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Basement Membrane Proteoglycans: Modulators *Par Excellence* of Cancer Growth and Angiogenesis

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Abstract

Proteoglycans located in basement membranes, the nanostructures underling epithelial and endothelial layers, are unique in several respects. They are usually large, elongated molecules with a collage of domains that share structural and functional homology with numerous extracellular matrix proteins, growth factors and surface receptors. They mainly carry heparan sulfate side chains and these contribute not only to storing and preserving the biological activity of various heparan sulfate-binding cytokines and growth factors, but also in presenting them in a more "active configuration" to their cognate receptors. Abnormal expression or deregulated function of these proteoglycans affect cancer and angiogenesis, and are critical for the evolution of the tumor microenvironment. This review will focus on the functional roles of the major heparan sulfate proteoglycans from basement membrane zones: perlecan, agrin and collagen XVIII, and on their roles in modulating cancer growth and angiogenesis.

INTRODUCTION

Basement membrane proteoglycans comprise a select group of high molecular-weight proteins which are almost universally decorated with heparan sulfate side chains (Yurchenco et al., 2004). Three main basement membrane heparan sulfate proteoglycans (HSPGs) have been well characterized: perlecan, collagen type XVIII and agrin. The first identified HSPG of basement membranes was perlecan, a modular proteoglycan with homology to growth factors and proteins involved in cell growth, lipid metabolism, adhesion, and homo- and heterotypic interactions (Hassell et al., 2003; Iozzo, 1998; Fuki et al., 2000). Collagen XVIII is a hybrid collagen/proteoglycan and a member of the multiplexin gene family together with the closely related collagen XV, which bears chondroitin sulfate side chains instead of HS chains (Oh et al., 1994a). Finally, the HSPG agrin represents an abundant constituent of most basement membranes and possesses a specialized function at the neuromuscular junction (Bezakova and Rüegg, 2003). The genes encoding these three HSPG protein cores are highly conserved and carry disparate biological functions, ranging from maintenance of basement membrane homeostasis to modulation of growth factor activity and angiogenesis. Moreover, some of these gene products are expressed in avascular tissues, such as cartilage, and in musculoskeletal and nervous tissues, where they modulate neuronal transmission and

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ocular development. Defects in some of these genes cause various human inherited disorders and often the disease phenotypes correlate well with those of mutant mice, flies and worms.

An emerging body of work supports the concept that these HSPGs have a dual function as pro- and anti-angiogenic factors (Iozzo and San Antonio, 2001). Via the HS chains, these proteoglycans can stimulate angiogenic signaling by sequestering, protecting and concentrating HS-binding growth factors such as FGF2, VEGF and PDGF, through which the HSGP-growth factor complex may be presented in a “biologically active” form to the cognate receptors. Alternatively, via proteolytic processing of their C-termini, these gene products can release powerful angiostatic fragments such as endostatin and endorepellin which can act in a paracrine function on sprouting endothelial cells, either locally or distantly.

This review will focus primarily on these three basement membrane HSPGs and will critically assess recent information related to their roles in regulating cancer growth and angiogenesis.

Perlecan, a multimodular and multifunctional proteoglycan

The designation for “perlecan” originates from studies using rotary shadowing electron microscopy which have shown a tortuous linear polymer with interspersed globular domains resembling a “string of pearls” (Hassell et al., 2003). Perlecan is a modular HSPG and is one of the largest single-chain polypeptides found in vertebrate and invertebrate animals (Iozzo, 1994; Iozzo et al., 1994; Iozzo, 1998; Whitelock et al., 2008). The five modules of perlecan are collated from protein units evolutionarily related to molecules involved in cell growth, lipid uptake and metabolism, intercellular interactions and adhesion (Fig.1). The perlecan protein core is ~470 kDa and, together with several O-linked oligosaccharides and as many as four HS chains, three in domain I and one potential in domain V (Hassell et al., 2003), it can reach a molecular weight of over 800 kDa. An intriguing question about perlecan’s evolutionary biology which has been recently raised (Farach-Carson and Carson, 2007) is: What is the advantage of linking 46 protein modules into a gigantic polypeptide over the synthesis of individual protomers? Perhaps, the answer lies in the generation of a protein scaffold, such as a long heterofunctional protein. The large size of perlecan’s protein core which spans ~200 nm in length, together with the three HS chains which could span an additional 60 nm in three dimensions, make perlecan an appropriate linker between extracellular matrix and cell surface receptors or complexes of receptors. The various modules of perlecan and the HS chains have a large repertoire of molecular interactions that include association with numerous HS-binding growth factors such as FGF2, FGF7,VEGF and PDGF, and other proteins which are constituents of basement membranes (Aviezer et al., 1994; Mongiat et al., 2000; Mongiat et al., 2001; Whitelock and Iozzo, 2005; Iozzo, 2005). Perlecan inhibits thrombosis (Nugent et al., 2000), and exhibits adhesive (Whitelock et al., 1999) or anti-adhesive (Klein et al., 1995) properties presumably by differentially affecting surface receptors such as $\alpha 2\beta 1$ integrin (Bix and Iozzo, 2005).

Our characterization of zebrafish perlecan provided the first genetic evidence linking perlecan function to developmental angiogenesis (Zoeller et al., 2008). By morpholino-

mediated perlecan knockdown we found that angiogenic blood vessel development of the intersegmental and sub-intestinal vessels was largely inhibited in the absence of perlecan. Vasculogenesis, the formation of the dorsal aorta and posterior cardinal vein, was observed to proceed normally, suggesting perlecan was only required for angiogenesis. A closer analysis revealed that the morphant vessels were non-functional, as evidenced by the lack of circulatory flow. Combined, these results suggested that perlecan functions at multiple levels during the angiogenic cascade, possibly influencing endothelial cell migration or proliferation and the events of lumen formation. The exact nature of perlecan function within these contexts is the current focus of our investigation.

Preceding the examination of zebrafish perlecan, analysis of murine perlecan expression in wild-type (Handler et al., 1997) and perlecan-deficient animals (Arikawa-Hirasawa et al., 1999; Costell et al., 1999) also supported a role for perlecan in vascular development. The perlecan-null mouse exhibits embryonic lethality associated with hemorrhage in the pericardial cavity or respiratory failure, and vessel modeling defects in the development of the great arteries and coronary artery pattern (Costell et al., 2002; González-Iriarte et al., 2003). The perlecan-null mouse does not exhibit any striking defects in developmental angiogenesis, a surprising observation given the widespread distribution of perlecan throughout the vascular basement membrane. Such observations brought to light the possibility of compensatory mechanisms which, perhaps mediated by collagen XVIII, could counter the lack of perlecan. The generation of a double knockout mouse (collagen XVIII and perlecan HS deficient) (Rossi et al., 2003), which exhibits compounded ocular defects compared to either alone, supports such principles. Furthermore, analysis of the perlecan HS-deficient mouse alone reveals altered growth factor modulation in numerous settings linked to angiogenesis (Tran et al., 2004; Zhou et al., 2004).

Beyond development, the role of perlecan has been defined within the context of tumor growth angiogenesis (Cohen et al., 1994; Iozzo et al., 1997; Mathiak et al., 1997). Structurally, perlecan co-localizes and supports new tumor blood vessel development (Iozzo et al., 1994). In addition to a structural role, perlecan serves a signaling function via the HS chains which are capable of binding and modulating numerous growth factors involved with angiogenesis. Perhaps the best studied are members of the FGF family, including the pro-angiogenic FGF2 (Nugent and Iozzo, 2000). Perlecan binds FGF2 via HS, promotes receptor activation and ultimately downstream signaling which supports mitogenesis and angiogenesis (Iozzo and Murdoch, 1996). Targeted deletion of perlecan-specific HS reduces matrix binding of FGF2 and results in enhanced smooth muscle cell proliferation (Tran et al., 2004), while the lack of HS inhibits wound healing and FGF2-induced angiogenesis and tumor growth (Zhou et al., 2004). Similarly, targeted knockdown of perlecan reduced growth factor response in prostate cancer cells (Savoré et al., 2005) as evidenced by decreased tumor growth and angiogenesis. Accordingly, these findings were similar to those observed in additional tumor cell lines (Aviezer et al., 1997; Sharma et al., 1998; Adatia et al., 1998) and supported by comparable observations in colon cancer cells harboring a somatic mutation in perlecan (Ghiselli et al., 2001). Taken together, these results implicate perlecan as a crucial component of growth factor regulation. The HSPG perlecan could be envisioned to coordinate a matrix gradient, protect growth factors from the environment, concentrate and/or present ligand to receptor, all within a context-specific fashion.

A recent report has further established the role of perlecan HS and tumor angiogenesis, with specific reference to VEGF-VEGFR2 modulation. Using hepatoblastoma xenografts treated with anti-VEGFR therapy, vessel recovery over time was associated with an increase in perlecan and heparanase expression around tumor vessels (Kadenhe-Chiweshe et al., 2008). Accordingly, the potential heparanase-mediated release of HS-bound VEGF (Reiland et al., 2004) or proteolytic protein core processing liberating growth factor-bound fragments (Whitelock et al., 1996), was found to support VEGFR2 activation and downstream survival signaling (Kadenhe-Chiweshe et al., 2008).

Collagen XVIII, an hybrid proteoglycan

Collagen type XVIII possesses features of collagens and proteoglycans and represents a member of the “multiplexin” family (Marneros and Olsen, 2005). The multiplexins, which include collagens XV and XVIII, are characterized by multiple triple-helix domains with interruptions (Oh et al., 1994a). Structurally, collagen type XVIII (Fig. 1) consists of ten interrupted collagenous domains, flanked by noncollagenous domains at the N- and C-termini. Collagen XVIII also harbors three Ser-Gly consensus binding sites for the attachment of HS glycosaminoglycan chains (Dong et al., 2003) and is an HSPG (Halfter et al., 1998). Collagen XVIII expression can be detected throughout the vascular and epithelial basement membranes of human and mouse tissues, with an overall distribution similar to that of perlecan (Marneros and Olsen, 2005).

Collagen XVIII is a homotrimer comprised of three identical α1 chains.The human *COL18A1* gene, localized to chromosome 21 and spanning 105 kb, comprises 43 exons (Oh et al., 1994b) which generate three protein variants derived from alternative promoter usage and splicing events (Elamaa et al., 2003; Saarela et al., 1998; Suzuki et al., 2002). The human and mouse α1(XVIII) collagen amino acid sequences exhibit 79% identity (Rehn et al., 1994) and an overall general conservation. Accordingly, the function of collagen XVIII has been explored through the analysis of a murine collagen XVIII knockout. Characterization of the *Col18a1*$^{-/-}$ mouse revealed that collagen XVIII is not required for viability or fertility but is essential for proper eye development (Fukai et al., 2002). Basement membrane thickening in a sub-line of *Col18a1*$^{-/-}$ mice also suggests a role for collagen XVIII during maintenance of the basement membrane, and revealed that ~20% of *Col18a1*$^{-/-}$ mice exhibit hydrocephalus as a consequence of alterations in the epithelial basement membrane of the choroid plexuses (Utriainen et al., 2004).

Ocular defects associated with collagen XVIII-deficient mice include disruption of the iris manifested by rupture of the posterior iris pigment epithelial cell layer (Robinson et al., 2006; Ylikärppä et al., 2003), iris basement membrane thickening (Robinson et al., 2006), improper macrophage-like cell migration (Robinson et al., 2006), abnormal flattening of the ciliary body (Robinson et al., 2006; Ylikärppä et al., 2003), and excessive deposits below the retinal pigment epithelium which compromise function and result in visual impairment (Marneros et al., 2004). Interestingly, these observations are in line with the phenotype associated with Knobloch syndrome caused by a mutation in the human *COL18A1* gene (Menzel et al., 2004). Despite the widespread expression of collagen XVIII throughout virtually all vascular basement membranes, evidence of no significant vascular defects in the

Col18a1$^{-/-}$ mouse, with the exception of the eye, suggests that collagen XVIII is not essential for vascular development (Fukai et al., 2002). The phenotype associated with the eye vasculature is characterized by delayed regression of the hyaloid vessels and disrupted retinal vascular outgrowth (Fukai et al., 2002). These observations support a central role for collagen XVIII specifically during ocular vessel development and maturation (Hurskainen et al., 2005).

The lack of collagen XVIII enhances angiogenesis in aortic explants derived from the *Col18a1*$^{-/-}$ mice when compared to wild-type animals which is associated with altered endothelial cell adhesion to the matrix (Li and Olsen, 2004). Collagen XVIII has also been linked to neovascularization and maintenance of vascular permeability during atherosclerosis since targeted deletion of collagen type XVIII increases these processes during disease progression (Moulton et al., 2004). Additionally, the *Col18a1*$^{-/-}$ animals display enhanced angiogenesis during wound healing (Seppinen et al., 2008), but do not experience enhanced tumor growth (Fukai et al., 2002). Thus, collagen XVIII has been suggested to play a negative regulatory, but not an essential, role during angiogenesis in certain contexts.

Agrin, an assembling proteoglycan

The third basement membrane HSPG, agrin, was first isolated from the electric organs of the Pacific electric ray as an agent responsible for acetylcholine receptor (AChR) clustering, and thus the eponym agrin from the Greek "*ageirein*" meaning "to assemble" (Nitkin et al., 1987). Subsequently, agrin was shown to be expressed in mammalians with similar AChR-clustering activity. Most work on deciphering agrin function in the mammalian body has hence been focused on agrin's contribution to the differentiation of the postsynaptic apparatus in neuromuscular junctions. Mice deficient in all agrin forms (Lin et al., 2001), as well as mice lacking nerve-specific agrin and hypomorphic for all other forms of agrin (Gautam et al., 1996), die neonatally due to respiratory failure secondary to improper excitation of the diaphragmatic muscle. Apart from its involvement in neuromuscular synapses, agrin is also important for establishing and maintaining both central nervous system and immunological synapses (Bezakova and Rüegg, 2003; Zhang et al., 2006). However, little is known about agrin's function outside the synaptic location.

Structurally, agrin shares with perlecan a rather intriguing multimodular organization (Fig. 1), and more complexity to agrin can be added by at least four sites of alternative splicing. The agrin N-terminus can be spliced to generate either a Type II transmembrane form of agrin which is expressed in brain, or a basement membrane-associated form containing the N-terminal-agrin (NtA) domain which is widely expressed throughout the body. The latter form gains via the NtA-domain high affinity for the laminin γ1 chain's coiled-coil domain. After the initial N-terminal domain lie a stretch of nine follistatin-like (FS) repeats, also known as Kazal-type protein inhibitor domains. The last two repeats are separated by an insertion of two laminin EGF-like (LE) domains. The central part of agrin after the initial FS repeats is comprised of two Ser/Thr (S/T)-rich domains of which the last one can be alternatively spliced to generate an X+/− form (Bezakova and Rüegg, 2003). The two S/T domains are interspersed by a sperm protein enterokinase and agrin (SEA) module

(Bezakova and Rüegg, 2003). SEA modules are patterns of secondary structure found in sperm protein, enterokinase, perlecan, agrin, and mucin-like glycoprotein, suggested to be involved in O-glycosylation. The specific function of each unit in the briefly-described domain stretch is largely unknown; however, taken together the agrin N-terminal part can be considered as a structural building block enabling correct presentation of the agrin signaling C-terminus. One important aspect of the N-terminal and central agrin backbone is that it carries HS chains, and rotary shadowing electron microscopy has revealed three attachment sites for HS chains (Denzer et al., 1998). Agrin can also be modified with chondroitin sulfate chains, at least *in vitro* (Winzen et al., 2003). An agrin fragment comprised of all the above described domains inhibits neuronal outgrowth independently of HS and chondroitin sulfate modifications (Baerwald-De La Torre et al., 2004). A portion containing just the FS-like repeats requires HS modification to inhibit neurite outgrowth, whereas the portion containing the LE repeats does not need to be modified by HS to inhibit neurite outgrowth (Baerwald-De La Torre et al., 2004). With the involvement of the HS-chains agrin has been shown to bind FGF2, thrombospondin, β-amyloid peptide, N-CAM, and the protein tyrosine phosphatase δ (Burgess et al., 2002). It is important to note that the contribution of HS chains from agrin is not essential for life since mice that are deficient in full-length agrin, but muscle-specifically express a genetically-engineered miniaturized form of agrin lacking the HS attachment sites, are viable and fertile (Lin et al., 2008).

The C-terminal portion of agrin contains, in concordance with perlecan and collagen XVIII, the major receptor interaction sites. With regard to its modular organization, the C-terminus of agrin shows close homology to perlecan domain V/endorepellin. Both harbor three LG repeats separated by EGF-like repeats. A structural difference in arrangement between the agrin and perlecan C-terminus is that the agrin LG1 domain is preceded by an EGF-like repeat and only one EGF-like repeat separates the LG2 and LG3 domains. The agrin C-terminus can be alternatively spliced at two different sites producing Y+/− (in chicken, named A+/−) and Z+/− (in chicken B+/−) agrin (Burgess et al., 2002). This splicing generates agrin forms with major differences in receptor affinity. In non-neuronal tissue Y(−)Z(−)-agrin is the predominant form. The agrin receptors will be reviewed below.

The organs richest in agrin content are brain, lung, and kidney (Gesemann et al., 1998; Groffen et al., 1998). Apart from various synapses as mentioned above, agrin is strongly expressed around the blood vessels of the brain, retina (Witmer et al., 2001), lung, and kidney; recently it has become evident that leukocytes also express agrin (Zhang et al., 2006). The low but specific expression seen in other organs can be accounted for by the contribution of agrin from the vasculature (Groffen et al., 1998). Changes in the agrin content of the vasculature can be seen in pathological conditions. Agrin staining of microvessels in Alzheimer’s brains show ragged, punctuated, and irregular patterns and a wider diameter as compared to vessels in non-diseased brains (Verbeek et al., 1999). Furthermore, agrin expression is highly variable in human glioblastomas; it is completely absent in most small blood vessels, but remains in the basement membrane of most larger-vessels (Warth et al., 2004). Interestingly, agrin-negative vessels are predominantly found in the central areas of the tumor (Warth et al., 2004). Agrin can be considered to be a marker of tumor angiogenesis in the liver. In the healthy human liver, agrin can be detected in minor amounts in the basement membranes of blood vessels and bile ducts (Tátrai et al., 2006;

Groffen et al., 1998). In contrast, agrin is markedly deposited in proliferating bile ductules, in the newly-formed septal vessels in hepatic cirrhosis and the angiogenic network of malignant hepatocellular carcinomas (Tátrai et al., 2006). Activated myofibroblasts, vascular smooth muscle cells, and epithelial cells all likely contribute to the aberrant production of agrin (Tátrai et al., 2006). Also cholangiocarcinoma, a primary liver carcinoma originating from the bile ductular cells, shows agrin overexpression in the newly-formed blood vessels (Batmunkh et al., 2007). In the early stages of cholangiocarcinoma, agrin is highly expressed but then decreases or is absent from the later stages of poorly-differentiated cholangiocarcinomas (Batmunkh et al., 2007). This suggests that agrin might be crucial in supporting the initial growth of the tumor.

In sum, agrin's effect on tumor angiogenesis is likely context dependent. Agrin expression seems to be protective against disorganized angiogenesis in glioblastomas, whereas in various hepatic malignancies it seems to support tumor angiogenesis, at least in the initial stages of tumor development.

C-terminal portions of basement membrane proteoglycans with angiostatic activity

The processing of extracellular matrix proteins, especially those derived from basement membrane zone components, generates fragments which possess anti-angiogenic properties (Sund et al., 2005; Nyberg et al., 2005; Clamp and Jayson, 2005; Whitelock et al., 2008). The focus of the next three sections will seek to explore the current literature describing the C-terminal regions of type XVIII collagen, perlecan and agrin.

Endostatin, the C-terminal anti-angiogenic fragment of collagen type XVIII

Collagen type XVIII harbors the C-terminal anti-angiogenic fragment, endostatin (Zatterstrom et al., 2000). Endostatin is proteolytically derived from the NC1 domain (Fig. 1) of the collagen type XVIII protein core (Ferreras et al., 2000). The NC1 domain consists of an N-terminal trimerization region, a central hinge region sensitive to proteolytic activity and the C-terminal endostatin domain (Sasaki et al., 1998) (Fig. 2). Endostatin has been linked to cell surface receptors including integrins α5β1, αvβ3 and αvβ5 (Rehn et al., 2001; Sudhakar et al., 2003), glypican (Karumanchi et al., 2001) and VEGFR2 (Kim et al., 2002).

Endostatin anti-angiogenic properties were originally characterized in a mouse tumor model. Essentially, endostatin treatment significantly reduced tumor growth via interference with tumor angiogenesis (O'Reilly et al., 1997). Subsequent investigations have further explored endostatin within the context of tumor angiogenesis and even expanded to include choroidal neovascularization (Marneros et al., 2007) and wound healing (Seppinen et al., 2008). Taken together, the endostatin anti-angiogenic effect includes a total gene expression reprogramming (Abdollahi et al., 2004) which ultimately disrupts endothelial cell migration (Kuo et al., 2001; Sudhakar et al., 2003) and survival (Dhanabal et al., 1999). Endostatin's signaling network is vast but clearly shows a linkage to pathways involved in angiogenesis such as those evoked by VEGF signaling and thrombospondin. Specifically, endostatin down-regulates several key components of the VEGF signaling cascade and, at the same

time, stimulates the synthesis of thrombospondin, a powerful angiostatic protein (Abdollahi et al., 2004). These findings reveal an integrated role for endostatin during vascular remodeling that, together with endostatin-evoked suppression of c-myc (Shichiri and Hirata, 2001), would ultimately reinforce its angiostatic effects (Wickström et al., 2005). It is not surprising that high levels of circulating endostatin reduce tumor burden and block the formation of pulmonary metastases (Sauter et al., 2001), and that high serum levels of endostatin are found in patients with Down syndrome caused by trisomy of chromosome 21, where collagen XVIII-encoding gene is located (Zorick et al., 2001). The latter can provide an explanation for the relatively decreased incidence of various solid tumors observed in Down syndrome patients.

Endorepellin, the C-terminal anti-angiogenic fragment of perlecan

In a search for novel proteins interacting with perlecan's domain V using the yeast two-hybrid system, we identified the C-terminal portion of collagen XVIII, including endostatin (Mongiat et al., 2003). It was readily discovered that perlecan's domain V blocked endothelial cell migration and capillary morphogenesis both *in vitro* and *in vivo*, and thus we named this fragment "endorepellin" to signify its repulsive activity against endothelial cells (Mongiat et al., 2003). Endorepellin interacts specifically with the α2β1 integrin (Bix et al., 2007; Bix et al., 2004), a key receptor involved in angiogenesis (Senger et al., 2002; Sweeney et al., 2003). In endothelial cells, endorepellin triggers a signaling cascade that leads to disruption of the actin cytoskeleton and thus to cytostasis (Bix et al., 2004; Iozzo, 2005; Bix and Iozzo, 2005; Bix and Iozzo, 2008). Using a proteomic approach, several key proteins involved in angiogenesis including β-actin were significantly down-regulated by exposing endothelial cells to recombinant endorepellin (Zoeller and Iozzo, 2008). Importantly, systemic delivery of human recombinant endorepellin to tumor xenograft-bearing mice causes a marked suppression of tumor growth and metabolic rate mediated by a sustained down-regulation of the tumor angiogenic network (Bix et al., 2006). Experiments using siRNA-mediated block of endogenous α2β1 integrin or α2β1-null animals have definitively proven that this is a key receptor for endorepellin, and thus for the perlecan protein core, and have further demonstrated that endorepellin targets the tumor xenograft vasculature in an α2β1 integrin-dependent manner (Woodall et al., 2008). Interestingly, endorepellin has been detected as a released product in the upper proliferating zone of fetal growth plate (West et al., 2006), suggesting that cartilage endorepellin might counteract blood vessel invasion in cartilage.

The distal laminin-like globular domain, LG3 (Fig. 2), possesses most of the biological activity (Bix et al., 2004) and can be released from the parent molecule by BMP-1/Tolloid-like metalloproteinases (Gonzalez et al., 2005) which recognize an ND dipeptide, Asn^{4196} and Asp^{4197}, which is highly conserved across species including human, mouse, *Drosophila* and zebrafish (Zoeller et al., 2008). This highly-conserved region within the perlecan protein core together with the high conservation of BMP-1/Tolloid-like metalloproteinases suggests that liberation of LG3 might be of physiological importance. Mutations in LG3 producing molecules with lower or no affinity for calcium (Gonzalez et al., 2005) disrupt LG3 angiostatic activity. It is noteworthy that the proximal two globular domains of endorepellin, LG1 and LG2, might be occupied by a number of high-affinity ligands such as α-

dystroglycan (Fig. 2) and endostatin within basement membrane zones and on cell surfaces (Bix and Iozzo, 2005; Bix and Iozzo, 2008). In contrast, LG3 might be relatively accessible and thus likely to be released by partial proteolysis, a process that is common to most LG domains of laminin.

Over a decade ago, Oda et al. (Oda et al., 1996) reported the presence of perlecan's LG3 fragments in the urine of end-stage renal patients. The LG3 fragments had N-terminal residues identical to those found in endothelial cells by us. Following this initial observation, a number of investigators have detected LG3 in several pathological conditions (Table 1). For instance, similar LG3 fragments have been found elevated in the urine of patients with chronic allograft nephropathy (O'Riordan et al., 2008), and in the amniotic fluid of pregnant women (Gianazza et al., 2007) with a marked increase in women with symptoms of premature rupture of fetal membranes (Vuadens et al., 2003; Thadikkaran et al., 2005) and those carrying trisomy 21 (Down syndrome) fetuses (Tsangaris et al., 2006). In addition, endorepellin fragments have been detected in normal human blood (Adkins et al., 2002), in the urine of children with sleep apnea (Krishna et al., 2006), in the secretome of pancreatic and colon carcinoma cells (Grønborg et al., 2006; Gonzalez et al., 2005), and in the media conditioned by apoptotic endothelial cells (Raymond et al., 2004; Laplante et al., 2005). In endothelial cells, the released LG3 interacts with the α2β1 integrin receptor of fibroblasts and triggers a signaling cascade that leads to activation of an anti-apoptotic pathway and potentially to a fibrogenic response (Laplante et al., 2006). Recently, it has been shown that caspase-3 activation triggers extracellular cathepsin L release which in turn cleaves endorepellin (Cailhier et al., 2008). In fibrotic diseases, endothelial cell apoptosis precedes the recruitment of fibroblasts, and thus release of LG3 could affect not only angiogenesis, but also the production of collagen and the overall sclerotic response. This is another example of cell-specific context in which cryptic perlecan fragments might exert diverse effects.

We propose that endorepellin/LG3 is liberated via partial proteolysis during tissue remodeling and cancer growth thereby representing an additional layer of control for angiogenesis (Iozzo, 2005). One possibility is that tumor growth might be enhanced *in vivo* by a lack of circulating LG3. In line with this idea, circulating LG3 levels are reduced in patients with breast cancer (Chang et al., 2008), suggesting that reduced titers might be a useful biomarker for cancer progression and invasion. Recent studies using α2β1 integrin-null mice have reported an increased angiogenesis in the granulation tissue of wounded animals (Grenache et al., 2006; Zweers et al., 2007). Tumor angiogenesis is dependent on the "cancer cell context" in α2β1 integrin-null animals further corroborating the importance of cross-talk between the invading cancer cells and the tumor microenvironment (Zhang et al., 2008).

An endorepellin-like fragment in agrin

Most of the receptor binding and hence direct biological activity is confined to the C-terminal end of the agrin protein core. Intriguingly, this part can be proteolytically processed and released from the mature protein (Fig. 2). Accountable for this processing is the synaptic serine protease neurotrypsin (Reif et al., 2007). Neurotrypsin is primarily expressed in the

pre-synaptic membrane of the neurons of the amygdala, cerebral cortex, and hippocampus, which are also regions with a particular high agrin content (Donahue et al., 1999). This protease has been suggested to be important for synapse reorganization and plasticity since a lack of neurotrypsin leads to mental retardation. Mice overexpressing neurotrypsin in motoneurons mimic agrin deficiency with broad, immature synapse endplates (Reif et al., 2007). Agrin has two cleavage sites for neurotrypsin at its C-terminus: one between the N-terminal S/T domain and the SEA domain and a second found just before the third LG-domain. This generates 110-, 90- and 22-kDa agrin fragments (Matsumoto-Miyai et al., 2009; Reif et al., 2007) (Fig. 2). There is evidence that processing of agrin also occurs outside the brain; agrin in kidney tubular basement membranes does not contain the C-terminal part, whereas in the glomerular basement membranes as well as in larger blood vessels of the kidney agrin is still intact (Groffen et al., 1998). Notably, agrin LG3 as well as perlecan LG3 have been found in the amniotic fluid of women with premature rupture of fetal membranes (Vuadens et al., 2003). The onset of neurotrypsin expression and its maximal expression corresponds to the onset and the maximal release of agrin fragments. In neurotrypsin-deficient mice no cleavage of agrin occurs, whereas released C-terminal agrin fragments can readily be detected in brain and kidney but not in the lungs of wild-type mice (Reif et al., 2007). These results indicate that this enzyme is the main protease responsible for agrin C-terminal processing.

Multiple receptors (Fig. 2) recognize the C-terminal region of agrin, both intact or released, and the specificity for receptor recognition is dependent on the agrin splice variant. Agrin LG2 binds the αvβ1 and an integrin β1 binding site contained in the EGF4-LG3 domains (Burgess et al., 2002). The highly glycosylated receptor α-dystroglycan shows affinity towards a large portion of the C-terminal stretch, and the binding is negatively influenced by the presence of the Y and Z inserts (Scotton et al., 2006). Recently, two novel agrin receptors were identified: the α3 Na^+/K^+ -ATPase and LRP-4. A 20-kDa agrin fragment at the C-terminus binds to and inhibits the α3 Na^+/K^+ receptor. Agrin inhibition of α3 Na^+/K^+ activity leads to membrane depolarization and increased action potential frequency both *in vitro* and *ex vivo*. The identification of α3 Na^+/K^+ as an agrin receptor suggests the possibility that agrin might be involved in cardiac disease since cardiomyocytes express both α3 Na^+/K^+ and agrin (Hilgenberg et al., 2006). Lrp4 was identified as the long-sought agrin receptor responsible for MuSK phosphorlyation and subsequent AChR clustering in muscles. LRP4 forms a complex with MuSK and the most C-terminal LG domain and the Z-splicing insert is enough for binding Lrp4 and to induce MuSK phosphorylation and the following AChR clustering (Kim et al., 2008). Lrp4 is also present in many developing tissues that do not express MuSK but the outcome of agrin ligation to Lrp4 in such a setting is currently unknown (Kim et al., 2008).

There is still very limited knowledge on how agrin signals through the integrin receptors and how these interactions influence muscle and other organs. More studies are needed to evaluate the role agrin plays in non-muscular tissues and the precise impact and function of the proteolytically-released C-terminal endorepellin-like fragment. Given the structural similarities to endorepellin, the dual processing, the high expression of agrin in blood vessels and its affinity for multiple receptors, it can be speculated that the endorepellin-like domain of agrin might also regulate blood vessel homeostasis.

Conclusions and future directions

The three members of the basement membrane HSPG family described in this review have a long evolutionary history insofar as they are expressed in lifeforms ranging from worms to man, thus encompassing >500 million years of evolution. It is not surprising, therefore, that parts of these ancestral molecules carry intrinsic information and bioactivities that are quite powerful when released from the parent molecule. A common and expanding theme is that endostatin, endorepellin and likely agrin LG3 represent members of a family of cryptic domains residing within larger parent molecules that often act in a dominant negative manner. For example, both endostatin and endorepellin can affect endothelial cell biology via integrin receptors and eventually both lead to endothelial cell dysfunction and inhibition of angiogenesis. Both molecules are found in the blood and in several biological fluids, including urine and amniotic fluid, where they can be indicative of blood vessel damage. These molecules might eventually become biomarkers for diseases such as cancer, as byproducts of the high turnover rate of malignant cells, or perhaps for osteoarthritis where continuous remodeling of bone and cartilage occurs. Another view is that circulating levels of the C-terminal HSPGs might be continuously released to add an additional layer of control for abnormal vessel sprouting during various pathologies including cancer, rheumatoid arthritis and vascular retinopathies.

The discovery of these bioactive fragments has opened new avenues of research and interest in developing antitumor agents. Utilization of HSPG fragments either as protein- or peptide-based pharmacological agents might represent a good therapeutic rationale, especially if provided in combination with other tumor suppressive compounds. Finally, a defined mechanism of action for each of these fragments must be elucidated prior to large clinical trials along with delineation of appropriate biomarkers for efficacy and toxicity testing.

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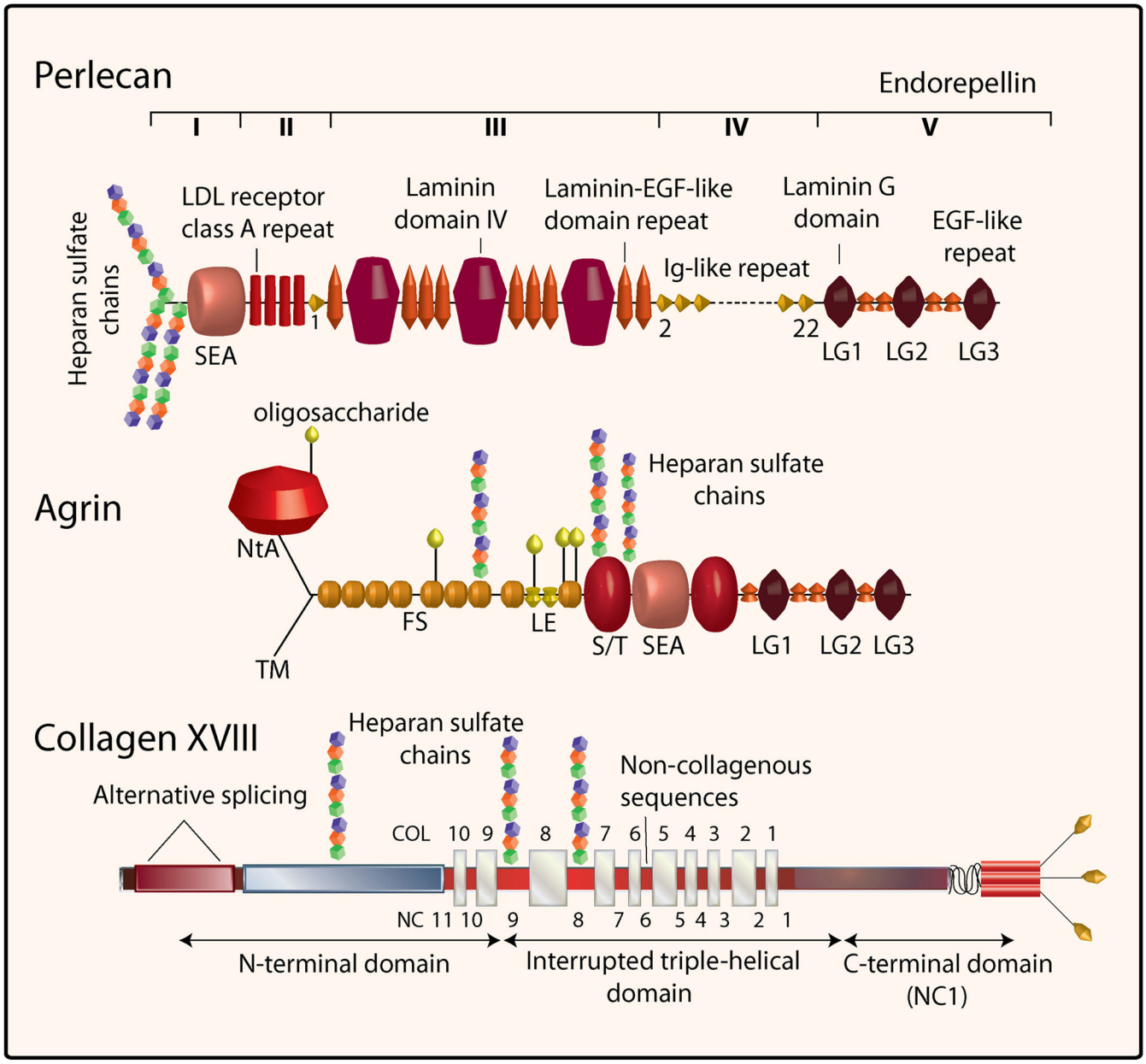

Fig. 1.

Structural domains of human perlecan, agrin and collagen XVIII. The five domains of perlecan are in Roman numerals from the N- to the C-terminus. The other domains of agrin and collagen XVII are listed or detailed in the text.

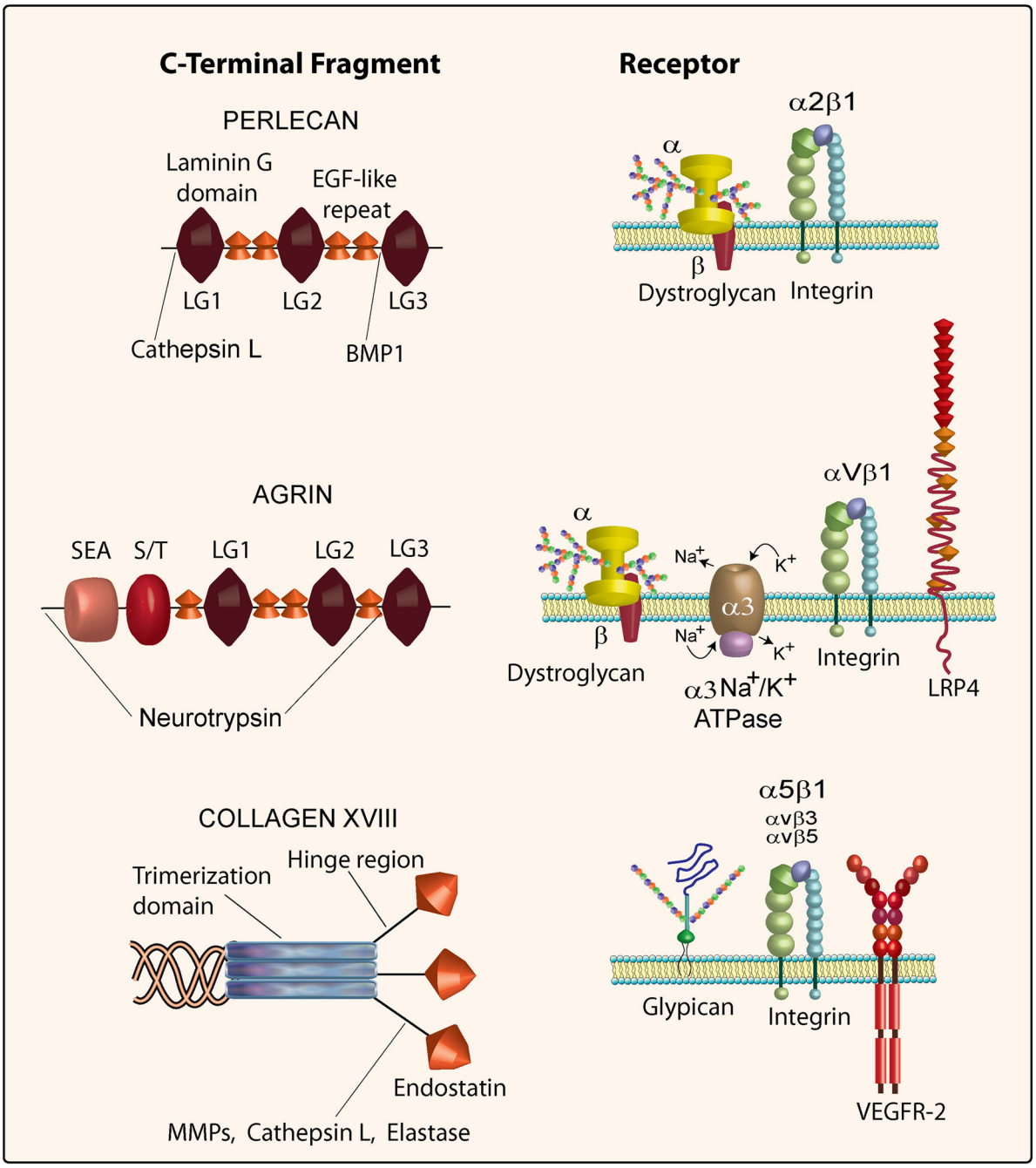

Fig. 2.

Schematic representation of the C-termini of perlecan, agrin and collagen XVIII (left) and their respective cognate receptors (right). Also annotated are the locations of various protease cleavage sites generating intact modules or fragments thereof from the three polypeptides. For additional details see the text.

Table 1.

Presence of perlecan's LG3 module in various tissues and biological fluids, and potential functional implications.

Location	Condition	Functional Implication	Reference
Urine	End-stage renal disease	Biomarker for vascular injury	(Oda et al., 1996)
Urine	Chronic allograft nephropathy	Biomarker for immune-mediated vascular injury	(O'Riordan et al., 2008)
Amniotic fluid *	Premature rupture of fetal membranes	Biomarker for fetal ischemia and vascular injury	(Vuadens et al., 2003; Thadikkaran et al., 2005)
Amniotic fluid	Mothers carrying Down Syndrome fetuses	Biomarker for abnormal fetal development and vascular injury	(Tsangaris et al., 2006)
Blood	Normal subjects and breast cancer patients	Reduced in breast cancer patient: role as anti-angiogenic factor	(Chang et al., 2008)
Secreted by endothelial cells	Endothelial cells undergoing apoptosis	Potential function as a paracrine inducer of fibrosis	(Raymond et al., 2004; Laplante et al., 2005; Laplante et al., 2006; Cailhier et al., 2008)
Secreted by various cancer cells	Pancreatic and colon carcinoma cells	Potentially linked to the highly turnover rate of transformed cells	(Grønborg et al., 2006; Gonzalez et al., 2005)
Urine	Children with sleep apnea	Potential biomarker of transient brain ischemia	(Krishna et al., 2006)

* Agrin LG3 was also found in the amniotic fluid of pregnant women with premature rupture of fetal membranes.