Secreted gliomedin is a perinodal matrix component of peripheral nerves

Yael Eshed,1 Konstantin Feinberg,1 David J. Carey,2 and Elior Peles1
1Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel
2Weis Center for Research, Geisinger Clinic, Danville, PA 17822

The interaction between gliomedin and the axonodal cell adhesion molecules (CAMs) neurofascin and NrCAM induces the clustering of Na⁺ channels at the nodes of Ranvier. We define new interactions of gliomedin that are essential for its clustering activity. We show that gliomedin exists as both transmembrane and secreted forms that are generated by proteolytic cleavage of the protein, and that only the latter is detected at the nodes of Ranvier. The secreted extracellular domain of gliomedin binds to Schwann cells and is incorporated into the extra-cellular matrix (ECM) in a heparin-dependent manner, suggesting the involvement of heparan sulfate proteoglycans (HSPGs). Furthermore, we show that the N-terminal region of gliomedin serves as an oligomerization domain that mediates self-association of the molecule, which is required for its binding to neurofascin and NrCAM. Our results indicate that the deposition of gliomedin multimers at the nodal gap by binding to HSPGs facilitates the clustering of the axonodal CAMs and Na⁺ channels.

Introduction

The presence of voltage-gated Na⁺ channels at the nodes of Ranvier ensures fast saltatory propagation of action potentials in myelinated nerves. The accumulation of these channels at nodes is tightly regulated by the overlaying myelinating Schwann cells (Poliak and Peles, 2003; Salzer, 2003; Schafer and Rasband, 2006). In the peripheral nervous system (PNS), the nodal axolemma is contacted by an ordered array of microvilli that project radially from the outer collar of two adjacent myelinating Schwann cells. These Schwann cell microvilli are embedded within a poorly defined filamentous matrix (i.e., the gap substance) that was referred to as the “cement disc” by Ranvier (Landon and Hall, 1976). The nodal gap substance consists of proteoglycans and nonsulfated mucopolysaccharides, which contribute to the ability of a wide variety of metallic cations to label the nodes of Ranvier (Zagoren, 1984). Proteoglycans that are present at peripheral nodes include versican (Apostolski et al., 1994; Melendez-Vasquez et al., 2005), NG2 (Martin et al., 2001), and syndecans (Goutebroze et al., 2003; Melendez-Vasquez et al., 2005), as well as hyaluronic acid and its binding protein hyaluronectin, which are associated with proteoglycans in the ECM (Apostolski et al., 1994; Delpech et al., 1982). Several ECM and ECM-associated proteins are also enriched at PNS nodes, such as collagen α4(V) (Melendez-Vasquez et al., 2005), laminin α2β1γ1 and α5β1γ1 (Occhi et al., 2005), dystroglycan, and some members of the dystrophin–glycoprotein complex (Occhi et al., 2005; Saito et al., 2003). Schwann cell–specific ablation of dystroglycan (Saito et al., 2003), and to a lesser extent of laminin γ1 (Occhi et al., 2005), causes disruption of microvillar organization and reduction in nodal Na⁺ channel clustering, suggesting that the microvilli play a direct role in node assembly. This notion is further supported by observations demonstrating that Schwann cell microvillar processes align with nascent nodes (Tao-Cheng and Rosenbluth, 1983; Melendez-Vasquez et al., 2001).

At the nodal axolemma, Na⁺ channels associate with two cell adhesion molecules (CAMs), NrCAM and the 186-kD isoform of neurofascin (Davis et al., 1996). Growing evidence suggests that during development, Na⁺ channels are recruited to clusters containing these axonodal CAMs that were first positioned by glial processes (Lambert et al., 1997; Lustig et al., 2001; Custer et al., 2003; Eshed et al., 2005; Sherman et al., 2005; Koticha et al., 2006; Schafer et al., 2006). Neurofascin and NrCAM interact with gliomedin, which is concentrated at the Schwann cell microvilli (Eshed et al., 2005). During myelination, gliomedin accumulates at the edges of myelinating Schwann cells, where it is associated with early clusters of Na⁺ channels. In myelinating cultures, both the expression and correct localization of gliomedin are essential for node assembly.
formation. Gliomedin is a type II transmembrane protein that is characterized by the presence of olfactomedin and collagen domains in its extracellular region, a domain organization shared by members of a specific subgroup of the olfactomedin proteins, termed colmedins (Loria et al., 2004). In addition, gliomedin contains a putative α-helical, coiled-coil sequence at its juxtamembrane region, which serves as an oligomerization motif in collagenous transmembrane proteins (Latvanlehto et al., 2003; Franzke et al., 2005). The olfactomedin domain of gliomedin was shown to mediate its interaction with neurofascin and NrCAM (Eshed et al., 2005). The aggregation of this domain using a secondary antibody was sufficient to induce nodelike clusters along the axons of isolated dorsal root ganglion (DRG) neurons. These observations led us to propose that the focal presentation of gliomedin to the axon during myelination causes the initial clustering of the axonodal CAMs into higher-order oligomers, which facilitates the recruitment of ankyrin G and Na⁺ channels (Eshed et al., 2005).

We report that gliomedin is cleaved from the cell surface by a furin protease, and then assembles into high–molecular weight multimers and incorporates into the ECM by binding to HSPGs. We propose that these unique features endow gliomedin its ability to cluster the axonodal CAMs, thereby facilitating node formation.

Results

Differential binding of gliomedin to DRG neurons and Schwann cells

We have previously shown that the OLF domain of gliomedin mediates its interaction with axonal neurofascin and NrCAM (Eshed et al., 2005). To examine the existence of additional ligands for gliomedin in peripheral nerves, we used a soluble Fc-fusion protein containing its extracellular domain but lacking its OLF domain (COL-Fc) in binding experiments on mixed DRG neurons/Schwann cell cultures (Fig. 1, A and B). Whereas OLF-Fc bound to neurons as expected, COL-Fc binding was detected to a subpopulation of Schwann cells that were aligned with axons. Binding experiments using cultures of rat Schwann cells, revealed that both COL-Fc (Fig. 1 E) and an Fc-fusion protein containing the entire extracellular domain of gliomedin (ECD-Fc; Fig. 1 C) labeled the cells, as well as areas between adjacent cells, suggesting that they interact with Schwann cell ECM. In contrast, OLF-Fc did not bind to Schwann cells (Fig. 1 D). To determine whether COL-Fc interacts with Schwann cell ECM, we have grown Schwann cells in the presence of ascorbate, which induces the formation of ECM fibrils and basal lamina-like structures (Eldridge et al., 1989). In ascorbate-treated Schwann cells, binding of COL-Fc was detected to the cell
surface, as well as to ECM deposits located between the cells (Fig. 1 F). COL-Fc still bound these ECM deposits in cultures that were pretreated with ammonium hydroxide and Triton X-100, a procedure that completely removed the cells from the slide but left the ECM fibrils intact (Fig. 1 G). To determine whether the interaction of COL-Fc with the ECM was specific to Schwann cells, we examined whether it binds astrocytes, which are known to accumulate similar ECM structures in culture (Ard and Bunge, 1988). As depicted in Fig. 1 (H and I), COL-Fc bound to the cell surface of astrocytes, but it did not bind to cell-free areas or to cultures pretreated with ammonium hydroxide and Triton X-100. These results demonstrate that the extracellular region of gliomedin contains distinct domains that mediate its interaction with both neurons and Schwann cells. They further suggest the existence of a novel glial ligand for gliomedin embedded within the Schwann cell ECM.

Gliomedin is found in both transmembrane and secreted forms

The domain organization of gliomedin is reminiscent of transmembrane collagens, all of which are type II transmembrane proteins that are cleaved from the cell membrane by furin-type endoprotease (Franzke et al., 2005). Hence, the ability of gliomedin to bind ECM deposits that are not cell surface–associated raises the question of whether it is secreted from Schwann cells. To examine this possibility, we immunoprecipitated gliomedin from cell lysates and media of cultured Schwann cells using an antibody to the cytoplasmic tail (Ab836), or an antibody directed to the olfactomedin domain (Ab320). As a control, we used human embryonic kidney (HEK)-293 cells that were transfected with a C-terminal, myc-tagged, full-length gliomedin cDNA. Immunoblots were then performed using an antibody to myc tag or an antibody that recognizes a short peptide sequence between the collagen and the olfactomedin domain (Ab720; recognition sites of the various antibodies is schematically depicted in Fig. 2 C). Ab836 and Ab320 immunoprecipitated an 89-kD protein from the lysates of Schwann cells that correspond to the transmembrane form of gliomedin (Fig. 2 A, middle). In transfected HEK-293 cells, the transmembrane form of gliomedin appeared as a doublet consisting of the 89-kD and a weaker ~92-kD band. In addition, Ab320 specifically precipitated a 91-kD protein from the medium of both cell types and a 45-kD protein from the transfected HEK-293 cells. The 45-kD protein was also detected in the medium of Schwann cells that were

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**Figure 2.** Gliomedin is a secreted protein generated by proteolysis. (A) Secretion of gliomedin by cultured cells. Media conditioned by cultured Schwann cells or HEK-293 cells transfected with a full-length, myc-tagged gliomedin cDNA were subjected to immunoprecipitation using antibodies that recognize the N- (Ab836) or C-terminal (Ab320) region of gliomedin (the region each antibody recognizes is shown in B). Western blotting was done using an antibody that recognizes a peptide located between the COL and OLF domains (MAb94) or to myc tag, as indicated. The location of molecular weight marker is shown on the right. (B) Glycosylation of gliomedin. Gliomedin was immunoprecipitated with Ab320 from cell lysate or the medium of HEK-293 cells transfected with a full-length, myc-tagged gliomedin cDNA. Glycosylated protein about 12% SDS gel, and immunoblotted with an antibody to myc tag. Note that the core protein of the secreted gliomedin form (62 kD) is smaller by 11 kD than the transmembrane form found in the cell lysate (73 kD). (C) Identification of cleavage sites in gliomedin. (right) Western blot analysis of protein-A–purified material from medium (CM) of HEK-293 cells expressing a C-terminal Fc-fusion protein of a full-length gliomedin, using an antibody to human Fc; the presence of the Fc adds 30 kD. A schematic presentation of the two gliomedin fragments isolated is depicted on the left, together with the domain organization of the protein, the location of the cleavage sites (arrowheads), and the regions recognized by the different antibodies. The second proteolytic cleavage site, which is located just N-terminal to the OLF domain (PNDD), was identified by N-terminal sequencing, whereas the first furin site (RNKR) within the NTR was identified by mutagenesis. The locations of R91 and R94 are labeled with asterisks. Color frames mark the different domains (gray, transmembrane; purple, coiled coil; blue, collagen; red, olfactomedin). (D) Secretion of the extracellular domain of gliomedin requires cleavage at a furin site. The media or cell lysates of HEK293 expressing a C-terminal, myc-tagged, full-length gliomedin, using an antibody to human Fc; the presence of the Fc adds 30 kD. A schematic presentation of the two gliomedin fragments isolated is depicted on the left, together with the domain organization of the protein, the location of the cleavage sites (arrowheads), and the regions recognized by the different antibodies. The second proteolytic cleavage site, which is located just N-terminal to the OLF domain (PNDD), was identified by N-terminal sequencing, whereas the first furin site (RNKR) within the NTR was identified by mutagenesis. The locations of R91 and R94 are labeled with asterisks. Color frames mark the different domains (gray, transmembrane; purple, coiled coil; blue, collagen; red, olfactomedin). (E) Only a secreted gliomedin is detected at the nodes of Ranvier. Teased rat sciatic nerves were immunolabeled using the different antibodies to gliomedin (red) and an antibody to Caspr (green), as indicated. Bar, 5 μm.
maintained in culture for longer periods of time (Fig. 2 A, right). Treatment of gliomedin immunocomplexes with N-glycosidase revealed that the core protein of the secreted form is smaller by \( \sim 11 \) kD than the transmembrane protein (62 vs. 73 kD; Fig. 2 B). This analysis demonstrates that gliomedin is secreted from Schwann cells as a major 91-kD and a minor 45-kD protein.

To directly determine the cleavage sites in gliomedin, we used an Fc-fusion protein in which the Fc region was fused to the C-terminal region of the full-length gliomedin. The rationale behind this approach was that because gliomedin is a type II transmembrane protein, cleavage of the protein should result in the release of an Fc-tagged protein to the medium, which could be purified using protein A agarose. Two distinct bands of \( \sim 116 \) and 80 kD were purified from the medium of HEK-293 expressing this construct and were subjected to N-terminal amino acid sequencing (Fig. 2 C). The N-terminal sequence of the lower band was identified as DDTLV, which corresponds to position 38–138 for functional sites using the Eukaryotic Linear Motif server (http://elm.eu.org/; Puntervoll et al., 2003), revealed the presence of a putative furin cleavage site (RNKR) at position 91–94. To determine whether this proteolytic site is important to the processing of gliomedin, we have constructed a mutant of the full-length gliomedin by replacing arginine at position 91 with glycine and arginine at position 94 with alanine (R91G94A). This mutant was transfected to HEK-293 cells, and the medium of these cells was analyzed 2 d later for the presence of gliomedin (Fig. 2 D). Mutating these arginines caused a marked reduction in the secretion of gliomedin to the medium, and was accompanied by an accumulation of the 92-kD transmembrane form in the cells. These results indicate that proteolytic cleavage of the mature 92-kD form by a furin-like enzyme mediates the shedding of the extracellular domain of gliomedin from the cell surface. In addition, and less frequently, another cleavage of the molecule at position 278 may occur, which separates the olfactomedin domain from the collagen triplex. Immunolabeling of teased rat sciatic nerves demonstrated a nodal staining of gliomedin using Ab320 and Ab720, but not when Ab836 was used (Ab836 does recognize gliomedin in Schwann cells; see Fig. 6 L), demonstrating that only the extracellular domain of gliomedin was detected at the nodes (Fig. 2 E). Furthermore, the transmembrane form of gliomedin

![Figure 3](https://jcb.rupress.org)
was not detected at nascent nodes during their development at sites that were labeled with the antibodies to the extracellular domain (unpublished data). Thus, we concluded that the main form of gliomedin found at the nodes of Ranvier is the cleaved form that contains the entire extracellular domain, including its collagen and olfactomedin domains.

Ascorbic acid induces the multimerization of gliomedin

The presence of a protease-resistant collagen domain in gliomedin indicates that it may assemble into homotrimers or higher-order multimers after cleavage from the cell surface. To test this possibility, we immunoprecipitated gliomedin from Schwann cells that were grown in the absence or presence of ascorbate, which stimulates the deposition of fibrillar collagen by Schwann cells (Chernousov et al., 1998). Under reducing conditions, only a monomeric form of gliomedin with an apparent molecular weight of 89 kD protein was detected in both untreated and ascorbic acid–treated cells (Fig. 3 A). However, in the presence of a chemical cross-linker (BS3), only high-molecular weight multimers of gliomedin were detected in ascorbic acid–treated cells (Fig. 3 A). These multimers were detected using an antibody to the extracellular, but not to the cytoplasmic tail, of gliomedin (Fig. 3 B, right). Collectively, these results demonstrate that ascorbate treatment of Schwann cells induced the secretion, cleavage, and oligomerization of gliomedin.

Multimerization of gliomedin is required for its interaction with axonodal CAMs

To determine which region in gliomedin mediates its self-association, we examined the ability of purified Fc-fusion proteins containing its entire extracellular domain (ECD-Fc), olfactomedin domain (OLF-Fc), ECD lacking the olfactomedin domain (COL-Fc), ECD lacking the coiled-coil domain (ECDdCC-Fc), and ECD lacking its N-terminal linker region (ECDdNTR) to precipitate a full-length myc-tagged gliomedin from transfected HEK-293 cells (Fig. 3 C). Similar amounts of the Fc-fusion proteins were used, as detected by immunoblotting with an antibody to human Fc (unpublished data). As an additional control, we used HEK-293 cells expressing a myc-tagged PSD93. Whereas ECD-Fc and COL-Fc specifically pulled-down gliomedin, ECDdCC-Fc was less efficient (estimated 50% reduction),

![Figure 4](https://jcb.rupress.org/)
and only a weak signal was obtained using ECDdNTR-Fc. OLF-Fc was unable to recognize and precipitate gliomedin (Fig. 3 D). To further explore the functional significance of these observations, we examined whether self-association of gliomedin is required for its interaction with neurofascin and NrCAM. To this end, COS7 cells expressing neurofascin (NF186) were incubated with the different Fc-fusion proteins as either dimers (i.e., caused by the presence of the Fc) or as multimers by preclustering them with a secondary antibody to human Fc (Fig. 3 E). It was found that, in contrast to ECD-Fc and ECDdCC-Fc, which bound to NF186-expressing cells without preclustering, only multimeric forms of ECDdNTR-Fc and OLF-Fc were able to recognize neurofascin. Similar results were obtained using DRG neurons (unpublished data). Thus, although the olfactomedin domain of gliomedin mediates its direct interaction with the axonal CAMs (Eshed et al., 2005), multimerization of the extracellular domain of gliomedin, which is conferred by other sequences present at the N-terminal region of the molecule, is essential for this interaction to take place.

Secreted gliomedin multimers are incorporated into the Schwann cell ECM

The observation that a secreted extracellular domain of gliomedin can bind to Schwann cells, has prompted us to examine whether the endogenous gliomedin is incorporated into the Schwann cell ECM. Thus, we immunolabeled cultured rat Schwann cells with antibodies to gliomedin, together with antibodies to the known components of Schwann cell ECM, laminin (Fig. 4, E–G), and the α4(V) collagen chain (Fig. 4, A–C). In Schwann cells grown in the absence of ascorbic acid, gliomedin was mostly present on the cell surface, whereas α4(V) immunoreactivity was detected on the cell surface and between the cells. Growing the cells in the presence of ascorbic acid for 48 h resulted in a dramatic incorporation of gliomedin into fibrillar ECM deposits, where it was found to be colocalized with α4(V), mainly in the larger fibrils (Fig. 4, A–C). Similar results were obtained using antibodies to perlecan and the α1(V) chain of collagen (unpublished data). Previous studies have shown that after ascorbic acid treatment, α4(V) incorporates into detergent-insoluble ECM material (Chernousov et al., 1998). To examine whether gliomedin was incorporated into similar structures, we immunoprecipitated gliomedin from ascorbic acid–treated Schwann cells that were extracted with a modified RIPA buffer, or with a RIPA buffer containing 1% SDS (Fig. 4 D). Cells grown in the absence of ascorbic acid were used as a control. Western blot analysis of these immunoprecipitants showed that the inclusion of SDS significantly increased the amount of gliomedin detected in the lysates of ascorbic acid–treated cells, but had no effect on the immunorecovery of gliomedin from untreated cells. A large amount of gliomedin was detected in the insoluble material obtained after the extraction of ascorbic acid–treated cells, with the modified RIPA buffer lacking SDS (Fig. 4 D, bottom), whereas the addition of SDS to the lysis buffer significantly reduced the amount of gliomedin in the insoluble pellet.

To ascertain that gliomedin was incorporated into Schwann cell ECM, cultures of Schwann cells grown in the presence of ascorbic acid were washed with ammonium hydroxide and Triton X-100 to remove the cells, and then immunolabeled using antibodies to gliomedin and laminin (Fig. 4, E–G). In non-treated cultures, gliomedin immunoreactivity was detected on the surface of the cells, as well as in fibrillar structures that were also labeled for laminin (top). After cell removal, most of the gliomedin immunoreactivity was associated with laminin-positive fibrils, further supporting the conclusion that it is incorporated into the ECM network. Notably, the extracellular domain of neurofascin (NF155-Fc; NF155 and NF186 are used interchangeably, as they both bind gliomedin) bound to ECM deposits that remained after Schwann cell removal, demonstrating that this axonal CAM still recognizes the ECM-incorporated multimers of gliomedin (Fig. 5 A). The binding of Fc-fusion proteins containing the extracellular domain of Necl1/SynCAM3, which is a CAM that interacts with Schwann cells, was abolished after the removal of the cells (Fig. 5 C), demonstrating that the ammonium hydroxide and Triton X-100 wash used did not create nonspecific Fc-binding sites on the slides. Finally, similar experiments done using rat astrocytes revealed that although these cells secrete large quantities of gliomedin to their culture medium (not depicted), NF155-Fc binding was only detected on the cell surface (Fig. 5 B), indicating that gliomedin was not incorporated into the ECM produced by these cells. This conclusion is further supported by the observation that the extracellular domain of gliomedin did not bind astrocyte ECM, which suggests that Schwann cells produce a specific ECM ligand for gliomedin (Fig. 1 I). Collectively, these results demonstrate that upon ascorbic acid treatment, gliomedin is secreted.

![Figure 5. Binding of neurofascin to ECM-bound gliomedin.](image-url)
from the cells and forms high-molecular weight multimers that are entrapped in the Schwann cell ECM.

**Association of gliomedin with Schwann cell ECM is mediated by heparan sulfate proteoglycans**

At the perinodal space, gliomedin is concentrated on the Schwann cell microvilli (Eshed et al., 2005), which are embedded in the proteoglycan-rich perinodal matrix. To examine whether the association of gliomedin with Schwann cells ECM is mediated by heparan sulfate proteoglycans (HSPGs), we used NF155-Fc as a specific affinity reagent to detect gliomedin in ascorbate-treated Schwann cells that were incubated with heparin.

As depicted in Fig. 6 A, in the absence of heparin, gliomedin was present on both the cell surface and in ECM deposits between adjacent cells. In cultures incubated for 30 min with heparin, gliomedin was released from the ECM and was only present on the cell surface (Fig. 6 B). The removal of gliomedin from Schwann cell ECM by heparin was even clearer using cultures that were pretreated with ammonium hydroxide and Triton X-100 to remove the cells (Fig. 6, C and D). In contrast to gliomedin, heparin had no effect on the distribution of α4(V) (Fig. 6, E–J) or other Schwann cell ECM components, such as laminin (unpublished data). Immunolabeling with an antibody to the cytoplasmic domain of gliomedin indicated that, in heparin-treated Schwann cells, NF155-Fc bound to the transmembrane form of gliomedin present on the cell surface (Fig. 6, K–M).

The efficient removal of secreted gliomedin by heparin indicates that its association with Schwann cells ECM requires HSPGs.

**Discussion**

Gliomedin was isolated as a glial ligand for neurofascin and NrCAM, which are two immunoglobulin CAMs that associate with Na⁺ channels at the nodes of Ranvier (Eshed et al., 2005). It was shown that immunopatching the olfactomedin domain of
Gliomedin contains four basic amino acids RNKR at position 91–94 between the transmembrane and collagen domains that match the consensus site for furin, which is a proprotein convertase that cleaves after the C-terminal arginine residue in the sequence R-X-K/R-R (Thomas, 2002). Mutating the two arginines at position 91 and 94, resulted in a marked reduction in the secretion of gliomedin to the medium, suggesting that, similar to other collagenous transmembrane proteins, gliomedin is processed by a furinlike enzyme. Immunolabeling rat sciatic nerves using antibodies to different regions of the molecule demonstrated that the transmembrane form of gliomedin is completely absent from nodes, indicating that the processing of gliomedin also occurs in vivo. The finding that only the cleaved form of gliomedin is present at nodes is further supported by previous immunoelectron microscopy data showing that gliomedin immunoreactivity was also present between the microvilli at the nodal gap (Eshed et al., 2005). At present, it is not clear whether the furin-dependent processing of gliomedin occurs in the trans-Golgi, at the cell surface, or at both sites, as was recently demonstrated for collagen XIII (Vaisanen et al., 2006).

The N-terminal sequence of the smaller gliomedin fragment found in the medium of transfected HEK-293 cells unequivocally identified as DDTLVG, demonstrating that the second cleavage of gliomedin occurs between asparagine 277 (N277) and aspartic acid 278, which is located N-terminal to the olfactomedin domain. This sequence corresponds well to the known cleavage site of the bone morphogenetic protein/Tolloid-like metalloproteinases, which process several collagens, as well as a variety of other ECM proteins (Ge and Greenspan, 2006). However, it should be emphasized, that the major proteolytic product of gliomedin detected in Schwann cell medium was the 91-kD fragment containing both the collagen and the olfactomedin domains. In contrast, we detected only a small amount of the 45-kD olfactomedin fragment in Schwann cell media grown for long periods of time in culture, indicating that the second proteolytic cleavage is much less common. In support, we did not detect the 45-kD protein in the medium of astrocytes and we could not extract it from nerve lysates (unpublished data).

Multimers of gliomedin are required for its interaction with the axonal CAMs

Previous experiments have demonstrated that the olfactomedin domain of gliomedin mediates its interaction with neurofascin or NrCAM (Eshed et al., 2005). We extend these findings and note an important difference between the ability of the olfactomedin domain to bind neurofascin as a single domain (OLF), or as part of the entire extracellular domain of gliomedin (ECD). We show that although ECD-Fc could bind to neurofascin as a soluble dimer (dimers are enforced by the Fc tag), binding of OLF-Fc required its multimerization with a secondary antibody. The N-terminal sequence of the smaller gliomedin fragment found in the medium of transfected HEK-293 cells unequivocally identified as DDTLVG, demonstrating that the second cleavage of gliomedin occurs between asparagine 277 (N277) and aspartic acid 278, which is located N-terminal to the olfactomedin domain. This sequence corresponds well to the known cleavage site of the bone morphogenetic protein/Tolloid-like metalloproteinases, which process several collagens, as well as a variety of other ECM proteins (Ge and Greenspan, 2006). However, it should be emphasized, that the major proteolytic product of gliomedin detected in Schwann cell medium was the 91-kD fragment containing both the collagen and the olfactomedin domains. In contrast, we detected only a small amount of the 45-kD olfactomedin fragment in Schwann cell media grown for long periods of time in culture, indicating that the second proteolytic cleavage is much less common. In support, we did not detect the 45-kD protein in the medium of astrocytes and we could not extract it from nerve lysates (unpublished data).

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the collagen domain of gliomedin is resistant to digestion by trypsin and chemotrypsin (unpublished data), which are a common characteristic of collagen triple helices (Bruckner and Prockop, 1981). Second, using a chemical cross-linker (BS3), we demonstrated that gliomedin is found as trimers in Schwann cells treated with ascorbate, which is known to stimulate prolyl-
hydroxylation of collagens, which is required for the formation of intramolecular hydrogen bonds and stabilization of their triple helical conformation (Trackman, 2005). Furthermore, we also detected multimers of gliomedin, suggesting that the formed homotrimers may be further assembled into higher-order structures that are similar to various collagens (Myllyharju and Kivirikko, 2004). Third, and in keeping with the latter possibility, we found that treatment of Schwann cells with ascorbic acid markedly reduced the solubility of gliomedin. Finally, we show that gliomedin undergoes self-association, which is mediated by the N-terminal region of the protein and is required for its interaction with the axonal CAMs. Gliomedin contains a juxtamembrane α-helical, coiled-coil sequence at position R42-E61, which, in transmembrane collagens, directs correct chain association and initiates a zipperlike folding of the triple helix (Latvanlehto et al., 2003; McAlinden et al., 2003). Self-association of gliomedin was reduced by the deletion of this coiled-coil sequence, but was almost completely abolished when the entire N-terminal domain was deleted. This result indicates that although the juxtamembrane coiled-coil sequence may facilitate trimerization, adjacent noncollagenous sequences within the N-terminal domain are required for the recognition and association of gliomedin monomers. In keeping with such a role, we found that binding of a soluble extracellular region of gliomedin lacking the N-terminal domain (ECCdNTR-Fc) to neurofascin required its multimerization.

Incorporation of gliomedin into Schwann cell ECM
Previous immunofluorescence and immunoelectron microscopy analyses of peripheral nerves demonstrated that gliomedin is localized to the nodes of Ranvier (Eshed et al., 2005). The finding that gliomedin is a secreted protein, thus, poses an important question as to how it is concentrated at the nodal gap. Binding experiments using mixed Schwann cells/neuron cultures showed that a secreted extracellular domain of gliomedin binds to both cell types. Although the olfactomedin domain mediated its binding to neurons, the N-terminal and collagen domains of gliomedin were required for its interaction with Schwann cells, suggesting the existence of another ligand for gliomedin at the perinodal space. Using isolated rat Schwann cells, we demonstrated that the extracellular domain of gliomedin binds to Schwann cell ECM. Moreover, we found that endogenous gliomedin secreted from Schwann cells was incorporated into fibrillar matrix deposits that were labeled for collagen V, laminin, and perlecan. We also showed that a soluble extracellular domain of neurofascin strongly bound to these matrix deposits, indicating that the embedding of gliomedin within the ECM does not diminish its ability to interact with the axonal CAMs. These results suggest that gliomedin is a specific component of the Schwann cell perinodal matrix, which was originally described by Ranvier as the cement disc (Landon and Hall, 1976). This conclusion is further supported by the recent identification of gliomedin as an ECM protein synthesized by follicular papilla, which is a group of specialized mesenchymal cells in the hair follicle (Cao et al., 2005). It is also of interest to note that all of the known olfactomedin-containing proteins have been identified as ECM proteins (Snyder et al., 1991; Bal and Anholt, 1993; Yokoe and Anholt, 1993; Hillier and Vacquier, 2003; Zeng et al., 2004; Ando et al., 2005; Furutani et al., 2005), which, similarly to gliomedin, have restricted tissue distributions and thus may comprise a unique subgroup of ECM elements as recently suggested (Liu et al., 2006).

The accurate deposition of many ECM proteins is often regulated by their binding to HSPGs (Bernfield et al., 1999). We showed that the binding of exogenous gliomedin to Schwann cells was inhibited by heparin, suggesting that it binds HSPGs. In agreement, incubation of ascorbate-treated Schwann cells with heparin completely removed gliomedin from the ECM. We also showed that the extracellular domain of gliomedin directly bound to heparin–Sepharose through its collagen domain. Consistent with this observation are two putative heparin-binding sequences located in the collagen-like domain of gliomedin (amino acid positions 176 and 253), both composed of a BBXB motif (where B represents a basic residue). Several HSPGs were shown to be expressed by Schwann cells, including syndecans (Carey et al., 1992), perlecan and glypican (Rothblum et al., 2004), agrin (Yang et al., 2001), and collagen XVIII (Halttunen et al., 1998), of which only syndecans were shown to be present at the nodes of Ranvier (Goutebroze et al., 2003; Melendez-Vasquez et al., 2005). Whether gliomedin interacts with any of these HSPGs, and whether such interaction is important for its localization at nascent nodes, is of great interest and will be determined in future studies.
Implications on nodes formation

The accumulation of Na\(^+\) channels at the nodes of Ranvier in the PNS depends on the presence of myelinating Schwann cells and requires the interaction of these channels with NrCAM and neurofascin, as well as with Blot spectrin and the cytoskeletal adaptor protein ankyrin G (Kordeli et al., 1995; Berghs et al., 2000; Malhotra et al., 2000; Ratcliffe et al., 2001; Komada and Soriano, 2002; Custer et al., 2003; Lemailliet al., 2003; Sherman et al., 2005). It was previously suggested that these channels are being recruited by ankyrin G to the axonal CAMs, which were first clustered by gliomedin present on glial processes that contact the nodal axolemma (Eshed et al., 2005; Schafers and Rashband, 2006). Based on our current results demonstrating that gliomedin is a secreted protein, one should consider a modification of this model. Thus, we propose that during the development of peripheral nerves, gliomedin is released from the cell surface by proteolytic cleavage and is then entrapped at the edges of myelinating Schwann cells by HSPGs that are enriched at these sites. Binding of gliomedin to HSPGs may serve two purposes. First, it would allow the buildup of a tremendously high local concentration of gliomedin at the nodal gap, and second, it would create a meshlike array of gliomedin that would facilitate high avidity binding and further clustering of neurofascin and NrCAM present on the axonema. Heparan sulfate glycosaminoglycan residue could be provided by transmembrane syndecans that are present on microvilli processes, as well as by secreted HSPGs that are found at the basal lamina overlaying the nodal gap. In keeping with the latter possibility, it has recently been demonstrated that the composition of the basal lamina at the nodes is different from that of the internodal region and contains specific ECM components, such as laminin α5β1γ2 (Occhi et al., 2005). Alternatively, gliomedin may interact with secreted HSPGs, such as perlecain, which would be immobilized at the nodal gap by dystroglycan that is present on the Schwann cell microvilli and is required for the normal clustering of nodal Na\(^+\) channels (Saito et al., 2003; Occhi et al., 2005). Once accumulated at the nascent nodes, gliomedin multimers would avidly bind to and induce the clustering of the axonal CAMs, thereby facilitating the assembly of nodal complexes containing Na\(^+\) channels.

Materials and methods

Tissue culture methods

Dissociated rat DRG cultures were grown in Neurobasal medium (Invitrogen) supplemented with 2% FCS and 50 ng/ml NGF (Neurobasal medium; Alomone Labs) for 5 d before being used in binding experiments. Purified DRG neurons were established by treating dissociated mixed cultures with two cycles (2 d each) of NB medium containing 10 \(\mu\)M uridine/10 \(\mu\)M 5-Fluoro 2-deoxyuridine (Sigma-Aldrich) to eliminate fibroblasts and Schwann cells. Schwann cells isolated from P4 rat sciatic nerve were grown in Schwann cell proliferation medium (DME, 3% FBS, 10% NDF, 5\% FCS, 20\% FCS, and 2 mM PMSF, and protease inhibitors) or in a 30-min incubation with the conditioned media at RT, cells were fixed and washed with 4% PFA for 5 min at RT. In the case of nonclustered binding, coverslips were incubated with a Cy3-conjugated anti-human Fc antibody after fixation for 30 min at RT. For antibody labeling, cells were fixed in 4% PFA, washed with PBS, and incubated in blocking solution (PBS, 10% normal goat serum, 0.1% Triton X-100, 1% glycine) for 30 min. Primary antibodies diluted in blocking solution were added for 1 h at RT, followed by washing with PBS and incubation with secondary antibodies diluted in blocking solution for 40 min. Coverslips were then washed, mounted in elvanol, and analyzed on a microscope (Eclipse E1000; Nikon; objective 60\% /1.4 NA) equipped with a camera (ORCA-ER; Hamamatsu). Fluorescence images were acquired using Openlab software (Improvision) and figures were mounted using PhotoShop software (Adobe). In the case of laminin and collagen V staining, live cultures were incubated with primary antibodies for 45 min, followed by washing and fixing. Secondary antibodies were added after a 30-min blocking as described for nonclustered cells. To assess the effect of heparin on the gliomedin labeling, Fc fusion binding or antibody labeling were performed after a 30-min treatment with 10 \(\mu\)g/ml heparin in PBS supplemented with Ca\(^{2+}\) and Mg\(^{2+}\). Teased sciatic nerves were prepared and immunolabeled as previously described (Eshed et al., 2005).

Immunoprecipitation, pull-down, and immunoblot analysis

For immunoprecipitation, Schwann cells or transfected cell--conditioned media and cell extracts (lysed in 50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM MgCl\(_2\), 10% glycerol, 0.5 mM EDTA, 2 mM PMSF, and 0.5 mM Pefabloc) were either mixed with a Cy3-conjugated anti–human Fc antibody for 30 min before the binding procedure or were not mixed (“nonclustered”). After a 30-min incubation of the cells with the conditioned media at RT, cells were fixed and washed with 4% PFA for 5 min at RT. In the case of nonclustered binding, coverslips were incubated with a Cy3-conjugated anti–human Fc antibody after fixation for 30 min at RT. For antibody labeling, cells were fixed in 4% PFA, washed with PBS, and incubated in blocking solution (PBS, 10% normal goat serum, 0.1% Triton X-100, 1% glycine) for 30 min. Primary antibodies diluted in blocking solution were added for 1 h at RT, followed by washing with PBS and incubation with secondary antibodies diluted in blocking solution for 40 min. Coverslips were then washed, mounted in elvanol, and analyzed on a microscope (Eclipse E1000; Nikon; objective 60\% /1.4 NA) equipped with a camera (ORCA-ER; Hamamatsu). Fluorescence images were acquired using Openlab software (Improvision) and figures were mounted using PhotoShop software (Adobe). In the case of laminin and collagen V staining, live cultures were incubated with primary antibodies for 45 min, followed by washing and fixing. Secondary antibodies were added after a 30-min blocking as described for nonclustered cells. To assess the effect of heparin on the gliomedin labeling, Fc fusion binding or antibody labeling were performed after a 30-min treatment with 10 \(\mu\)g/ml heparin in PBS supplemented with Ca\(^{2+}\) and Mg\(^{2+}\). Teased sciatic nerves were prepared and immunolabeled as previously described (Eshed et al., 2005).

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GLIOMEDIN IS A PERINODAL MATRIX COMPONENT • ESHED ET AL. 561

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