determination and on Dpp in the developing eye and antennal discs [1, 4]. Overall, these and other studies reveal that different cell types can take advantage of glypican-mediated regulation to control signal supply during distinct developmental processes. It is also likely that glypicans control the activity of different ligands in a stage- and/or tissue-specific manner.

Glypicans for signal reception and spreading

One major question in the field is how glypicans control extracellular signaling. Once secreted, regulatory signal peptides bind to cell membrane receptors and activate specific intracellular cascades, thus determining cell fate [29]. It is important to note that extracellular signals can act both at short and long distances by either acting on cells near the producing source or on those more distant to it. Subsequently, short- and long-range signaling trigger distinct biological outcomes according to the spatial position of targeted cells [29]. Changes in time and local concentration of signal availability are another mechanism repetitively used for regulating cell fate. In this context, concentrating ligand at their targets and/or prolonging ligand availability establishes a direct link between strength-length of receptor activation and biological read out [29]. It is generally accepted that glypican binding modulates the kinetics of receptor activation by potentiating action of signal peptides [3]. For example, glypicans can capture secreted factors, after they have stopped being produced, in order to increase their concentration and/or availability near to the cognate receptor (Fig. 1). The differential binding affinity can then favor receptor interaction. Alternatively, glypicans might either stabilize ligand–receptor interaction or allow ligands to form multivalent complexes that efficiently activate the receptor (Fig. 1 [3]).

On top of this, genetic and embryological studies have shown that glypicans play a more general role in spreading and polarization of extracellular signals. For example, in the wing imaginal discs, *Wg* is secreted at the prospective wing margin and spreads symmetrically along the dorsoventral axis to form a concentration gradient in receiving tissue, where it activates short- and long-range target genes. In this biological system, the glypican dally-like

modulates Wg in both a negative and a positive way. In particular, dally-like reduces Wg near the wing margin while extending its range towards more distant cells [30, 31]. This striking behavior of dally-like is consistent with a mechanism by which certain glypicans function by sequestering a significant fraction of extracellular signal from its cognate receptors when levels are high while favoring signal accumulations when levels are lower [19, 30]. In this context, glypicans may contribute to sharpen activity gradients and modulate long-range transport of signals [19, 30].

Glypican cleavage and intercellular trafficking for signal control

How can glypican binding shape the range of signal activity? One possibility is given by the discovery of the alpha/beta-hydrolase Notum that cleaves dally-like within the GPI anchor (Fig. 1 [19]). The evidence indicates that Notum-mediated cleavage of dally-like allows its shedding from the cell surface. In this model, Wg would also be released from cells but as a dally-like-bound form, which is unavailable for signaling. Interestingly, Notum production is highest where Wg activity is high [19, 32], suggesting that dally-like cleavage would be spatially regulated. In this scenario, Notum could also establish a gradient of clearance of dally-like and Wg from the cell membrane, with a peak where Wg and Notum levels are highest. Spatial regulation of dally-like activity by Notum can also explain how dally-like can contribute to potentiate Wg long-range signaling. In particular, in regions where Wg and Notum are low, some dally-like would remain on cell surfaces where it would enhance cellular responses to Wg. Recent studies have identified xHtrA1 as a new serine protease that through proteoglycan cleavage releases cell surface-bound Fgfs and stimulates Fgf long-range signaling [33]. Although the molecular nature of the proteoglycan substrate of xHtrA1 is still unknown, Gpc4 is a potential candidate. Thus, the identification of functional interactions between distinct proteases and glypicans provides information about another level of regulation of signal supply. It will be interesting to examine to what extent these proposed mechanisms apply and are conserved in mammals, and whether or not they can determine specificity in glypican functions. In conclusion, these studies show that glypicans can function both as low-affinity co-receptors or they can be converted from a membrane-tethered co-receptor to secreted signal antagonists by cell surface shedding.

Lipid-linked signal peptides like Wg and Hh can be released from the plasma membrane on lipoprotein particles that then act as vehicles for intercellular transfer [34]. In *Drosophila*, glypicans such as dally bind lipoprotein particles via their HS moieties and recruit them to the disc wing [34]. These findings suggest yet another mechanism for signal spreading where the membrane-anchored forms of glypicans play the role of attracting signal-bearing lipoprotein particles. Interestingly, dally and dally-like continue to associate with lipoprotein particles after cellular shedding, and released glypicans on such particles can positively influence signaling [22]. For example, it has been proposed that lipoprotein-bound glypicans could promote transfer of signals to their receptor by reducing affinity of such particles for cell surface HS [22]. Alternatively, particles with multiple copies of glypicans and signal peptides would have the potential to induce receptor cross-linking [22]. In the situation where receptor-mediated endocytosis would restrict the range of signal spreading, this mechanism could provide yet an additional way to shape signaling gradients.

Similar to other HSPGs, glypicans might also control ligand diffusion by trapping them and allowing 'sliding' along the HS chain located on neighboring cells (Fig. 1 [3]). Endocytosis has also been involved for transport of glypicans via planar transcytosis (Fig. 1 [3, 22]). Interestingly, Wingless is specifically internalized from the apical and basal surfaces but not the lateral one of the disc epithelium, suggesting that the apical and basal surfaces are more endocytically active than other regions [35]. Overexpression of dally-like switches Wg localization predominately to the lateral surface, where it can diffuse without being endocytosed [35]. Thus, glypicans also appear to polarize signal peptides within an epithelium, which in turn can influence the trafficking events leading to gradient formation.

Glypicans in human diseases

Glypicans are among the most abundant HSPGs in the developing nervous system (NS) and are expressed in embryonic and adult neural stem cells [36–38]. Embryological manipulations in *Xenopus embryos* have begun to provide insight into their role in brain patterning [27], suggesting that mutation in glypican genes can underlie human NS disorders. The fact that Gpc4 is present at high levels in the developing kidney and regulates hepatocyte growth factor-mediated morphogenesis in renal epithelial cells [39] also suggests the involvement of different glypicans in renal pathologies. However, the interest for glypicans in molecular medicine has been booster by the discovery that mutations in the human GPC3 and GPC4 genes are associated with the Simpson-Golabi-Behmel syndrome (SGBS) [5, 40, 41]. Clinical manifestations of SGBS include both prenatal and postnatal overgrowth and morphological abnormalities [42]. Lack of gpc3 in mice recapitulates several phenotypes of the SGBS patients such

as developmental outgrowth and dysplastic kidneys, thus providing strong evidence that modifications of Gpc3 function are implicated in the development of SGBS [24, 43]. At cellular level, the tissue overgrowth syndrome of gpc3-deficient mice appears to be a consequence of increased cell proliferation, which is consistent with the possibility that Gpc3 acts as a negative regulator of cell division [44]. However, Gpc3 also induces apoptosis in a cell type-specific manner [42], suggesting that enhanced cell survival may also contribute to the overgrowth defects [42]. It is important to note that glypicans, like other HSPGs, can act as carriers for cellular uptake of growth-promoting polyamines such as spermine [45, 46]. It has been proposed that glypicans bind poly-amines to their HS side chains by electrostatic interaction. After transport to endosomes, HS moieties are degraded by nitric oxide. This is expected to weaken HS interaction with polyamines and results in their unloading and possibly exit from endosomes to elicit functions. The mechanisms underlying poly-amine uptake have been analyzed in several systems and discussed in previous reports [46, 47]. Further understanding of the in vivo role of glypican-mediated polyamine uptake will come from studies in animal models or patients affected by glypican deficiency. Interestingly, loss-of-function mutations in the GPC3 gene are associated with a high incidence of neuro-blastoma and testicular gonadoblastoma in SGBS patients [48, 49]. Loss of GPC3 has also been found associated with cancers, such as mesothelioma, ovary, and breast cancers [42]. As discussed above GPC3 is an inhibitor of cell proliferation and can induce apoptosis, thus the effects of loss of GPC3 on tumor development are compatible with its function as a tumor suppressor. However, its role in cancers appears to vary depending on the cellular context and signal implicated. Indeed, GPC3 overexpression acts as an oncogene in hepatocellular carcinomas where it is considered as a new diagnostic molecular marker [49, 50]. Moreover, GPC3 can also be a potential marker for malignant transformation, as its expression appears to be restricted to malign, and not benign, hepatocellular conditions [49, 50]. Interestingly, *GPC3* is not expressed in the adult liver whereas it is found in its embryonic counterpart [49]. Whether GPC3 functions as an oncofetal protein in hepatocellular carcinomas remains to be established, although overexpression studies in cultured cells begin to support this possibility [51]. Other glypicans, like *GPC1* and *GPC5*, are also overexpressed in human cancers like gliomas and rhabdomyosarcomas [52, 53] where they might function as oncogenes. Overall, these studies show that the involvement of glypicans in cancer can range from tumor suppressors to onco-genes. Possibly, these opposing actions are consequences of the ectopic signals produced by either their loss-or gain-of-function.

Outlook

The involvement of glypican genes in human congenital malformations and cancers has raised the question whether they can be targets of molecular therapies. Given the ability of HS chains to bind to a multitude of regulatory proteins, potential treatments of diseases involving HSPGs is aimed at targeting such interactions. It has been shown that complexes could be disrupted by addition of competitive saccharide ligands mimicking the HS moiety or by peptide competitors [54]. Although both strategies can offer prospectives for drug development, their clinical application still requires a better understanding of the specificity involved in HS–protein interactions. Immunotherapies based on glypican peptides are increasingly considered as a complementary approach for treatment of cancers linked to glypican over-expression. In particular, preclinical studies have shown that specific Gpc3 peptides can induce peptide-reactive cytotoxic T lymphocytes in transgenic mice without inducing autoimmunity [55]. Moreover, these cytotoxic T lymphocytes can reduce tumor mass when implanted in mice carrying Gpc3-positive tumors [55].

HSPGs play an important role in uptake of cell-penetrating peptides, and cellular HSPGs enhance their translocation into cells [56, 57]. To date, cell-penetrating peptides such as Antp (aa 43–58) are coupled to liposomes to increase the efficiency of liposome cellular uptake and of the entrapped molecules they may carry [56]. It is important to note that liposomes are considered potential carriers for release of cytotoxic agents in cancer cells because their application enhances efficacy of delivery and favors selectivity, which in turn reduces toxic effects [58]. Thus, HSPGs and possibly glypican-mediated uptake of cell-penetrating peptide-modified liposomes could represent an interesting novel mechanism for enhancing cell-specific delivery of a large variety of liposome-entrapped therapeutic drugs.

Embryonic stem cell (ESC)-based therapies can also provide attractive alternatives for cancer immunotherapies. For example, ESC-derived dendritic cells function as specialized antigen-presenting cells like those derived from bone marrow [59]. These cells could elicit potent protective and anti-tumor effects once they are genetically modified to express different human *GPC* genes, as previously shown for those expressing human *GPC3* [59]. However, when designing therapies for pathologies involving glypicans, it is also important to take into account that these diseases are often linked to glypican loss-of-functions rather then gain-of-functions. Nevertheless, alterations in cellular responses to regulatory signals underlie glypican-triggered dysfunction. We think that a better understanding of glypican involvement in normal and pathological processes, as well as the identification of

the associated signal, should provide a wider clinical spectrum for the development of targeted therapies.

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