Fibronectin Type III-like Domains of Neurofascin-186 Protein Mediate Gliomedin Binding and Its Clustering at the Developing Nodes of Ranvier*

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The cell adhesion molecules (CAMs) of the immunoglobulin superfamily (Ig-CAMs) play a crucial role in the organization of the node of Ranvier in myelinated axons. In the peripheral nervous system, Gliomedin (Gldn) secreted by Schwann cell microvilli binds NgCAM-related CAM (NrCAM) and Neurofascin-186 (NF186) and direct the nodal clustering of voltage-gated sodium channels (Nav). NF186 is the single axonal Gldn partner to ensure Nav clustering at nodes, whereas NrCAM is only required in glial cells (Feinberg, K., Eshed-Eisenbach, Y., Frechter, S., Amor, V., Salomon, D., Sabanay, H., Dupree, J. L., Grumet, M., Brophy, P. J., Shrager, P., and Peles, E. (2010) Neuron 65, 490–502). The olfactomedin domain of Gldn is implicated in the interaction with nodal Ig-CAMs. However, the interacting modules of NrCAM or NF186 involved in Gldn association are unknown. Here, we report that fibronectin type III-like (FnIII) domains of both Ig-CAMs mediate their interaction with Gldn in pulldown and cell binding assays. Using surface plasmon resonance assays, we determined that NrCAM and NF186 display similar affinity constant for their association with Gldn (Kd of 0.9 and 5.7 nM, respectively). We characterized the FnIII domains 1 and 2 of NF186 as interacting modules that ensure association with Gldn. We found that the soluble FnIII domains of NF186 (FnIII-Fc) bind on Schwann cells and inhibit Gldn and Nav clustering at heminodes, the precursors of mature nodes in myelinating cultures. Our study reveals the unexpected importance of FnIII domains of Ig-CAMs in the organization of nodes of Ranvier in peripheral axons. Thus, NF186 utilizes distinct modules to organize the multimeric nodal complex.

The organization of the node of Ranvier is essential for proper nerve conduction along myelinating fibers. The axonal subdomains, which are formed by contacts with myelinating glial cells, include the nodal gap, the flanking paranodal junctions, the juxtaparanodes, and the internodes. Selective axoglial contacts, mediated by extracellular matrix components and cell adhesion molecules (CAMs),2 direct the assembly of the nodal complex and are responsible for voltage-gated sodium channel (Nav) and potassium Kv7 channel clustering (2–4). In peripheral axons, two cell adhesion molecules of the immunoglobulin superfamily (Ig-CAMs), the axonal 186-kDa isoform of Neurofascin (NF186) and NrCAM, first accumulate at high density and precede the Nav clustering at heminodes, which will fuse to form the mature node (5, 6). NF186 and NrCAM interact with the olfactomedin domain of Gliomedin (Gldn), a N-glycosylated trimeric molecule secreted by Schwann cells (SCs) and incorporated into the extracellular matrix (7–9). It has recently emerged that axo-glial antigens, especially Neurofascin (NF), are specific immune targets in inflammatory demyelinating diseases (10, 11). Autoantibodies to NF and Gldn have been identified in experimental allergic neuritis, a model of Guillain-Barré syndrome (12). In this model, antibody-mediated alteration of NF186 and Gldn precedes disruption of Nav clusters and paranodal retraction/de-myelination pointing to a crucial role of this adhesive complex for nodal integrity.

Several genetic studies analyzed the role of NF186 and Gldn and provided new insights into the mechanisms underlying nodal formation (1, 13, 14). Using conditional genetic ablation, Bhat and co-workers demonstrated recently that NF186 is required for nodal assembly (14). Using combined dorsal root ganglia (DRG) neurons/SCs co-cultures from different knockout animal models, Peles and co-workers found that NF186 is the single axonal partner of Gldn to ensure Nav clustering at nodes (1). Interestingly, NrCAM is only required in glial cells and not on the axonal side, to influence Gldn clustering. In Gldn-null mice, Nav clustering is affected during development at heminodes but not in mature nodes, even if SC microvilli are

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disorganized (1). Thus, it seems that NF186 may interact with other partners at the node that may compensate for the loss of Gldn or that paranodes may be sufficient to restrict Nav at mature nodes.

At paranodes, the glial isoform of NF, NF155, influences the formation of septate-like junctions through its association with the axonal Caspr/Contactin complex (15, 16). Using different genetic models, several studies demonstrated the distinct roles of glial NF155 and neuronal NF186 in the formation of the paranodes and nodes, respectively (13, 14, 17, 18). These two isoforms contain distinct domains due to alternative splicing: i.e. NF186 encompasses a juxta-membrane mucin-like domain and NF155 an additional Fibronectin type III-like (FnIII) domain. However, the two NF splice variants both share the ability to bind multiple ligands including Gldn and Contactin. Therefore, it is of importance to determine whether NF utilizes distinct modules to organize and stabilize specific axonal sub-domains of myelinated fibers. The mucin-like domain specific for NF186 is not implicated in Gldn binding, and no functional activity has been reported for this domain (19). Recently, we showed that the Ig5–6 domains of NF bind Contactin and are required for the formation of paranodal junctions whereas they are dispensable for Gldn binding and nodal assembly (20).

In the present paper, we show that the FnIII domains of both NrCAM and NF186 are implicated in Gldn binding. Our data indicate that a module composed of FnIII domains 1 and 2 of NF186 mediates interaction with Gldn. Moreover, functional analysis using myelinating cultures of DRG neurons indicates that treatment with soluble FnIII domains of NF186 prevents the clustering of Gldn at heminodes, as observed with full-length NF186.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The Contactin, Contactin-Fc, NF155-Fc, NrCAM-Fc, and GFP-tagged NrCAMΔFnIII (deletion of amino acids 608–998) constructs have been described previously (15, 21). The Gldn-Fc construct was a gift from Dr. E. Peles (The Weizmann Institute, Israel). HA-tagged NF186 was from Dr. V. Bennett (Duke University, Durham, NC), and NF186-Fc was from Dr. M. Grumet (Rutgers University, New Brunswick, NJ). Rat Gliomedin (NM_181382.2) was amplified by PCR from a rat sciatic nerve cDNA library and subcloned into pcDNA3 at KpnI-EcoRV sites with Myc epitope inserted at the C terminus. The HA-tagged NF186 deleted for the six Ig domains (amino acids 33–584; HA-NF186ΔIg), the Ig5–6 domains (amino acids 385–584; HA-NF186ΔIg5–6), the three FnIII domains (amino acids 613–894; HA-NF186ΔFnIII), and the Ig5–6 and FnIII domains (amino acids 385–894; HA-NF186ΔIg5–6ΔFnIII) were generated by QuikChange mutagenesis (Agilent Technologies). The FnIII domains-Fc construct (FnIII1,2,4-Fc) was generated by PCR amplification of the sequence coding for the signal peptide, HA tag, and FnIII domains and insertion in the HindIII-NheI sites of pIgPlus. The FnIII domains-Fc constructs deleted for FnIII1 or FnIII4 (FnIII2,4-Fc and FnIII1,2-Fc) were generated by QuikChange mutagenesis (deletion of amino acids 613–696 and 810–894, respectively). GFP-NrCAM in pEGFP-C1 was generated by PCR amplification of the four FnIII domains (amino acids 632–1022) of NrCAM12 and Agel insertion in NrCAMΔFnIII. All mutant constructs were verified by sequencing (Beckman Coulter Genomics).

**Antibodies**—The rabbit anti-Caspr antibody (antiserum SL51) was described previously (22). The TRITC-conjugated goat anti-human Fc and the mouse anti-pan-Nav and mouse anti-Brevican mAbs were purchased from Sigma. The mouse anti-myelin associated glycoprotein (MAG) mAb was from Millipore. The rabbit anti-Gldn, the goat anti-GFP, and the rat anti-myelin basic protein (MBP) antibodies were from Abcam, and the rat anti-HA mAb was from Roche Diagnostics. The HRP-conjugated secondary antibodies were from Jackson ImmunoResearch and Alexa Fluor-conjugated secondary antibodies were from Invitrogen.

**Cell Culture**—N2a and HEK293 cells grown in DMEM containing 10% FCS (Invitrogen) were transiently transfected using jetPEI (Ozyme). Dissociated DRG neurons and SCs mixed cultures were prepared from embryonic day 16 Wistar rats. All animal procedures were carried out according to the animal care and experimentation committee rules approved by the CNRS. DRG were mechanically dissociated with 0.25% trypsin (Invitrogen) diluted in L-15 medium and plated at densities of 100,000 cells on Matrigel-coated 18-mm glass coverslips (BD Biosciences). Mixed cultures were maintained for 7 days in Neurobasal medium supplemented with 2% B-27, 1% penicillin-streptomycin, 0.3% glutamine (Invitrogen), and 100 ng/ml NGF (Euromedex). Myelin formation was induced by adding 50 μg/ml L-ascorbic acid (Euromedex) for an additional period of 10–18 days (23).

**Soluble Fc Binding and Immunostaining**—All the Fc chimera were purified from the supernatant of transfected HEK293 cells and used as described previously for binding experiments on transfected N2a cells (15, 22). Fc binding was also performed on mixed cultures of DRG neurons and SCs 10–14 days after myelination induction. After washing and fixation with cold methanol, cells were incubated in blocking solution (5% normal donkey serum in PBS buffer) for 1 h and incubated with rabbit anti-Gldn antibody (1:100) or rat anti-HA (1:200) and then with secondary antibodies. After washing in PBS, cells were mounted in Mowiol (Calbiochem) and examined using an ApoTome AxioObserver Z1 inverted microscope under the control of Axiovision software (Carl Zeiss MicroImaging GmbH).

**Soluble Fc Perturbation of Myelination**—Purified Fc-chimera (5 μg/ml) were added to DRG neurons/SCs mixed cultures at the time of myelin induction (day 7) and replenished every 2 days. After 14–18 days, cultures were fixed in cold methanol and immunostained for MAG and Gldn, or for MBP, Nav, Caspr, or Gldn. Confocal image acquisition was performed on a TCS SP2 laser-scanning microscope equipped with 63×/1.32 N.A. oil immersion objective (Leica Microsystems). Fluorescence images were collected automatically as frame-by-frame sequential series, with an average of three frame scans. Maximal projections of 10–15 Z-stacks were produced for image analysis.

**Pulldown Assay and Immunoblotting**—HEK293 cells transiently transfected with HA-NF186, HA-NF186ΔFnIII, HA-NF186ΔIg, GFP-NrCAM, or GFP-NrCAMΔFnIII, were homogenized on ice in lysis buffer containing 1% Nonidet P-40

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and 1% Triton X-100 as described previously (15). NrCAM binding on Glnd-Fc was analyzed by pulldown assays using protein A-Sepharose as described previously (20). For NF186 binding assays, solubilized proteins (1.5 mg per condition) were incubated with Glnd-Fc-chimera (10–50 μg) covalently immobilized onto activated agarose beads using the Pierce co-immunoprecipitation kit (Thermo Scientific). Recombinant human Brevican (40 nM) (R&D Systems) was used for binding assays with Glnd-Fc, NF186-Fc, or NF186ΔFnIII-Fc linked to agarose beads. Inactivated agarose beads incubated with Glnd-Fc were used as negative control. Proteins retained by affinity were analyzed by SDS-PAGE and Western blotting. Immunodetection was performed with primary antibodies (anti-Gldn, 1:1,000; anti-HA, 1:1,000; anti-Fc, 1:1,000; anti-GFP, 1:1,000; anti-Brevican 1:1,000), HRP-conjugated secondary antibodies (1:5,000), and chemiluminescence method (Roche Diagnostics) using ChemiGenius 2XE (Syngene).

Surface Plasmon Resonance—Surface plasmon resonance (SPR) was performed on a Biacore 3000 apparatus (GE Healthcare) at 25 °C with PBS as a running buffer. Purified Fc-chimeras were coupled to CM5 sensor chips using standard amine coupling chemistry (approximately 300–600 resonance units for NF186-Fc or NF186ΔFnIII-Fc, and 700–1200 resonance units for NrCAM-Fc and FnIII1,2,4-Fc). The first flow cell was subjected to the chemical step of coupling to serve as an internal control. Alternatively, Fc alone was coupled to the sensor chip as a negative control. The single-cycle kinetic method was used to measure the affinity of Glnd with Ig-CAMs as described previously (24). Glnd-Fc was serially diluted 2-fold in running buffer yielding concentrations ranging from 2.5 to 40 nM and samples injected sequentially at 30 μl/min in increasing concentrations over both the ligand and the reference surfaces. Injections were carried out using Kinject command with an association time of 120 s and a dissociation time of 60 s except for the last injection where the dissociation time was extended to 10 min. Blank run injections of PBS were performed in the same conditions before Glnd-Fc injections. Subtracted sensorgrams were globally fitted with the 1:1 titration kinetic binding model from Biaevaluation 4.1 software.

RESULTS

FnIII Domains of NrCAM Are Required for Interaction with Glnd—Previous studies demonstrated that the olfactomedin domain of Glnd mediates its interaction with NF and NrCAM (7, 8). To dissect the basis for Ig-CAMs/Glnd association further, we investigated whether the Ig or FnIII domains of NrCAM may be critical for these interactions. HEK293 cells were transfected with GFP-NrCAM or GFP-NrCAMΔFnIII, which is deleted of the four FnIII domains. Cell extracts (Fig. 1A, Input) were incubated with Glnd-Fc immobilized onto protein A-Sepharose beads. GFP-NrCAM strongly precipitated with Glnd-Fc and not with Fc used as negative control (Fig. 1A). In contrast, GFP-NrCAMΔFnIII did not precipitate with Glnd-Fc (Fig. 1A). Next, Glnd-Fc binding assays were performed on N2a cells transiently transfected with GFP-NrCAM or GFP-NrCAMΔFnIII (Fig. 1B). NrCAM surface expression was not modified by deletion of FnIII repeats as illustrated in Fig. 1, B and C, showing the fluorescence for GFP. Glnd-Fc strongly bound N2a cells transfected with GFP-NrCAM but not with GFP-NrCAMΔFnIII (Fig. 1B) in agreement with the results obtained in pulldown experiments. In contrast, deletion of FnIII domains of NrCAM had no effect on its association with Contactin (Fig. 1C).

FnIII Domains of NF186 Are Implicated in Interaction with Glnd—We then assessed whether the FnIII domains of NF186 may be implicated in its interaction with Glnd, as we observed for NrCAM. Recently, we showed that the Ig5–6 domains are not required for the interaction between NF186 and Glnd (20). We generated mutant forms of NF186 with either deletion of the three FnIII domains (HA-NF186ΔFnIII) or deletion of the six Ig domains (HA-NF186ΔIg). HA-NF186, HA-NF186ΔFnIII, and HA-NF186ΔIg were detected on immunoblots with an apparent molecular mass higher than the predicted one, likely due to glycosylation (Fig. 2A, Input). We performed pulldown assays by incubating extracts from HEK293 cells transfected with the different forms of NF186 with Glnd-Fc covalently immobilized onto activated agarose beads. Inactivated agarose beads incubated with Glnd-Fc were used as control (Fig. 2A, CTL). As shown in Fig. 2A, HA-NF186 precipitated with Glnd-Fc (lane 2) and not with control agarose beads (lane 1). Both HA-NF186ΔFnIII (lane 3) and HA-NF186ΔIg (lane 4) precipitated with Glnd-Fc. HA-NF186ΔFnIII precipitated with Glnd-Fc was detected as a doublet (lane 3). The lower band indicated with an asterisk was strongly enriched in the precipitate compared with total extract and may result from proteolytic cleavage (25). The mucin-like domain is not implicated in Glnd binding (7, 19, 20). Thus, we can hypothesize that two binding sites may be involved in interaction with Glnd in regard to pulldown experiments, one involving the FnIII repeats and the other the Ig domains. By comparison with NrCAM in which the FnIII repeats were required for Glnd binding, it seems that NF186 may bind Glnd in a more complex way. Because the proteoglycan Brevican has been described to bind NF186, but not NrCAM (26), it might be implicated in mediating complex association between NF186 and Glnd. We used recombinant human Brevican (40 nM) in pulldown experiments with Fc-chimeras (Fig. 2B). Brevican was precipitated with beads covalently linked with Glnd-Fc (lane 2), NF186-Fc (lane 3), and NF186ΔFnIII-Fc (lane 4) but not with control beads (lane 1). However, we were not able to detect Brevican in HEK293 cell extracts suggesting that other proteoglycans might be implicated.

As a complementary approach, Glnd-Fc binding assays were performed on N2a cells transiently transfected with HA-NF186, HA-NF186ΔFnIII, or HA-NF186ΔIg (Fig. 2D). All NF186 mutants were detected at the cell surface as illustrated in Fig. 2C by immunofluorescence staining of the extracellular HA epitope. Glnd-Fc strongly bound N2a cells transfected with either HA-NF186 or HA-NF186ΔIg. In contrast, Glnd-Fc did not bind cells expressing NF186ΔFnIII by opposition with observations made in pulldown experiments (Fig. 2D). Deletion of the FnIII domains of NF186 should not affect its interaction with Contactin, which only requires the Ig domains (27). As expected, Contactin-Fc strongly bound N2a cells expressing HA-NF186ΔFnIII and did not bind cells expressing HA-NF186ΔIg (Fig. 2E).
Ig1–4 Domains of NF186 Mediate Its Interaction with NrCAM—In addition, we performed binding assays using NrCAM-Fc on N2a cells transfected with HA-NF186 constructs. NrCAM-Fc strongly bound to NF186 (Fig. 3) as reported in Lustig et al. (6). Previous studies in the chick indicated that NrCAM interacts with NF via its Ig domains (28). To map the modules of interaction in the rat, we analyzed NrCAM-Fc binding on HA-NF186 deleted from the six Ig, the Ig5–6 or the Ig5–6, and FnIII domains. All of the constructs were detected at the cell surface of N2a transfected cells using anti-HA immunostaining (Fig. 3). Deletion of the Ig5–6 and FnIII domains of NF186 had no effect whereas total deletion of the Ig domains prevented NrCAM-Fc binding (Fig. 3). Thus, we mapped the Ig1–4 domains of NF186 as implicated in its interaction with NrCAM.

FnIII Domains of NF186 Are Sufficient for Gldn Binding—To determine whether FnIII domains may be not only critical, but sufficient for interaction with Gldn, we generated Fc fusion proteins for the ectodomain of NF186 (NF186-Fc), the ectodomain of NF186 deleted for the FnIII domains (NF186ΔFnIII-Fc), and a Fc fusion only containing the three FnIII domains of NF186 (FnIII1,2,4-Fc). All NF186-Fc fusion proteins produced in HEK293 cell supernatant were detected by immunoblotting with an apparent molecular mass higher than expected due to N-glycosylation (Fig. 4A). As illustrated in Fig. 4B, FnIII1,2,4-Fc protein strongly bound N2a cells expressing the transmembrane form of Gldn as did NF186-Fc. The FnIII1,2,4-Fc did not bind N2a cells expressing Contactin (Fig. 4C). This result indicates that the FnIII domains of NF186 are sufficient for Gldn binding. In contrast, NF186ΔFnIII-Fc was unable to bind Gldn expressed on N2a cell surface (Fig. 4B) whereas it bound Contactin (Fig. 4C). To precisely define the modules of NF186 implicated in Gldn interaction, we generated Fc fusion proteins fused with FnIII1,2 or FnIII2,4 domains. Deletion of FnIII1 abolished binding on Gldn-expressing N2a cells, whereas deletion of FnIII4 had no effect (Fig. 4B). Thus, the FnIII domains 1 and 2 of NF186 are sufficient for Gldn binding.
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**FIGURE 2.** FnIII repeats of NF186 are implicated in its interaction with Gldn. A, extracts (Input) from HEK293 cells were transfected with HA-NF186 (lanes 1 and 2), HA-NF186ΔFnIII (lane 3), or HA-NF186ΔIg (lane 4). HA-NF186 and both HA-NF186ΔFnIII and HA-NF186ΔIg were precipitated with Gldn-Fc but not with control (CTL) beads. The red asterisks indicate a low band of HA-NF186ΔFnIII enriched after precipitation with Gldn-Fc (lane 3). B, recombinant Brevican (Bcan) was precipitated with Gldn-Fc (lane 2), NF186-Fc (lane 3), or NF186ΔFnIII-Fc (lane 4) but not with control (lane 1) beads. C–E, N2a cells transfected with HA-NF186, HA-NF186ΔFnIII, or HA-NF186ΔIg were surface-labeled for HA (C) or incubated with Gldn-Fc (D) or Contactin-Fc (E) and TRITC-conjugated anti-Fc antibodies (red). Deletion of the FnIII domains prevented Gldn-Fc binding, but the deletion of the Ig5–6 or FnIII domains had no effect. All images were obtained using identical microscopic settings. The data illustrated are representative of three independent experiments. Scale bar, 20 μm.

**SPR Measurements of Gldn Interaction with Ig-CAMs**—The direct interaction of the FnIII domains of NF186 with Gldn was confirmed by SPR experiments. As shown in Fig. 5A, Gldn-Fc bound to FnIII1,2,4-Fc and NF186-Fc immobilized on the sensor chip but not to NF186ΔFnIII-Fc. No interaction was revealed even at high concentrations of Gldn-Fc (800 nM) over immobilized NF186ΔFnIII-Fc (data not shown), in accordance with our observations in cell binding assays. The affinity of NF186 and FnIII domain recombinant proteins was measured by injecting increasing concentrations of Gldn-Fc over immobilized NF186-Fc or FnIII1,2,4-Fc (Fig. 5B). Binding analysis indicated that NF186-Fc and FnIII1,2,4-Fc strongly associated with Gldn-Fc with an apparent affinity constant ($K_D$) of 5.7 ± 0.8 nM ($k_{on} = 1.2 \pm 0.3 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 7.2 \pm 2.7 \times 10^{-4} \text{s}^{-1}$) and 2.2 ± 1 nM ($k_{on} = 1 \pm 0.1 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 2.2 \pm 1.4 \times 10^{-4} \text{s}^{-1}$), respectively ($n = 3$, Fig. 5B). Altogether, these data indicate that the in vitro interaction of NF186 with Gldn is mediated by its FnIII domains.

Next, we compared the binding affinity of NF186 and NrCAM for Gldn. NrCAM-Fc associated with Gldn-Fc with a $K_D$ of 0.9 ± 0.3 nM ($k_{on} = 3 \pm 0.8 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, $k_{off} = 3.1 \pm 1.6 \times 10^{-4} \text{s}^{-1}$) ($n = 3$, Fig. 5B). Thus, NF186-Fc and NrCAM-Fc affinities for Gldn-Fc are in the low nanomolar range with NrCAM-Fc exhibiting a six times greater affinity than NF186-Fc.

**Soluble FnIII Domains of NF186 Inhibit Gldn Clustering at Heminodes in Myelinating Culture**—To assess the functional relevance of the interaction between the FnIII domains of NF186 and Gldn, we investigated the ability of the NF186-Fc deletion mutants to bind Gldn endogenously expressed by SCs. Binding assays were performed using myelinating cultures of DRG neurons 10–14 days after myelin induction. We detected NF186-Fc binding on SCs positive for Gldn and no binding with NF186ΔFnIII-Fc at a concentration of 50 μg/ml (Fig. 6A). FnIII1,2,4-Fc and FnIII1,2-Fc strongly bound SCs in co-clusters with Gldn whereas FnIII2,4-Fc (all at 10 μg/ml) did not (Fig. 6A). To study the importance of the FnIII domains of NF186 in Gldn clustering during myelination, perturbation experiments were performed using DRG neurons/SCs myelinating mixed cultures. NF186-Fc, FnIII1,2,4-Fc, or Fc were used as control (all tested at 5 μg/ml) were added in the culture medium at the

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onset of myelin induction. After 18 days, cells were immunostained with antibodies against Gldn and MAG (Fig. 6B) or MBP (Fig. 6D) as markers of myelin, and the presence of Gldn clusters was evaluated at the sides of myelinated segments under the various conditions. MAG immunostaining enabled us to localize the sites of nodes and heminodes that displayed Gldn clustering after treatment with control Fc. The percent-
percentage of heminodes with Gldn clustering was dramatically reduced in myelinating cultures treated with FnIII1,2,4-Fc compared with control Fc-treated cultures (50/1006 5.3 versus 98.7/1006 1.3%, Fig. 6C). This alteration of Gldn clustering in the presence of FnIII1,2,4-Fc was similar to that induced with NF186-Fc (53.1/1006 7.4%, Fig. 6C). In addition, the recruitment of Nav was not detected at the side of myelinated segments when Gldn clustering was prevented (Fig. 6D). In contrast, immuno-

FIGURE 4. FnnIII domains of NF186 are sufficient for its association with Gldn. A, supernatants from HEK293 cells transfected with NF186-Fc, NF186ΔFnnIII-Fc, FnnIII1,2,4-Fc, FnnIII2,4-Fc, or FnnIII1,2-Fc were analyzed by immunoblotting using anti-Fc antibody. The Fc chimeras migrated with an apparent molecular mass higher than expected (148, 94, 68, 59, and 59 kDa, respectively). B and C, N2a cells transfected with Gldn (B) or Contactin (C) were incubated with NF186-Fc, NF186ΔFnnIII-Fc, FnnIII1,2,4-Fc, FnnIII2,4-Fc, or FnnIII1,2-Fc and TRITC-conjugated anti-Fc antibodies (red). Deletion of the FnnIII domains prevented binding onto Gldn- but not Contactin-expressing cells. The FnnIII1,2 domains were sufficient for binding onto Gldn-expressing cells. All images were obtained using identical microscopic settings. Scale bar, 30 μm. The data illustrated are representative of three independent experiments.

FIGURE 5. SPR measurement of Gldn interaction with Ig-CAMs. A, Gldn-Fc (40 nM) was injected over immobilized FnnIII1,2,4-Fc, NF186-Fc, and NF186ΔFnnIII-Fc. B, a set of concentrations of Gldn-Fc (2.5–40 nM) was sequentially injected over immobilized NF186-Fc, FnnIII1,2,4-Fc, and NrCAM-Fc. The black traces represent the specific binding obtained after subtraction of a blank, run and the gray traces represent the fit of the data to a kinetic titration 1:1 interaction model. The data illustrated are representative of three inde-

DISCUSSION

During myelination of peripheral nerves, several adhesion-based mechanisms act in concert to allow Nav clustering and stabilization at the node of Ranvier, including the axo-glial contacts at the nodal gap and paranodes (1, 13, 14, 29). The two NF splice variants that are present at the nodes and paranodes may use distinct interacting modules for their association with their

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respective partners. We previously identified the Ig5–6 domains of glial NF155 as being selectively required for Contactin binding and paranodal junction formation (20). In the present study, we provide new insights into the molecular mechanisms underlying the interaction between the Ig-CAMs, NF186, and NrCAM, and their glial ligand Gldn. We found that NF186 and NrCAM both interact with Gldn via their extracellular Fn III domains with similar affinity constant. We demonstrated that Fn III repeats are functional interacting modules implicated in nodal organization because the soluble Fn III domains of NF186 prevented Gldn and Nav clustering at heminodes of myelinated sensory neurons in culture.

**Fn III Domains of NF186 and NrCAM Mediate Direct Interaction with Gldn**—Using cell binding and label-free SPR assays, we found that the Fn III domains of NF186 mediate its direct interaction with Gldn. Mutant deletion analysis indicated that the first Fn III domain is required for Gldn binding whereas the fourth domain is dispensable. Therefore, the Fn III,2 domains of NF186 may be the minimal modules involved in interaction with Gldn. Interestingly, the second Fn III domain of NF186 has been implicated in interaction with the β1 subunit of Nav, in addition to the first Ig domain (30). Hence, Fn III domains may be implicated in multiple interactions within the nodal complex. NrCAM is another Ig-CAM present at the node of Ranvier in the peripheral nervous system also known to interact with Gldn (5, 7, 8). Here, we show that NrCAM uses the same modules, the Fn III domains, to interact with Gldn. It must be noted that pair-wise comparison between Fn III domains of L1 family members indicates that the first Fn III domains of NF and NrCAM display the highest similarity (25). The consensus motif within the Fn III domains of NrCAM and NF186 involved in Gldn binding still remains to be precisely identified. Conversely, it has been reported that the same interacting domain of Gldn, the olfactomedin domain, interacts with both

![FIGURE 6. Soluble Fn III domains of NF186 inhibit Gldn and Nav clustering at heminodes in myelinating culture. A. DRG neurons/SCs mixed cultures were induced for myelination with ascorbic acid after 7 days in vitro. Ten days after myelin induction, cells were incubated with NF186-Fc or NF186ΔFn III-Fc (50 μg/ml), Fn III1,2,4-Fc, Fn III1,2-Fc, or Fn III2,4-Fc (10 μg/ml) preclustered with TRITC anti-Fc at 37 °C for 30 min. Cells were fixed and immunostained for Gldn, and nuclei were stained with Hoechst (blue). NF186-Fc, Fn III1,2,4-Fc, and Fn III1,2-Fc (red) co-clustered with Gldn expressed by SCs (green). B–D, DRG neurons/SCs mixed cultures were incubated from day 7 with control Fc, NF186-Fc, or Fn III1,2,4-Fc (5 μg/ml) for 18 days. B, double-staining for Gldn (green) and MAG (red) as a marker for myelinated segments. Maximal projection of 10–15 Z-stacks was obtained using identical confocal settings. Green and white arrowheads indicate heminodes with or without Gldn clustering, respectively. C, Gldn clustering at heminodes was quantified (250 internodes analyzed per condition in each set of culture). Means ± S.E. (error bars) of results obtained in four independent myelinating cultures are shown. ***, p < 0.001 compared with control Fc (ANOVA, followed by Mann-Whitney U test). D, triple staining for MBP (blue), Caspr or Gldn (red), and Nav (green). Note that Fn III1,2,4-Fc prevented the clustering of Gldn and Nav at heminodes but not at mature nodes. Scale bars, 10 μm in A and D, 20 μm in B.**
Ig-CAMs (8). We determined that NrCAM and NF186 bind Gldn with high affinity (apparent $K_d$ of 0.9 and 5.7 nM, respectively). Our data slightly differ with the report of Feinberg et al. (1), in which a mouse NrCAM-Fc was used in cell binding assays that apparently displays a lower affinity for Gldn than NF186-Fc. However, this construct only contains two FnIII domains (6). Worth noting, FnIII domains are frequently considered as a signal-transducing module. As already described among CAMs, it has been demonstrated that FnIII domains of Contactin are required for intracellular activation and modulation of tyrosine phosphorylation through raft-mediated association with Fyn kinase (31). It would be important to explore the intracellular pathways linked to Gldn binding on NrCAM and NF186 FnIII domains and whether it might regulate tyrosine phosphorylation of the cytoplasmic tail and binding of the scaffolding molecule ankyrin G.

Surprisingly, we detected an interaction between the Ig domains of NF186 and Gldn in pulldown assays. In contrast, the Ig domains of NrCAM do not allow any association with Gldn. It should be noted that soluble purified proteins are used in cell binding and SPR assays, in contrast with cell lysates in pulldown experiments. In this respect, we can hypothesize that the Ig domains of NF186 may associate with Gldn in an indirect manner via an intermediate protein only present in solubilized protein extracts, whereas FnIII repeats clearly promote direct binding. NF186 and Gldn have been reported to bind matrix-associated proteoglycans that may bring together the two proteins. The Ig domains of NF186 interact with the chondroitin sulfate proteoglycan Brevican, whereas NrCAM does not (26). On the other hand, Gldn has been described to bind heparan sulfate proteoglycan (8), and we show here that Gldn may also interact with Brevican.

**Soluble FnIII Domains of NF186 Prevent Formation of Heminodes during Myelination**—We found that the soluble FnIII domains of NF186 (FnIII-Fc) are able to bind SCs and are functionally active. Perturbation experiments indicate that FnIII-Fc significantly disrupts the clustering of Gldn and Nav at heminodes during myelination to the same extent as NF186-Fc (7, 19). The FnIII-Fc effect may result from blockade of the endogenous interactions between NF186 and Gldn in perturbing assays. However, the soluble FnIII domains of NF186 may also prevent the interaction between NrCAM and Gldn on the glial side (Fig. 7C). Our data are in apparent contradiction with the distinct roles of Ig and FnIII domains in the targeting of NF186 at the node of Ranvier as analyzed in nucleofected DRG myelinating cultures (32). These authors found that the Ig domains of NF186 are required for its targeting to nodes and heminodes, whereas deletion of FnIII domains induces a minor effect. Our biochemical analyses argue against a major role of Ig domains in promoting the interaction with Gldn. Alternatively, the Ig domains of NF186 may be implicated in heterophilic cis- or trans-interactions with NrCAM or in interaction with other ligands, such as proteoglycans as intermediate partners implicated in Gldn clustering. We showed previously that the Ig5–6 domains of NF186 are not required for Gldn binding and nodal organization as established in knock-in mice (20). The specific role of Ig1–4 and FnIII domains of NF186 has now to be evaluated in vivo by genetic approach (Fig. 7A).

**Model of Multimolecular Adhesive Complex at the Node of Ranvier**—It was demonstrated recently that deletion of axonal NF186 results in alterations of nodal organization in vivo in both the central and peripheral nervous systems (14). Similarly, Feinberg et al. (1) found that the initial clustering of Gldn at heminodes requires axonal NF186 in myelinating culture of DRG. In contrast, axonal NrCAM is dispensable whereas secreted glial NrCAM seems to play a critical role and has been proposed to increase the affinity of Gldn for its association with NF186 (1). These data do not rule out the hypothesis that the glial cis-interaction between Gldn and NrCAM may facilitate the heterophilic binding of glial NrCAM to axonal NF186. Our cell binding assays indicate that soluble NrCAM interacts strongly with NF186 expressed at the cell membrane, even without pre clustering. Because Gldn is secreted as trimers (8, 9), formation of a cis-complex between NrCAM and Gldn may...
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consist in a preclustering step, increasing avidity for axonal NF186 (Fig. 7B). NrCAM exhibits high affinity for Gldn (which is even higher than the affinity of NF186 for Gldn), so that we could consider that NrCAM may stably associate in cis with Gldn through its FnIII domains on the glial side. This complex in turn might promote multimeric association, first via Gldn binding on NF186 FnIII domains and second, via NrCAM interacting with NF186 Ig1–4 domains (Fig. 7B). The model proposed in Fig. 7B does not take into account the Ig-CAM conformation, especially the accessibility of FnIII domains for trans-partners. Interestingly, it has been proposed that the Ig and FnIII domains of L1 may adopt a closed globular conformation. The authors postulated that ligation with Ig domains of L1 (e.g. L1-L1) may result in a permissive open conformation that would favor L1 clustering and subsequent integrin recruitment via FnIII domains (33, 34). The NF186 structure has been resolved for Ig domains, which are organized in a typical horseshoe structure (35). We can hypothesize that a conformational change of Ig domains (e.g. after NrCAM-NF186 binding) may favor Gldn binding to NF186 FnIII domains. Further experiments will be required to elucidate the precise architecture of the nodal Gldn-NF186-NrCAM complex.

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