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Structural deciphering of the NG2/CSPG4 proteoglycan multifunctionality

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ABSTRACT: The chondroitin sulfate proteoglycan 4 (*CSPG4*) gene encodes a transmembrane proteoglycan (PG) constituting the largest and most structurally complex macromolecule of the human surfaceome. Its transcript shows an extensive evolutionary conservation and, due to the elaborated intracellular processing of the translated protein, it generates an array of glycoforms with the potential to exert variant-specific functions. CSPG4-mediated molecular events are articulated through the interaction with more than 40 putative ligands and the concurrent involvement of the ectodomain and cytoplasmic tail. Alternating inside-out and outside-in signal transductions may thereby be elicited through a tight functional connection of the PG with the cytoskeleton and its regulators. The potential of CSPG4 to influence both types of signaling mechanisms is also asserted by its lateral mobility along the plasma membrane and its intersection with microdomain-restricted internalization and endocytic trafficking. Owing to the multitude of molecular interplays that CSPG4 may engage, and thanks to a differential phosphorylation of its intracellular domain accounted by crosstalking signaling pathways, the PG stands out for its unique capability to affect numerous cellular phenomena, including those purporting pathologic conditions. We discuss here the progresses made in advancing our understanding about the structural-functional bases for the ability of CSPG4 to widely impact on cell behavior, such as to highlight how its multivalency may be exploited to interfere with disease progression.—Tamburini, E., Dallatomasina, A., Quartararo, J., Cortelazzi, B., Mangieri, D., Lazzaretti, M., Perris, R. Structural deciphering of the NG2/CSPG4 proteoglycan multifunctionality. FASEB J. 33, 3112–3128 (2019). www.fasebj.org

KEY WORDS: extracellular matrix · structure-function relationship · macromolecular interactions · signal transduction

PHYLOGENESIS AND POST-TRANSLATIONAL TRAITS OF NG2/CSPG4

In the early 1980s, approaches of reversed immune-proteomics (*i.e.*, the exploitation of mAb raised by immunization with whole cells or cell membrane preparations to identify biologically and clinically relevant surface antigens) led to the discovery of a unique cell membrane-associated proteoglycan (PG) that was concurrently named (melanoma) cell surface proteoglycan or (M)CSP, high molecular weight-melanoma associated antigen or HMW-MAA (1–14), and nerve-glia antigen 2 (NG2, because of its glial association in rat brain) (15). Biochemical analyses of the antigen in melanoma cells demonstrated that it was a highly glycosylated macromolecule with a facultative glycanation giving rise to polydisperse macromolecules with molecular masses varying from 240,000 to 700,000 Da (5, 11, 13, 14, 16, 17).

Subsequent molecular and immunochemical studies provided detailed information about its generalized distribution pattern in healthy and pathologic tissues. It became clear that the PG [hereafter referred to as NG2/chondroitin sulfate PG 4 (CSPG4)] is a rather ubiquitous surface component of immature and/or highly dynamic cell phenotypes throughout the human body. Recent investigations also unveil that the PG becomes frequently upregulated upon neoplastic transformation in a variety of cancer types (18–20). NG2/CSPG4 is also known to be

ABBREVIATIONS: cdc42, cell division cycle 42; CS, chondroitin sulfate; CSPG4, chondroitin sulfate proteoglycan 4; ECM, extracellular matrix; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAG, glycosaminoglycan; MMP, matrix metalloproteinase; MT-MMP, membrane-type metalloproteinase; NG2, nerve-glia antigen 2; OPC, oligodendrocyte progenitor cell; PDGF, platelet derived growth factor; PDGFR, platelet derived growth factor receptor; PG, proteoglycan; Rho, Ras homolog family member

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frequently modulated during progression of the tumors and to be abundantly expressed in highly aggressive cancer cell subsets, metastatic formations, and injured tissues (especially the CNS). Accordingly, NG2/CSPG4 stands out as one of the most characteristic theranostic factors in at least 2 disease conditions, cancer and neurologic disorders.

Primary sequence determination of the rodent (21) and human (22) orthologs initially highlighted the uniqueness

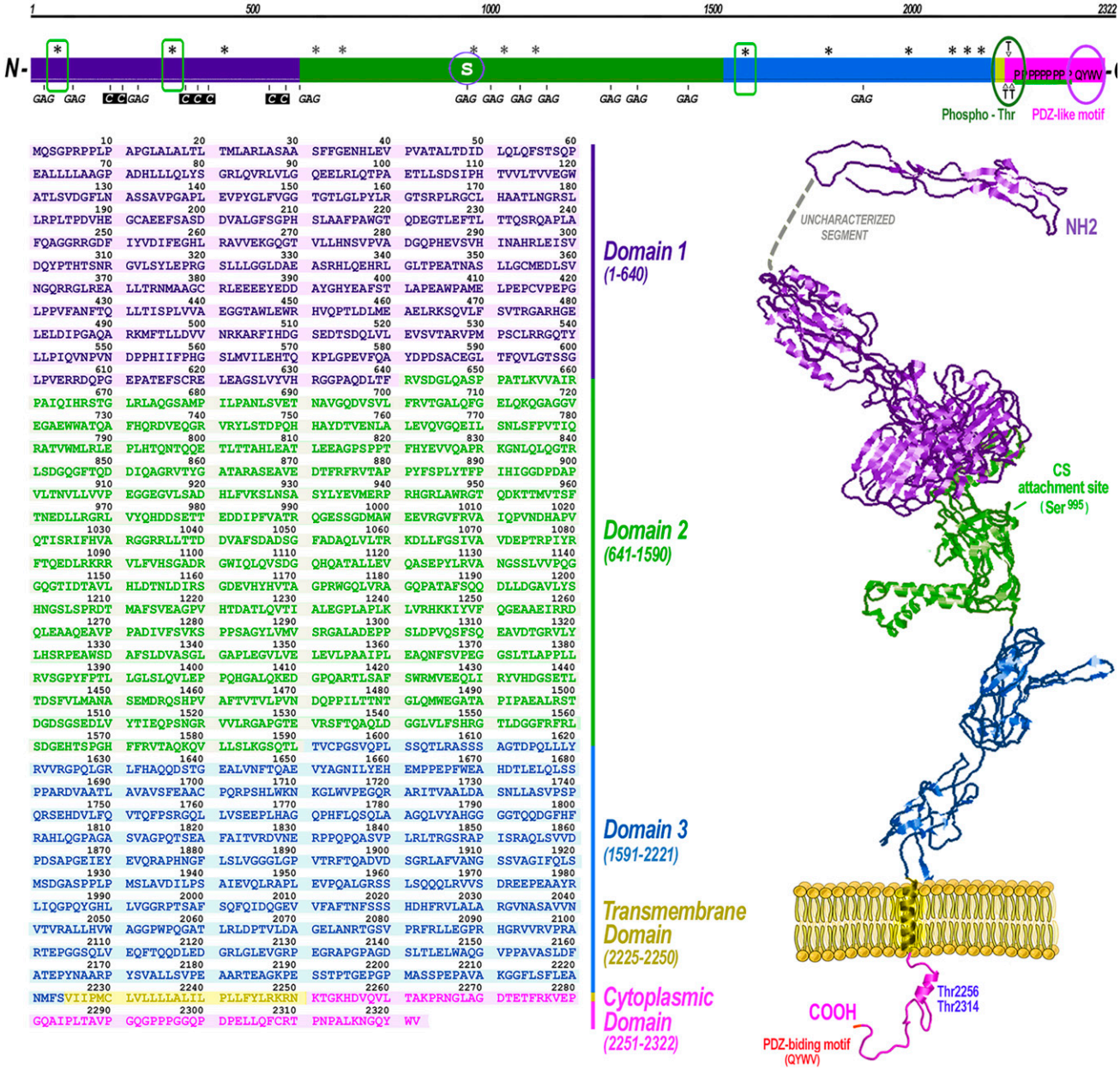

Figure 1. Primary sequence (left panel) and structural model of the subdomain organization (central panel), alongside the comprehensive portrait of predicted glycosylation sites of human NG2/CSPG4. In the upper panel, GAG indicates the position of the potential sites for GAG attachment, of which that corresponding to serine residues S995 (Ser^{995}) matches the conserved S999 shown by biochemical, proteomic, and mutagenesis analysis to be the prime glycanation site in the rodent ortholog. C, cysteine residues potentially involved in disulfide bonding. Asterisks denote the relative position of putative *N*-glycosylation sites, of which boxed ones correspond to those that we have thus far found by proteomic analyses to be ubiquitously occupied in melanoma and soft-tissue sarcoma cell lines (unpublished results). The green circle highlights the cytoplasmic threonine residues (Thr^{2256} and Thr^{2314}) prone to phosphorylation (Phospho-Thr), and the pink circle identifies the canonical PDZ-like motif (QYWV) of the cytoplasmic tail. In the central panel, the relative position of the 3 structurally-functionally assigned domains is reported along with their estimated length (number of amino acids within brackets). A total of 15 unique *CSPG* repeats (each roughly 100 aa-long) are present between residues 420 and 2135 of the ectodomain, whereas the outermost N-terminal segment of the ectodomain encompasses 2 contiguous Laminin-G–type repeats. The plasma membrane–proximal region of the PG further accomodates 2 potential MMP cleavage sites whose proteolytic utilization leads to the release of virtually the entire ectodomain of the macromolecule from the cell surface. An additional, MT1-MMP/MMP14–susceptible cleavage site has been pinpointed within the D1 subdomain (*i.e.*, within residues 490–500).

of the *CSPG4* gene product. The contiguous human cDNA spans 8071 nt and embodies an open reading frame of 2322 aa that build up a polypeptide of 250,537 Da (**Fig. 1**). The predicted protein encoded by a 10 exon-gene is an integral membrane molecule with a large N-terminal extracellular domain (2221 aa), encompassing numerous glycosylation sites and up to 3 putative glycosaminoglycan (GAG) attachment sites. Experimental evidence derived from studies on ectopic expression of the rodent NG2/CSPG4 ortholog in tumor cells pinpoint the serine residues S999 (conserved in human NG2/CSPG4 at position 995) as the predominant one implicated in chondroitin sulfate (CS) attachment (23). Whether this holds true for other cell types and species remains to be demonstrated.

The *CSPG4* nucleotide sequence further predicts a single transmembrane domain (25 aa) and a short cytoplasmic tail (76 aa) harboring 2 conserved threonine residues prone to differential phosphorylation and a similarly highly conserved, canonical PDZ binding motif (QYWV) located more C-terminally. The human *NG2/CSPG4* gene resides on chromosome 15:24q and was originally identify within the region 75,966,663–76,005,189 (24). Refined positioning [using Ensembl (*https://useast.ensembl.org/index.html*) and GeneLoc (*https://genecards.weizmann.ac.il/geneloc/index.shtml*); ID: ENSG00000173546; Location: Chromosome 15:75966663-76005189:-1.] places it at 75,674,322 [starting point; base pair (bp) from the end of the short arm (pter)] to 75,712,848 (ending point; bp from pter; **Fig. 2**). The gene embodies a CpG island flanking the transcriptional start site, which has been proposed to contribute to the transcriptional regulation of the gene by promoter methylation (25). Binding sites for a variety of transcription factors, including yin and yang 1 transcription factor, helix-loop-helix transcription factor 3, paired box 2, NF-κB, forkhead box protein I1, LIM domain only 2, and homeobox protein H6 family member 3 (*http://www.genecards.org/cgibin/carddisp.pl?gene=CSPG4&keywords=CSPG4*), have been predicted to be dispersed throughout the *CSPG4* gene. Combined amino acid sequence and transmission electron microscopy/rotary shadowing analyses of the isolated PG (26) initially portrayed a subdomain arrangement of the extracellular portion of the NG2/CSPG4 core protein entailing a globular N-terminal subdomain (D1), characterized by intramolecular disulfide bonding, a flexible segment containing alternating α-helical and β-sheet regions (D2), and a membrane proximal subdomain (D3) assuming an extended globular conformation (Fig. 1). An extension of these analyses identified a unique set of repeated (a total of 15) 100 aa-long modules along the ectodomain of NG2/CSPG4 that were denominated CSPG and found to be shared to different extents by proteins identified in fly, worms, a cyanobacterium, and sea urchin, in which they were found within the blastocoelar matrix protein (gi:9837427). In other organisms the repeated modules were found in the protein KIAA1920 (gi:15620899), hypothetical proteins FLJ-22031 (gi:13640532), CG10275 (gi:7298451), C48E7.6 (gi:7497613), and protein HieB, [gi:9858192 (27)]. The same sequence analyses also disclosed 2 phylogenetically conserved laminin G-type motifs occupying most of the N-terminal globule [(27)] and known to harbor the potential to bind heparin and α-dystroglycan.

CSPG4 appears indeed to be an evolutionary conserved gene, being identified in 181 species (*https://www.ncbi.nlm.nih.gov/gene/?term=cspg4*) spanning from *Drosophila* (ortholog known as Perdido-Kon-tiki (28, 29)] to man. The phylogenetic analysis of *CSPG4* was performed using a traditional approach, involving a multiple sequence alignment constructed using Clustal-X, v.1.81 (*http://www.clustal.org/clustal2/*), followed by tree reconstruction using the Phylip Package (*http://evolution.genetics.washington.edu/phylip.html*) (**Fig. 3**). Throughout these species the *CSPG4* gene generates a protein product showing a significant degree of sequence identity. Furthermore, at least in certain species, the gene is believed to be transcribed early on during embryogenesis. This is, for instance, the case in the developing sea urchin in which NG2/CSPG4 has been implicated in the cell–extracellular matrix (ECM) interactions involved in convergent-extension gastrulation movements (30). Nucleotide sequence determination of the NG2/CSPG4 transcript in various human cell types and tissues highlights a significant degree of polymorphism (Fig. 2). The precise distribution pattern of these polymorphisms and to what extent they are actually translated into an array of NG2/CSPG4 protein isoforms remains to be determined.

Studies on the precise post-translational modifications of the NG2/CSPG4 core protein have primarily been addressed in malignant cancer cells and have afforded some basic information on the composition of the attached oligosaccharides and GAGs. These investigations have identified 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-β-4-*O*-sulfo-D-galactose and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-β-6-*O*-sulfo-D-galactose as the prevalent oligosaccharides, whereas the prevailing GAGs appear to be CS-A and -B isomers with putative masses of 30–60 kDa (5, 16, 17).

Correct trimming and processing of the *N*-linked oligosaccharides bound to the NG2/CSPG4 core protein precursor are believed to be essential for its proper glycanation, as well as for proper intercalation of the PG into the cell membrane (3, 16, 31–34). Thus, it is likely that the recurrent intracellular trapping of NG2/CSPG4 observed in neoplastic cells *in vitro* and *in situ* may be attributed to an imperfect glycosylation of the precursor polypeptide passing through the Golgi apparatus and/or to insufficient acidification of the vesicular transport compartment (3, 31–33). On the other hand, numerous cancer cell lines preferentially (if not exclusively) display non-glycanated forms of the PG on their surfaces, which are nonetheless correctly transported to the cell membrane (23, 31–33, 35). However, these GAG-free variants seem to differ substantially in their relative content of fucosylated and sulfated polyLacNAc-type *N*-glycans, in accordance with the relative metastatic potential of the cells displaying them on their surfaces (36, 37). NG2/CSPG4 molecular species ranging from 240 to 275 kDa in size are frequently found to be constitutively released by cancer cells, whereas secretion of 240 kDa-sized molecules was originally demonstrated upon treatment of cells with glycosylation

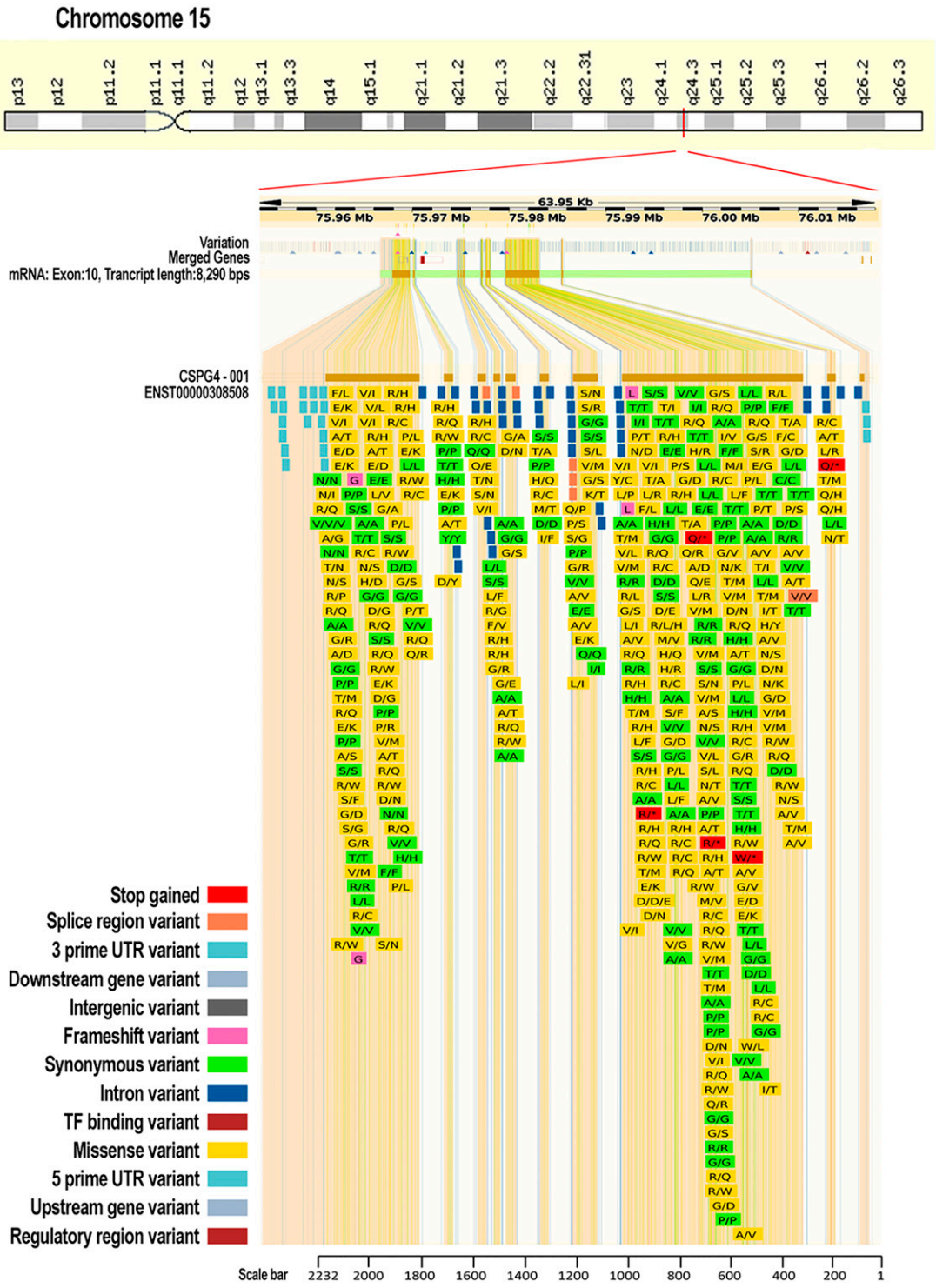

Figure 2. Chromosomal localization, nucleotide sequence variation, and predicted polymorphisms presently identified within the human *CSPG4* gene. The upper part of the figure shows a schematic representation of the human chromosome 15 the location of the NG2/CSPG4 gene. The main portion shows a magnification of the gene to better show the currently identified genetic variations (single nucleotide polymorphisms; SNPs). Color coding indicates the type variations generating the predicted array of genomic isoforms.

inhibitors (3). Thus, it is plausible that in transformed cells having defective glycosylation machineries, unglycosylated NG2/CSPG4 variants are directly released from the cells, without being previously intercalated into the plasma membrane.

In all species and cellular/tissue systems studied thus far, glycanated and nonglycanated variants of NG2/CSPG4 seem to coexist, but with a topographical bias toward distinct microdomains of the cell membrane (17, 23). Conversely, certain cell types may predominantly express nonglycanated variants (38–40), leaving covert in which cells, tissues, and biologic-pathologic contexts the macromolecule is actually expressed as a *bona fide* PG. Further investigations are also needed to define the cellular and

Figure 3. Phylogenetic tree derived from CSPG4 nucleotides sequences of 33 out of 181 organisms for which this genomic information is currently available. The tree embodies the pacific oyster (*Crassostrea gigas*) depicted as an outgroup because of its substantial evolutionary distance from *Homo sapiens*. The tree respects the speciation and is coherent with the tree of life.

molecular mechanisms that drive the expression of different glycosylation variants of the PG that are detectable in healthy and diseased tissues and in cultured cells, as well as to what extent these variants may engage in diverse molecular interactions.

SUBCELLULAR ORGANIZATION OF NG2/CSPG4 GLYCOFORMS IS DICTATED BY CELL POLARIZATION AND IMPLICATES MULTIPLE CYTOPLASMIC ADAPTOR AND RAS HOMOLOG FAMILY MEMBER GUANOSINE TRIPHOSPHATE HYDROLASE–EFFECTOR PROTEINS

Melanoma cells are recognized to be the cell type with the highest NG2/CSPG4 expression and on these cells 40,000–100,000 NG2/CSPG4 surface molecules have been estimated by immunologic means (5, 41). Similar values have been derived from immune-radioactive assessments of antigen abundance in melanoma lesions *in situ* (42). As predictable, these PG molecules are not evenly distributed along the surface of the cells. By contrast, they show distinct spatial organizations and highly dynamic rearrangements that appear to be determined by: *1*) the front-rear polarization of the cell, *2*) the nature of the interactive ECM substrate, and *3*) the interplays by which the PG may engage with the substrate components. In addition, because it is hypothesized that different NG2/CSPG4 glycoforms may arrange differently along the cell surface, they may be differentially involved in the cells' response to discrete environmental cues.

On stationary, largely unpolarized cells, putative glycoform-specific NG2/CSPG4 antibodies have immunolocalized NG2/CSPG4 variants in distinct subareas of the cell membrane. Glycanated variants concentrate in filopodial extensions, including the finest protrusions conventionally denoted "microspikes," where they seem to colocalize with β1, α3, and α4 integrin subunits, ezrin, and neural precursor cell expressed, developmentally down-regulated 9 (**Fig. 4** and **Table 1**) (17, 23, 43–47). However, NG2/CSPG4 molecules lacking GAG chains have also been noted in microclustered configurations confined to putative lipid rafts. This surface topography has also been noted with ectopically expressed NG2/CSPG4 molecules and diverse sets of anti-NG2/CSPG4 antibodies (48–50;

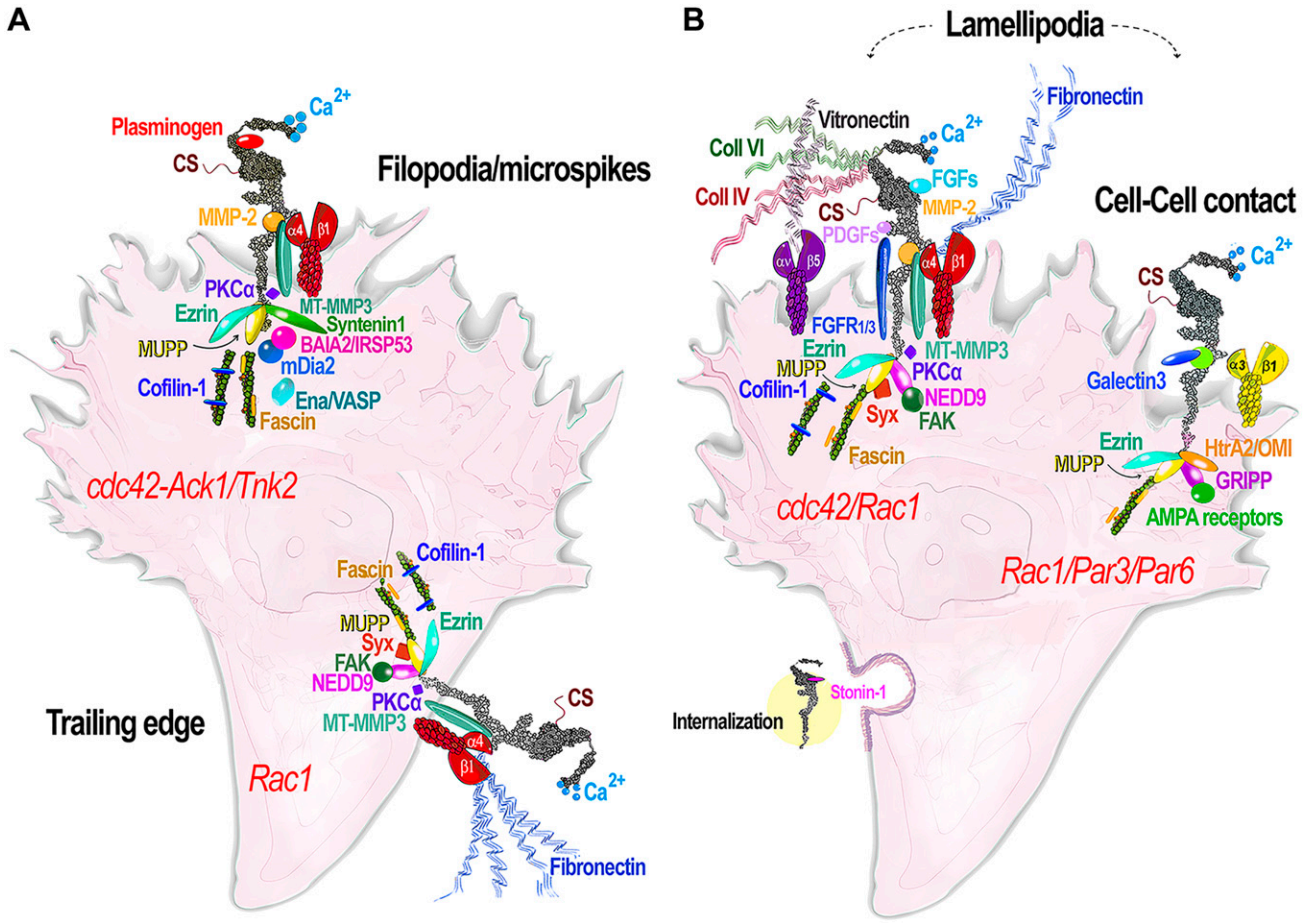

Figure 4. Schematic overview of the front-to-rear, polarized molecular interplays promoted in motile cells and upon cell-cell contacts by NG2/CSPG4, both at cell surface and intracellularly. The background drawing colored in pink is a schematized view of moving cell, as conventionally observed with phase-contrast light microscopy. It is used to differentiate NG2/CSPG4-mediated molecular interactions taking place in the anterior *vs.* posterior part of the cell. In the anterior part of moving cells, highly glycanated variants of the PG accumulate in microspikes and filopodial extensions (*A*), where they tightly link, *via* PDZ domain-containing adaptor molecules, to the actin microfilamentous network sustaining these membrane protrusions and their associated molecules. By direct or indirect binding, these NG2/CSPG4 variants influence the function of integrins, cluster at the leading edge of the cell and contribute to the control of cell adhesive phenomena by impacting on the activation of up-stream Rho/Rac-family cytoskeletal regulators. Filopodial NG2/CSPG4 also sequesters plasminogen and implicates the PG in the control of the urokinase-based matrix-degradation system. At the level of the lamellopodium (*A*, top), medium-to-low glycanated isoforms of the PG serve as a coreceptor for several ECM components, as well as coactivators of integrins. Interaction of the NG2/CSPG4 with the ECM, along with its integrin docking function, triggers intracellular signal cascades propagated through PCKα-mediated cytoplasmic phosphorylation of NG2/CSPG4 and activation of FAK to support cell survival. Accumulation of NG2/CSPG4 in lipid rafts is instrumental for its exertion of a growth factor receptor docking function and optimal transduction of growth factor-induced intracellular signals. Extracellular engagement of NG2/CSPG4 also leads to activation of molecules dictating cell polarity and activation of upstream regulators of microtubule dynamics, such as Rac-1. NG2/CSPG4 concentrating in focal adhesions is known to be internalized through stonin-1, and this internalization process is proposed to be critically involved in the regulation of the dynamics of cell-ECM junctions. NG2/CSPG4 associated with both the healthy and tumor vasculature of the CNS affects cell-cell interactions and the polarization of the interacting cells (*B*, top). In the rear end of the cell (*A*), NG2/CSPG4 similarly contributes to cytoskeletal rearrangements, as well as to the dynamics of the cell-substratum contacts and integrin receptors. Posterior extracellular engagement of NG2/CSPG4, along with its indirect association with the actin cytoskeleton, similarly leads to activation of Rac-1. Nonglycanated isoforms of the PG are the ones prevailing in this compartment of locomory cells (*i.e.*, trailing edge).

TABLE 1. *Summary of the molecular interplays exerted by NG2/CSPG4 in primary cellular events*

Cytoplasmic domain								
Cell adhesion/movement/polarity		Cell proliferation and growth		Cell-cell adhesion/polarity		Eptodomain		
Direct binding	Indirect linkage	Direct binding	Indirect linkage	Direct binding	Indirect linkage	Direct Binding	Indirect linkage	Effector
PKCα	Cofilin-1					Ca^{2+}	pro-MMP2	cdc42
Ezrin	Fascin					Plasminogen/tPA	Col II, Col IV	Ack1
MUPP-1	Ena/VASP					MMP-2	FAK	Tnk2
NEDD9						MT-MMP3/MMP16	Stonin-1	Rac1
Syntenin-1						MT1-MMP/MMP14	β1 integrin	PI-3K
BAIA/IRSP53						α2 integrin subunit		
mDia2						α4 integrin subunit		
						Fibronectin		
						Tenascin-C		
						Laminin 111		
						Perlecan		
						Col V, Col VI		
			ERK			Ca^{2+}	MT-MMP/MMP14	ERK1/2
			PKCα			FGFs	ADAM-10	PKCs
			PI-3K			PDGFAA		Phospholipase D
			Akt-1			FGFR1		
			mTOR			FGFR3		
						PDGFRα		
						PDGFRβ		
				Ezrin	Fascin	Ca^{2+}	P-selectin ?	Rac1
				MUPP-1	AMPA receptors	Galectin3		Par3
				GRIPP-1	Tiam-1	Galectin9		Par6
				Syntenin-1	Syx	Angiostatin		PKCζ
				HtrA2/OMI	γ-secretase	α3 integrin subunit		

Ack1, activated cdc42 kinase 1; ADAM-10, disintegrin and metalloproteinase domain-containing protein 10; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BAIA/IRSP53, brain-specific angiogenesis inhibitor 1-associated protein 2; Col, collagen; Ena/VASP, actin regulator/vasodilator stimulated phosphoprotein; GRIP-1, glutamate receptor interacting protein 1; HtrA2/OMI, HtrA serine peptidase 2; mDia2, diaphanous-related formin-3; mTOR, mechanistic target of rapamycin kinase; MUPP-1, multi-PDZ domain protein 1; Rac1, Rac family small GTPase 1; Syx, synectin-binding RhoA exchange factor; Tiam-1, T-cell lymphoma invasion and metastasis inducing Rho/Rac1-activating exchange factor; Tnk2, tyrosine kinase non receptor 2; tPa, tissue plasminogen activator.

unpublished results). In nascent filopodia and invadopodia, NG2/CSPG4 colocalizes with fascin-1, and we are hypothesizing that in these cell membrane protrusions, it may partner up with additional actin dynamizing proteins and adaptor or cell division cycle 42 (cdc42)–effector molecules (Fig. 4). Furthermore, in membrane microprotrusions, the intracellular domain of NG2/CSPG4 is preferentially phosphorylated in Thr^{2314} by a PKCα-dependent mechanisms and this phosphorylation event seems crucial in directing the PG, along with integrin α3β1 (and possibly other β1-class integrins), into these cell membrane domains (43, 44, 47). Whether NG2/CSPG4, in its phosphorylated or unphosphorylated state, acts as a promoter of the cell membrane protrusion leading to formation of filopodial/invadopodial extensions would need to be defined by further investigations.

In motile cells, NG2/CSPG4 assumes a bipolar subcellular arrangement characterized by enrichment of the PG in both ruffling membranes and retraction fibers of the trailing edge (23, 50). A concomitant macroclustering is noted in the lamellopodium, contiguously with dynamic focal adhesion placques and putative areas of cell-substrate contact. Intriguingly, in such areas, the cytoplasmic domain of NG2/CSPG4 is differentially phosphorylated on its more C-terminal threonine residue Thr^{2256} (43, 44, 47) and the PG undergoes extensive *stonin-1*-promoted internalization and endocytic intracellular trafficking (Fig. 4). This latter phenomenon seems to be required for the dynamics of the cell-substrate junctions and the motile properties of the cells (51). The finding, therefore, provides an explanation for the diffuse lamellopodial NG2/CSPG4 distribution noted in ruffling membranes of migrating cells (Fig. 4). Collectively, the currently available information on the cell surface distribution of the PG in response to actin cytoskeleton rearrangements suggest that the membrane organization of NG2/CSPG4 may be tightly linked to cell polarity, as well as being strongly dependent upon the molecular interactions ensued by the PG's cytoplasmic tail. Furthermore, because NG2/CSPG4 is known to act as a docking receptor for several growth factors (Table 1), it would not be particularly surprising to find concomitant microclusters of the PG in cell membrane subdomains accumulating growth factor receptors.

Immunomapping at the ultrastructural level and experiments carried out with glycanation-defective NG2/CSPG4 mutant molecules have added further support to a diverse distribution of NG2/CSPG4 glycoforms in diverse areas of the cell. They have documented that retraction fibers primarily contain NG2/CSPG4 variants lacking GAGs bound to residue S999, whereas those largely deprived of GAG chains are more widely distributed (17, 23). Taken together, these findings suggest that glycanation variants of NG2/CSPG4 polarize in a discrete manner along the cell membrane, adjusting their distribution in accordance with the polarization of the cell. Furthermore, in cells interacting with highly adhesive matrix components, such as fibronectin (to which NG2/CSPG4 is not known to bind directly), the PG is observed to align with stress fibers. By contrast, in cells adhering to matrix substrates for which NG2/CSPG4 may serve as a putative (co) receptor, the PG aligns with both contractile and noncontractile actin microfilaments. Hence, its membrane distribution is not delimited to areas overlaying microfilaments bundled by fascin but may be observed differentially organized throughout the lamellipodium (48, 50; unpublished results). Based upon a series of published findings and some of our more recent unpublished results, we propose a generalized view on the topographical surface arrangement of NG2/CSPG4 (Fig. 4). The PG organizes in macroclusters at the level of adhesion sites and in microclusters at the level of lipid rafts distant from the such sites.

Through the exploitation of the yeast 2–hybrid expression system it has been possible to delineate some of the actin cytoskeleton interactions engaged by the cytoplasmic domain of NG2/CSPG4 (Table 1). These investigations have independently identified the multi-PDZ domain protein 1, the glutamate receptor interaction protein, syntenin-1, and the mitochondrial HtrA serine peptidase 2 protein as direct binding partners of the NG2/CSPG4 cytoplasmic tail (52–55). More recent observations also show that, depending upon the phosphorylation pattern of this domain, the NG2/CSPG4–multi-PDZ domain protein 1 interaction coaxes the front-rear cell polarity signaling pathways (*i.e.*, involving the *Par* cell polarity protein complex), which are modulated by the signaling molecules synectin-binding Ras homolog family member A (RhoA) exchange factor and T-cell lymphoma invasion and metastasis inducing Rho/Rac family small GTPase 1–activating exchange factor. The interaction may thereby contribute to the directional migration of NG2/CSPG4–expressing oligodendrocyte progenitor cells (OPCs) toward sites of tissue damage in the injured CNS (56).

In immature glial cells establishing synaptic connections with neurons, the NG2/CSPG4–glutamate receptor interacting protein 1 interaction seems to provide a scaffold complex for the clustering of α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptors, such as to potentiate the neuron-glia recognition and signaling processes (53). The association of *syntenin-1* with NG2/CSPG4 also seems to be pivotal for OPC movement. The mechanistic explanation for this may converge upon the proposed role of *syntenin-1* in PG endocytosis and PG-mediated exosome (57), as well as on the importance assigned to the internalization of NG2/CSPG4 in the control of cell motility. Cumulatively, these findings unveil mechanistic leads to an extended function of the PG in the dual intra- and extracellular control of cellular interactions, including those dictating cell polarity and implicating phenomena of cell adhesion and migration.

BIDIRECTIONAL METALLOPROTEINASE-DEPENDENT INTERACTIONS OF THE NG2/CSPG4 ECTODOMAIN ACCOUNT FOR ITS CELL SURFACE RELEASE

The NG2/CSPG4 is frequently shed from cell surface, both under physiologic and pathologic conditions, and the fragments of the cleaved ectodomain may be retained in

the surrounding ECM to be further subjected to proteolysis. Massive shedding of the ectodomain may be induced by cleavage at the lower portion of the cell membrane-proximal D3 subdomain, releasing a virtually full-length ectodomain fragment in the range of 240–290 kDa (58). The shedding process may be modulated by PKC-dependent signal transduction and by the cell's interplay with the ECM substrate. Cell surface proteolysis has also been proposed to trim the ectodomain at the level of the membrane-proximal D1 subdomain loop (58), but sequence data are not currently available to support this suggestion.

A tantalizing hypothesis is that intact (nonglycanated) NG2/CSPG4 may be released from cells in exosomes, as recently described for other transmembrane PGs (50, 59). If occurring, such vescicle-based release would resolve the identity of the NG2/CSPG4 molecules of $M_{r:s}$ of 250–290 kDa reported to be shed into the culture media, *i.e.*, the PG may be secreted by the cell through exosomes in its intact form. Notwithstanding the putative artifactual nature of the surface release of NG2/CSPG4 by cultured tumor cell lines, fragmented forms of the PG have been extensively described in both healthy and diseased tissues (40, 60–64). The MW range of the prevalent proteolytic NG2/CSPG4 fragments identified both *in vitro* and *in vivo* spans from 130 to 275 kDa (40, 58, 61). This would suggest that proteolytic cleavage of the NG2/CSPG4 ectodomain may occur in sequential steps, starting from a cell surface-proximal proteolysis and continuing with more N-terminal fragmentations of the released protein. If not completely destroyed in the extracellular spaces, NG2/CSPG4 fragments may eventually enter the blood stream. In fact, such fragments are frequently found in peripheral blood of cancer patients for whom they may serve as an adjunct in the prognostication of the disease (65, 66).

GAG-bearing variants of melanoma NG2/CSPG4 interact with the latent forms of metalloproteinase [membrane-type matrix metalloproteinase 3 (MT-MMP3)/matrix metalloproteinase 16 (MMP16)] and may form ternary complexes, including both this metalloproteinase (MMP) and pro-MMP2 (Table 1). In such complexes, the former membrane-bound MMP activates the second one [*i.e.*, MMP2 (67, 68)]. This is likely to create an autodigestion loop leading to an NG2/CSPG4 surface cleavage pattern that may be balanced by the exogenous activity of tissue inhibitor of metalloproteinase 2 and 3 (60, 68). However, on macrophages and OPCs, the NG2/CSPG4 ectodomain may also be dislodged from the cell surface by MT1-MMP/MMP14, which finds its preferential cleavage site within the upper portion of the N-terminal D1 subdomain [*i.e.*, within residues 490–500 (64)]. The preventive effect on surface shedding observed with a spectrum of MMP inhibitors, along with *in vitro* digestion and colocalization studies, also implicates MMP9 and MMP13 in the proteolytic processing of NG2/CSPG4 (69–71).

In injured brain, NG2/CSPG4 is reportedly susceptible to disintegrin and metalloproteinase domain–containing protein 10 secreted by OPCs, whereas the C-terminal cytoplasmic tail may be further processed by a γ-secretase (72). Finally, an autodegradation loop analogous to that entailing the abovementioned MMP16-MMP2 complex seems to be operated by plasminogen and by tissue plasminogen activator. Accordingly, evidence has been afforded on the ability of NG2/CSPG4 to bind plasminogen (Table 1), *via* an interaction mediated by the D2 subdomain of the PG and the kringle domains 1–3 of the enzyme precursor (73, 74). Meanwhile, parallel *in vitro* and *in vivo* observations demonstrate that the extracellular protease tissue plasminogen activator also binds the same segment of the NG2/CSPG4 ectodomain (63). Thus, it could be assumed that bidirectional regulation of MMP-ECM protease activation and NG2/CSPG4 proteolysis endows the PG with the potential to influence biologic phenomena that are dependent upon its extracellular processing and surface shedding.

PERMISSIVE *VS.* NONPERMISSIVE MOLECULAR INTERACTIONS OF NG2/CSPG4 IN NEURITE OUTGROWTH AND REGENERATION OF THE NERVOUS TISSUE

The molecular interactions that the PG engages in the context of tissue injury and regeneration of the damaged CNS (*i.e.*, axon outgrowth and remyelination) are forged by conflicting data and counteracting interpretations of the experimental findings. Contusion lesions experimentally evoked in the rodent spinal cord are accompanied by a perilesional accumulation of a rare NG2/CSPG4-expressing OPC subset (also referred to as polydendrocytes), possibly accompanied by an ancillary population of Schwann-like cells (75–84). This can be confirmed by *in vivo* imaging of the OPC subset by means of NG2/CSPG4–based transgenic tagging, which also reveals that these cells originate a self-regulated and highly motile population that rapidly displaces to focal injury sites upon tissue insult (85).

An initial confusion about the actual role on neurite outgrowth exerted by NG2/CSPG4 displayed by injury-attracted OPCs probably stems from early studies in which an immunopurified variant of the PG was used as an immobilized substrate agent. As would have been predicted, isolated neurons failed to attach and extend their projections on the isolated NG2/CSPG4 molecule (86). This result was reproduced using astrocytes or meningeal cells overexpressing the PG (87, 88). Based upon the detection of some binding of isolated neurons to a recombinant fragment encompassing the central portion of the NG2/CSPG4 ectodomain, along with the failure of neurons to attach to fragments corresponding to juxtaposed regions, it was proposed that the presumptive inhibitory activity of the PG resided within subdomains D1 and D3 (89). This assignment was further substantiated by the restored synaptic transmission and neuronal excitability observed upon treatment of injured animals with subdomain-specific anti-NG2/CSPG4 antibodies, or by local injection of purified full-length NG2/CSPG4 (90–92).

In the *in vitro* settings, it was suggested that the inability of neurons to attach to NG2/CSPG4 substrates was attributed to Ca^{2+}-mediated intracellular signals, transduced

by an orphan G-protein–coupled 280 kDa-receptor (93). More recently, these presumptive signal transduction pathways have been proposed to involve activation of PKCζ and the polarity complex proteins Par3 and Par6 [also known as partitioning defective homologue 3/6 (PARD3/PARD6)]. (Table 1), leading to downstream activation of Rac family small GTPase 1and cdc42 (94). Notwithstanding that GPCRs are documented to be involved in chemorepulsive phenomena, the identity of the proposed neuronal NG2/CSPG4 receptor and the modes through which its ligand interaction would activate intracellular signaling events remains veiled. It remains similarly difficult to reconcile these findings with the extended series of *in vivo* observations implicating a dominating regeneration-inhibitory effect exerted by the CS chains of scar-enriched ECM PGs (95). Furthermore, parallel *in vitro* and *in vivo* experimental data indicate that NG2/CSPG4–expressing glial progenitors do not repel growing axons but, on the contrary, act permissively for their outgrowth. This function may be exerted by the PG by serving as a major source of ECM molecules with recognized growth-promoting activity and by the ability to counteract infiltrating macrophages (78, 81).

Further discordant pieces of data on the presumptive inhibitory effect of NG2/CSPG4 on nervous tissue regeneration are those derived from approaches of cellular therapy: both neural and mesenchymal stem cells with constitutive high expression of NG2/CSPG4 effectively contribute to the repair of damaged CNS tissues (96–98). Similar effects are seen with NG2/CSPG4–expressing embryonic stem cell–derived neural lineage cells (99). Finally, in NG2/CSPG4 knockout mice, loss of the PG causes loss of OPCs, impairs remyelination, and exacerbates the neurologic deficits induced by traumatic brain injury, but it does not lead to overt defects in neurite regeneration (100). Collectively, these latter observations sustain the idea that NG2/CSPG4 is essential for optimal nervous tissue repair (101, 102), rather than being a counteracting factor of this event. Recent studies also unveil a close association between NG2/CSPG4, Notch signaling, and *Prospero* expression during nervous tissue repair in *Drosophila* (103, 104), highlighting a putative evolutionary conservation of the regenerative function of the PG.

Lentil viral delivery of anti-NG2/CSPG4 short hairpin RNA probes in the same experimental rodent model of CNS damage as previously described have been shown to tangibly ameliorate axon growth and scar formation. However, in this experimental setting, the apparent increase in scar-associated NG2/CSPG4-expressing OPCs is paralleled by a decrease in reactive astrocytes and macrophages positive for heat-shock cognate 70KD protein 44KD ATPase N-terminal mutant with Cys 17 replaced by Lys (105, 106). Because unique and highly plastic macrophage subsets appearing in injured brain are widely known to upregulate NG2/CSPG4 (64, 77, 107–110), it is plausible that the observed promotion of CNS regeneration upon local knockdown of NG2/CSPG4 may be linked to an eradication of these macrophage populations and/or to the hampering of their glial cell interactions and the secretion of tissue repair–promoting factors. Thus, more stringent analyses of the reciprocal role of NG2/CSPG4–expressing and nonexpressing glial progenitors and macrophage/microglial cells may be the key to resolving the conundrum about permissive *vs*. nonpermissive NG2/CSPG4-mediated interactions in CNS repair.

FUNCTIONAL MULTIVALENCY OF THE NG2/CSPG4 ECTODOMAIN

The primary and higher order structures of the NG2/CSPG4 ectodomain shapes multivalent binding sites for structurally and compositionally diversified molecules (Table 1). These may range from those associated with the cell membrane to those involved in paracrine signaling (Fig. 4). Primary extracellular ligands of NG2/CSPG4 remain components of the ECM and early studies examining (or, rather, searching for) such interactions disclosed binding of the recombinant ectodomain of the PG to tenascin-C, laminin 111, perlecan, and collagen types II, V, and VI, but not other collagens (26, 49, 111–113). From a structural point of view, affinity for such diverse molecules appears rather remarkable considering that type II, V, and VI collagens have virtually nothing in common beyond being endowed with triple-helical repeats, which are evidently shared by other "non-NG2/CSPG4–interacting" collagens; these macromolecules are yet different from other ECM glycoproteins showing some binding to NG2/CSPG4, such as, for instance, perlecan. Among the putative ECM ligands, collagen type VI and perlecan seems to be a particularly relevant one, as also demonstrated by the ability of NG2/CSPG4 to mediate β1-class integrin-independent anchorage of cells to these macromolecules (38, 113–116). Furthermore, a critical role of the NG2/CSPG4-collagen type VI interaction in cell movement and signal transduction, along with a tight association of the 2 molecules *in vivo*, have incontrovertibly been documented by a plethora of investigations in different systems (50, 111, 114–122).

The central D2 subdomain (Fig. 1) has been identified as the region of the PG responsible for the collagen interaction. Collagen binding to NG2/CSPG4 does not implicate the CS chain or chains that may be attached to the D2 subdomain but is highly dependent upon the macromolecular configuration of the ligand (26, 39, 119, 120). Recombinant NG2/CSPG4 binds the α2(VI) chain, but not the α1(VI) chain or a proteolytic fragment corresponding to the N-terminal globular domain of the α3(VI) chain (119). However, a more accurate functional analysis of the NG2/CSPG4–collagen type VI interaction points to the tetrameric N- or C-terminal globular domain assemblies as the prevailing site responsible for the NG2/CSPG4–mediated cell attachment and migration of cancer cells and further identifies at least 2 different β1-class integrins cooperating with the PG in this process [one of which is the α2β1; the other remains unidentified (39)]. Detailed analyses of the interaction of NG2/CSPG4 highlights a remarkable analogy with the NG2/CSPG4–collagen VI: in both cases complexes of the 2 macromolecules can be

isolated from the same cell (113). This would suggest that the ECM interactions of NG2/CSPG4 may frequently involve components coexpressed with the PG.

Since no NG2/CSPG4–mediated cell adhesion to collagen type V, or to other fibrillar collagens, has thus far been experimentally substantiated, we have implicitly ruled out the possibility that binding of NG2/CSPG4 to triple-helical collagenous structures may be of functional significance. Incidentally, however, we and other investigators have observed a polarized cell surface distribution of NG2/CSPG4 in cells juxtaposed to collagen type IV fibrillar networks of perivascular basement membranes (40, 62), hinting at a potential interaction of the PG with this collagen type. In support of an NG2/CSPG4–collagen type IV interplay are the findings on the dependence of the PG for optimal cancer cell adhesion and migration in response to collagen type IV substrates (39, 123). Even in this case, the noncollagenous globular domain of the collagen appears critically involved in the NG2/CSPG4–mediated cellular interaction.

Experimental evaluation of the involvement of NG2/CSPG4 in cell-matrix interplays further unfolds an unprecedented role of the PG in the attachment and migration of cancer cells on vitronectin substrates (39; unpublished results). This finding largely parallels the previously proposed involvement of NG2/CSPG4 in the attachment and locomotion of melanoma cells on fibronectin substrates (124, 125). As the potential of NG2/CSPG4 to bind to such a diverse array of ECM components is deduced from experiments measuring adhesive responses of cells after knockdown of the PG, the use of NG2/CSPG4–null or immunosorted NG2/CSPG4–positive and –negative cell subsets, the effective molecular interaction between the PG and the variety of proposed ECM ligands remains to be more solidly demonstrated.

An alternative possibility may be that NG2/CSPG4 modulates the activity of the receptors for many of these ECM components and that this modulation is dispensable for stable cell adhesion and movement. In this context, a presumptive NG2/CSPG4–integrin cooperation would appear extremely evolutionary conserved, because it is seems crucial to muscle development in *Drosophila* (28, 29, 126). In mammalian species, at least 3 different integrins, including $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha4\beta1$ (Fig. 4), have thus far been co-immunoprecipitated with the NG2/CSPG4 from cells exhibiting a PG-integrin coaction in the context of cell attachment and migration (35, 39, 45, 47, 124, 125, 127–130). Aside from possible CS-mediated interactions of NG2/CSPG4 with the $\alpha4$ integrin subunit (35), a more consolidated evidence for an integrin interaction with the core protein of the PG remains to be fully documented. Conversely, a NG2/CSPG4–mediated activation of the integrin heterodimer can be demonstrated structurally and functionally to occur in both cis and trans orientation (47). Studies on a variety of healthy (*e.g.*, pericytes) and neoplastic cell types have provided relevant information on the NG2/CSPG4–integrin interplay and how this may specifically contribute to the PG's involvement in highly dynamic, integrin-dependent cellular phenomena, such as cell proliferation, motility, and survival.

The central portion of the NG2/CSPG4 core protein is also reportedly critical for the sequestering of growth factors, and the same region may be responsible for the concomitant association with corresponding receptors to form ternary complexes. This seems to be the case for at least platelet derived growth factor (PDGF)-AA (112, 131–133), several fibroblast growth factors (FGFs) (38, 134, 135), and their cognate receptors (Fig. 4). Binding studies and *in vitro* tests on cells have further established an affinity hierarchy among different FGF family members binding to NG2/CSPG4 and have disclosed binding constants of values close to the ones detected for FGF binding to heparin/heparan sulfates and for NG2/CSPG4 binding to PDGF-AA (38, 133, 135). Because the implicated extracellular portion of the PG does not show any similarity with the ligand binding sites of the growth factor tyrosine kinase receptors, the interaction must rely upon novel structural motifs that are likely to be spatially separated from the ones ruling in the growth factor-tyrosine kinase receptor interplays.

Contrary to what could be anticipated from a CS-PG, in which GAG attachment and FGF/PDGF binding sites are encompassed by the same segment of the core protein, NG2/CSPG4–mediated capturing and activation of these growth factors is entirely GAG-independent (38, 133, 135). On the other hand, CS chains of the PG have been proposed to be implicated in P-selectin–mediated homotypic interactions of breast cancer cells (136). However, at present, this finding seems questionable, because we do *not* detect glycanated variants of NG2/CSPG4 on the breast carcinoma cell lines allegedly reported to bind to P-selectin *via* a CS-dependent mechanism.

Direct binding of fibroblast growth factor receptor 1 (FGFR1) and FGFR3 to NG2/CSPG4 is well substantiated and is functionally validated in the context of pericyte sprouting and angiogenesis (38). Conversely, its importance in the context of oligodendrocyte maturation and tumor growth may only be speculated. Moreover, it is less certain for which of the PDGF-mediated signaling events NG2/CSPG4 is absolutely essential. The PG is documented to bind with high-affinity PDGF-AA but not with PDGF-BB (133), whereas other PDGF isoforms have not been tested. PDGF-AA/platelet derived growth factor receptor α (PDGFRα)-elicited signal transduction is strongly impaired in NG2/CSPG4–deficient cells (38, 131, 132, 137), but the PG seems to colocalize with both PDGFRα and PDGFRβ, albeit in different areas of the body, different cell types, and different tumor entities. Thus, although a functional correlation of the NG2/CSPG4–PDGFRα interaction is thoroughly asserted in normal murine smooth muscle cells, a physical association between NG2/CSPG4 and PDGFRβ awaits full documentation in human cells that are known to depend upon their NG2/CSPG4 for optimal PDGFRβ-mediated signal transduction (38). Of further interest is the fact that in the CNS, where PDGFRα is the most represented PDGF receptor on NG2/CSPG4–expressing OPCs, a direct role of the PG in PDGF signaling remains debatable. This is hinted at by the observation that brain tumors generated by retrovirally induced overexpression of PDGF form with equal frequency and to the same extent in wild-type and NG2/CSPG4 knockout mice (138).

Thus far, it has been arduous to prove the formation of heterotrimeric assemblies of NG2/CSPG4-FGF-FGFR or NG2/CSPG4-PDGF-PDGFR, leaving open the possibility that the mode of action of NG2/CSPG4 as a growth factor coreceptor is markedly different from that described for heparan sulfate-bearing PGs. Our hypothesis is that NG2/CSPG4 may serve as a relay molecule, which associates with growth factor receptors and presents the ligands to them in a highly controlled manner, but without purporting durable ternary complex formations. Such mechanism would be compatible with a contribution of NG2/CSPG4 to the shaping and perception of mitogen/morphogen gradients. Although not firmly demonstrated, it is likely that binding of growth factors to NG2/CSPG4 promotes dimerization of the mitogens and sets up for the optimal interaction of such ligand homodimer with the cognate dimerized receptors. In this context, the envisioned possibility is that the PG may similarly intervene in the process of dimerization of the receptors. Based upon experimental evidence, we have excluded any docking receptor function of NG2/CSPG4 for other growth factors, including nerve growth factor, hepatocyte growth factor, epidermal growth factors, and several TGF family members (38). However, whether other signaling molecules, beside PDGFs and FGFs (*e.g.*, cytokines, hedgehogs, and Wnts), may also interact with the central D2 subdomain of the NG2/CSPG4 core protein is another challenging issue.

The molecular interactions ensuing in the lower, membrane-proximal region of the NG2/CSPG4 ectodomain are still poorly defined. Nevertheless, relevant interactions seem to involve the segment of the PG immediately juxtaposed to the plasma membrane. For instance, galectin-3 has been shown to bind within the lower portion of the D3 subdomain and participate in ternary complexes including the glycoprotein and the $\alpha 3\beta 1$ integrin to promote endothelial-pericyte interactions during angiogenesis (47, 128, 139). Uniquely, the galectin-3–NG2/CSPG4 linkage seems to be mediated by *O*-glycosylation of 1 or more of the 6 threonine residues of the (rodent) PG, predicted *in silico* to be prone to post-translational modification. Thus, galectin-3-binding and potential higher-order integrin-NG2/CSPG4–galectin-3 arrangements would appear to be subordinate to the involvement of selected NG2/CSPG4 variants in such a complex formation.

SIGNALING NETWORKS IMPLICATING THE NG2/CSPG4 CYTOPLASMIC DOMAIN

By virtue of the high plasma membrane mobility, and through the potential of its cytoplasmic tail to associate with a wide range of cytoskeletal adaptor molecules, NG2/CSPG4 is linked to multiple crosstalking signaling cascades. These are comprised of 2 primary pathways: the cell survival pathway involving the PI3K–Akt-1–mechanistic target of rapamycin kinase axis and the pathway associated with cell polarization, adhesion and motility, and implicating focal adhesion kinase (FAK); neural precursor cell expressed, developmentally down-regulated 9; the Par cell polarity protein complex; Rho, Rac-1; cdc42; and some of their regulators, including activated cdc42 kinase 1, Cas scaffolding protein family member 1, and the synectin-binding RhoA Rho/Rac guanine nucleotide exchange factor Syx1 (39, 43–45, 47, 56, 127, 129, 130, 140, 141). Because signal transmission associated with cytoskeletal rearrangements and cell shape changes is strongly influenced by the transducing activity of several NG2/CSPG4–cooperating integrin receptors, it is at present impossible to separate the Akt/PKB–associated pathway involved in cell survival (142) from those regulating cell adhesion and motility. This is such as to delineate independent, or converging, FAK/Rho guanosine triphosphate hydrolase activations specifically accounted for by the PG, independent of integrin coaction (47).

The 2 NG2/CSPG4 cytoplasmic threonines prone to phosphorylation are known to be differentially modified by crosstalking signal transduction pathways entailing ERK1/2 (and possibly other ERKs) and PKCα (43–45). It seems rather logical that phosphorylation of Thr^{2314} by ERKs may be specifically connected with cell-cycle progression and cell proliferation events copromoted by NG2/CSPG4 (39, 44). By contrast, more cumbersome seems the dissection of the signal transduction pathways characterized by the FAK/PKCα cooption, which, in the case of PKCα, may implicate an upstream mobilization of second messengers and downstream regulation of phospholipase D. Furthermore, it is difficult to conceive how changes in phosphorylation status and modulation of cytoskeletal adaptor protein interaction may ensue entirely independently of extracellular engagement of the NG2/CSPG4 ectodomain. Therefore, it may be envisioned that the potentially multivalent role of NG2/CSPG4 in the control of different cellular phenomena may be governed by a delicate regulation of alternating inside-out and outside-in signaling mechanisms.

In the context of the regulation of cell polarity, cell adhesion, and cell migration/invasion, there is strong evidence in favor of NG2/CSPG4 cooperating with integrin receptors while acting as coreceptor for certain ECM components (38). Our working hypothesis is that the PG may act as an upstream molecular switch for the mechanotransduction events relayed by the nuclear translocation of yes associated protein 1/transcriptional coactivator with PDZ-binding motif. If this were the case, it would be expected that the involvement NG2/CSPG4 may influence the persistency of directionality in cells moving on softer, more flexible matrices *vs.* more rigid ones and would corroborate the proposed role of the PG in the control of cell polarity observed in the context of OPCs and pericytes. Based upon the wealth of previously discussed findings, this is likely to require differential phosphorylation of the NG2/CSPG4 cytoplasmic threonines and the concomitant mobilization of specific cytoskeletal adaptor proteins. Further understanding of the modes through which such processes are controlled by extracellular engagement of NG2/CSPG4 may be instrumental for a better understanding of how cells up-regulating the PG in the context of tissue regeneration may respond to motility-promoting cues, as well as how cancer cells with augmented levels of NG2/CSPG4 may acquire more invasive properties.

MECHANISTIC INSIGHTS INTO THE MULTIFUNCTIONALITY OF NG2/CSPG4 MAY PROVIDE LEADS TO UNDERSTANDING CELLULAR EVENTS REGULATING EMBRYONIC DEVELOPMENT, THE BALANCING OF HOMEOSTATIC PROCESSES, AND DISEASE PROGRESSION

Early embryonic and evolutionary conserved expression of NG2/CSPG4 suggests a pivotal role of the PG in developmental phenomena, especially those involved in morphogenetic structuring of tissues (*e.g.*, skin, skeletal muscle, cartilage and nervous tissues). Deletion of the NG2/CSPG4 gene in mouse partially confutes this idea but does not preclude that NG2/CSPG4 may be critical for embryonic development in other species. As in many other gene knockouts lacking an overt phenotype at birth (see *International Mouse Knockout Consortium*; *UC Davis Knockout Mouse Project Repository*; *Mouse Genome Informatics*), loss of NG2/CSPG4 nevertheless sets up homeostatic imbalances and the susceptibility of incurring into degenerative and neoplastic pathologies, as well as a higher probability of exhibiting developing deficits in the tissue regeneration capabilities. It is plausible to assume that these imbalances may be accounted by interruption of the *continuum* between the outside (extracellular environment) and the inside of the cell that loss of NG2/CSPG4 may lead to, alongside with the consequent defective responses of NG2/CSPG4–null cell to vital environmental cues in the absence of the PG. Such deficiencies may become detrimental in the pathophysiologic repair of injured tissues, especially when it implicates neovascularisation as a primary mechanism, but may be beneficial during tumor progression.

Whereas complete loss of NG2/CSPG4 may be causative of physiologic deficiencies, excessive cell surface shedding of the PG may act as a double-edged sword. In physiologic conditions it may adjust the density and facilitate accessibility of soluble signaling molecules accumulating in the proximity of the cell, and the released fragments may act positively upon the adjacent cells [as in the case of pericyte-endothelial cell interplays (47, 128)]. On the other hand, in the context of cancer formation and progression it may counteract anoikis and promote the malignant and invasive behavior of the neoplastic cells.

Much has yet to be learned about how NG2/CSPG4 coordinates responses to extracellular signals with the regulation of the internal machinery of the cell, such as to impact on a variety of cellular phenomena. Although the comprehensive repertoire of NG2/CSPG4 ligands remains to be established, and we need to better define the nature and dynamics of its molecular interactions, the wide range of intracellular ligands prospects the involvement of NG2/CSPG4 in the control of complex signaling networks. Presumably, particular attention should be given to the possibility that diverse glycoforms of the PG may engage in diverse interactions and thereby exert a diversified control of cellular phenomena in healthy and diseased conditions. FJ

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AUTHOR CONTRIBUTIONS

E. Tamburini, J. Quartararo, and B. Cortelazzi have collected the published scientific data and elaborated most of the paragraphs; A. Dallatomasina has compiled the databank information and performed *in silico* analyses; D. Mangieri has specifically contributed to the neuroscience paragraph; M. Lazzaretti has elaborated most of the schemes and figures; and R. Perris has proposed the content of the manuscript and contributed to its writing.

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