Neurofascin Is a Glial Receptor for the Paranodin/Caspr-Contactin Axonal Complex at the Axoglial Junction

Perrine Charles,[1,6] Steven Tait,[2,6]
Catherine Faivre-Sarrailh,[3] Gilles Barbin,[1]
Frank Gunn-Moore,[2]
Natalia Denisenko-Nehrbass,[4]
Anne-Marie Guennoc,[1] Jean-Antoine Girault,[4]
Peter J. Brophy,[2] and Catherine Lubetzki[1,5]
[1]INSERM U-495
"Biologie des Interactions Neurones/Glie"
UPMC
Hôpital de la Salpêtrière
75651 Paris Cedex 13
France
[2]Department of Preclinical Veterinary Sciences
University of Edinburgh
Edinburgh EH9 1RJ
United Kingdom
[3]CNRS UMR 6545
Institut de Biologie du Développement
13288 Marseille Cedex 9
France
[4]INSERM U-536
Institut du Fer à Moulin
17 Rue du Fer à Moulin
75005 Paris
France

Summary

In myelinated fibers of the vertebrate nervous system, glial-ensheathing cells interact with axons at specialized adhesive junctions, the paranodal septate-like junctions [1]. The axonal proteins paranodin/Caspr and contactin form a *cis* complex in the axolemma at the axoglial adhesion zone, and both are required to stabilize the junction [2, 3]. There has been intense speculation that an oligodendroglial isoform of the cell adhesion molecule neurofascin, NF155, expressed at the paranodal loop [4, 5] might be the glial receptor for the paranodin/Caspr-contactin complex, particularly since paranodin/Caspr and NF155 colocalize to ectopic sites in the CNS of the dysmyelinated mouse *Shiverer* mutant [5]. We report that the extracellular domain of NF155 binds specifically to transfected cells expressing the paranodin/Caspr-contactin complex at the cell surface. This region of NF155 also binds the paranodin/Caspr-contactin complex from brain lysates in vitro. In support of the functional significance of this interaction, NF155 antibodies and the extracellular domain of NF155 inhibit myelination in myelinating cocultures, presumably by blocking the adhesive relationship between the axon and glial cell. These results demonstrate that the paranodin/Caspr-contactin complex interacts biochemically with NF155 and that this interaction is likely to be biologically relevant at the axoglial junction.

[5]Correspondence: catherine.lubetzki@psl.ap-hop-paris.fr
[6]These authors contributed equally to this work.

Results and Discussion

The Extracellular Domain of NF155 Binds Specifically to Transfected Cells Expressing Paranodin/Caspr and Contactin

A fusion protein in which the whole of the extracellular region of NF155 was fused to the Fc region of human IgG was generated. This NF155-Fc fusion protein was then used as a probe for the interaction with CHO cells transfected with either contactin alone or paranodin/Caspr with contactin. While transfection with contactin alone is sufficient for expression of the protein at the cell surface, association with contactin is necessary for delivering paranodin/Caspr to the plasma membrane [6].

Surface binding of NF155-Fc was clearly visible on live cells expressing both paranodin/Caspr and contactin (Figure 1A), but not on cells transfected with contactin alone (Figure 1B). NF155 is a member of the IgG superfamily, but no binding of an Fc fusion protein involving another family member, MUC-Fc [7], was detected on the doubly transfected cells (Figure 1C). Our results are in contrast with data reported by Volkmer et al. [8] showing a direct interaction of contactin with the Ig domains of all the neurofascin isoforms expressed at the membrane of transfected COS cells.

Biochemical Interaction between NF155 and the Paranodin/Caspr-Contactin Complex

In order to investigate the association between NF155 and the axonal complex further, the NF155-Fc fusion protein was immobilized on a protein A-agarose column and incubated with brain detergent lysate. After thorough washing, the column was eluted with a buffer containing 1 M NaCl. The presence of paranodin/Caspr in the high-salt eluate demonstrated that paranodin/Caspr had bound to the extracellular domain of NF155 and suggested that these proteins interact biochemically within a complex (Figure 2). Furthermore, contactin co-purified with paranodin/Caspr; hence, it would appear that the functional ligand for NF155 in the axolemma is the *cis* complex of paranodin/Caspr with contactin. Neither protein bound to the control human IgG column (Figure 2). The specificity of these interactions was indicated by the fact that NF155-Fc bound neither to the axonal isoform of neurofascin, NF186 [5], nor to the transmembrane glial protein myelin-associated glycoprotein (MAG), which is also found in the myelin paranodal domain, but not at the axoglial junction (Figure 2).

Blocking Interaction between NF155 and the Paranodin/Caspr-Contactin Complex Inhibits Myelination

Thus far, we have shown that NF155 can interact with the paranodin/Caspr-contactin complex on the surface of heterologous cells. We have also demonstrated that these proteins interact biochemically in vitro. Nevertheless, it was important to demonstrate that these associations were likely to be of functional significance in axon-
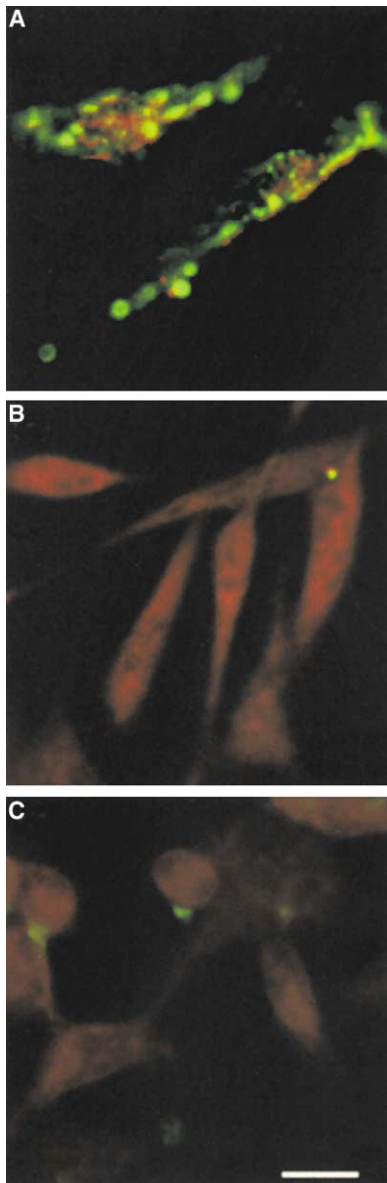
Figure 1. NF155 Interacts with the Paranodin/Caspr-Contactin Complex on Transfected Cells

(A) Binding of NF155-Fc using fluorescein-conjugated anti-Fc antibodies was detected on CHO cells expressing both paranodin/Caspr and contactin.
(B) Cells expressing contactin alone were not labeled.
(C) No binding of MUC-Fc to cells expressing paranodin/Caspr and contactin was observed.
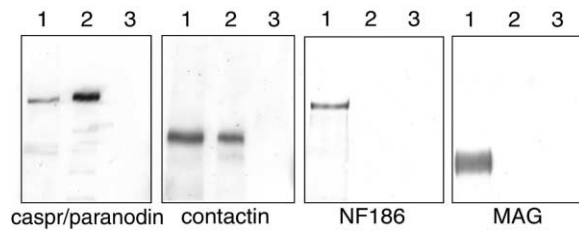The scale bar represents 20 μm.

Figure 2. Biochemical Interaction of NF155 with the Paranodin/Caspr-Contactin Complex In Vitro

NF155-Fc or human IgG linked to protein A-Sepharose was incubated with rat brain lysate, and bound proteins were eluted with 1 M NaCl. Caspr and contactin in the lysate (lane 1) were eluted from the NF155 column (lane 2), but not from the IgG column (lane 3). Neither the axonal isoform of neurofascin, NF186 [5], nor the transmembrane glial protein myelin-associated glycoprotein (MAG) bound to NF155-Fc or IgG.

internodes (p < 0.001) (Figure 3G). Support for the view that this inhibition is the result of interaction between NF155-Fc and the paranodin/Caspr-contactin complex is that negligible binding of the fusion protein to neurons was observed in the coculture before 15 days in vitro
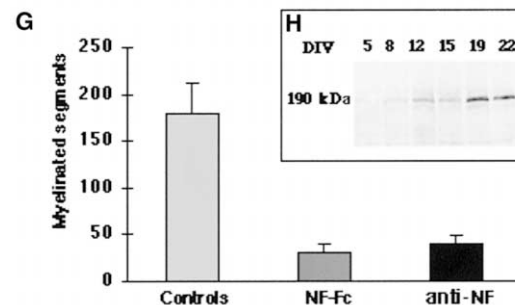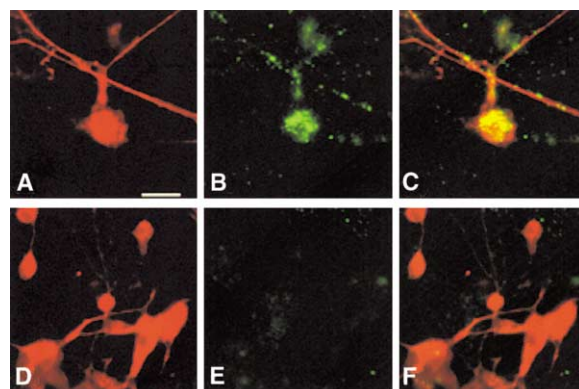

Figure 3. Blocking Interaction between NF155 and the Paranodin/Caspr-Contactin Complex Inhibits Myelination

(A–F) In neuron/oligodendrocyte cocultures, (B) NF155-Fc, but not (E) MUC-Fc, binds on (A and D) TuJ-1-positive neuronal processes and cell bodies. (C) and (F) illustrate merge colors of (A) and (B) and (D) and (E), respectively.
(G) NF155-Fc and antibodies against NF155 decrease myelination in vitro.
(H) Kinetics of paranodin/Caspr expression in myelinating cocultures; paranodin/Caspr is expressed a few days before the onset of myelination.
The scale bar represents 40 μm.

glial interactions. Hence, we exploited a well-characterized myelinating oligodendrocyte-neuron coculture system in which the extent of myelination can be quantitated [9]. NF155-Fc bound to neurons in this culture system (Figures 3A–3C), whereas the control fusion protein MUC-Fc did not (Figures 3D–3F). Note the binding of the NF155-Fc to both the cell bodies and processes of the neurons (Figures 3A–3C). The addition in culture medium of the NF155-Fc fusion protein prior to myelination caused a 93% decrease in the number of myelinated
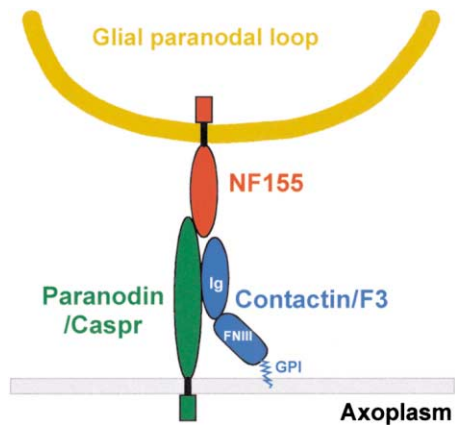
Figure 4. Model of NF155 Interaction with the Paranodin/Caspr-Contactin Complex at the Axoglial Junction

NF155 and paranodin/Caspr are transmembrane proteins, whereas contactin is anchored to the membrane by glycosylphosphatidyl-inositol (GPI). Paranodin/Caspr interacts with the region of contactin that encompasses immunoglubulin-like domains (Ig), and not the region encompassing fibronectin III repeats (FNIII) [6]. Our data reveal that NF155 binds directly to paranodin/Caspr or to a complex between this protein and contactin.

(DIV) (data not shown), which corresponded well with the kinetics of paranodin/Caspr production (Figure 3H).

A similar inhibition on myelination was noted using antibodies directed against the extracellular domain of NF155 (87% decrease in the number of myelinated segments, p < 0.005) (Figure 3G). The effect was concentration dependent and was reduced to a 38% inhibition with a 2-fold dilution of the antibody. In previous experiments with antibodies reacting to other oligodendroglial or axonal antigens, it was clearly demonstrated that antibody-induced changes of myelination are not, in this system, related to a steric, nonspecific effect of the antibody [10]. Moreover, the effect of neurofascin antibodies was not related to a decrease in the number of either neurons or oligodendrocytes (data not shown). Altogether, these results suggest that NF155-Fc fusion protein or antibodies against NF155 inhibit myelination by blocking the adhesive relationship between the axon and glial cell.

Contactin and paranodin/Caspr form a complex at the paranodal junction in both the CNS and PNS [11]. Indeed, contactin appears to be essential for the delivery of paranodin/Caspr to the cell surface in transfected cells [6]. In support of the importance of this interaction, it has been shown that paranodin/Caspr is not delivered to the axonal membrane in contactin-deficient mice [3]. Moreover, contactin is expressed at the membrane but is mislocalized and excluded from paranodes in paranodin/Caspr-deficient mice [2]. Axoglial paranodal junctions lack transverse bands and are severely disrupted in both mutant mice [2, 3], suggesting that the paranodin/Caspr-contactin-NF155 complex forms the core structure of paranodal junctions. Interestingly, in both mutants, some neurofascin is still detected at the paranode, indicating that the presence of its ligand is not essential for the targeting of NF155 to the glial paranodal loops; thus, NF155 most likely plays an essential role in the organization of paranodal junctions [2, 3].

The fact that the axoglial junctions of mice lacking ceramide galactosyltransferase are abnormal has suggested that galactocerebroside, or its sulphated derivative, may act as a glial receptor for paranodal axonal ligands [12, 13]. The importance of the lipid environment notwithstanding, contactin has been shown to interact with a variety of cell adhesion molecules in vitro, of which probably the best glial candidate is receptor protein tyrosine phosphatase zeta/β [14, 15]. We can hypothesize either the formation of a tripartite complex with paranodin/Caspr, contactin, and NF155, or a conformational effect of paranodin/Caspr on contactin, allowing its binding to NF155 (Figure 4). However, a direct interaction between paranodin/Caspr and NF155 cannot be excluded. In keeping with the recent viewpoint proposing a transbilayer/transcellular scaffold of proteins between the glial cell and axon acting as a molecular sieve to partition the sodium and potassium channels [16], we can speculate that the paranodin/Caspr-contactin-NF155 complex spans the extracellular cleft between axon and glial cell.

Experimental Procedures

DNA Constructs and Protein Expression

The extracellular domain of NF155 (amino acids 1–1214) was inserted into pIGplus (a gift from Prof. Pat Doherty), and a fusion protein comprising the NF155 extracellular domain with the Fc domain of human IgG was expressed in COS7 cells, purified on protein A-Sepharose (Sigma), dialyzed against PBS, and quantified by the BCA method (Perbio Science). The MUC-Fc construct was a gift from Dr. M.T. Filbin.

NF155-Fc Ligand Purification

Purification of the endogenous ligand for NF155 from rat brains with NF155-Fc fusion protein was carried out essentially as described [17], except that the lysis buffer contained 1% Triton X-100 and 60 mM octyl glucoside. NF155-Fc or human IgG (100 μg) bound to protein A-Sepharose (200 μl of 50% slurry) was incubated with brain lysate overnight with mixing at 4°C. The beads were collected by centrifugation and washed 3 times with 50 volumes of 20 mM HEPES (pH 7.4), 1 mM EGTA, 0.1 mM NaCl, 2.5 mM MgCl$_2$, 2 mM CaCl$_2$, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM PMSF. The beads were then packed into a column and washed extensively. Elution was in the same buffer containing 0.2 M NaCl (4 ml) followed by 1 M NaCl (4 ml). Proteins in the final eluate were precipitated with TCA, separated by SDS-PAGE on 6% gels, transferred to nitrocellulose, and detected by Western blotting.

Antibodies

Rabbit antibodies used were: SL51 against paranodin/Caspr ([18] or a gift from Dr. D.R. Colman); contactin (a gift from Dr. J.L. Salzer) [14]; MAG (generated against a KLH-coupled peptide corresponding to the C-terminal 20 amino acids of L-MAG); NF186 [5]; NFI2, generated against a KLH-coupled peptide corresponding to a peptide (CNNPYNDSSLRNHPDIYSAR) derived from the amino acid sequence of the small exon 8 present in NF155 between Ig domains 2 and 3, but absent in NF186 [19]. We used mouse monoclonal antibodies against myelin basic protein (MBP) (IgG1; Euromedex) and neuronal tubulin (TuJ-1, a gift from A. Frankfurter).

Cell Culture

Cocultures of oligodendrocytes and neurons were prepared from fetal mouse brains, and myelination occurred at 17–18 DIV. The extent of myelination was quantitated exactly as described previously [20]. Anti-neurofascin antibodies or fusion proteins were added to the culture medium before myelination at 10 DIV, and myelination was quantitated by counting the number of MBP-posi-

tive internodes at 24 DIV (peak of myelination). Fresh antibody or fusion protein was added twice a week when the culture medium was changed. The antibody was used at a dilution of 1/100, and the fusion protein was used at 1 μg/ml. Immunofluorescence was performed as described [9]. Live cells transfected with paranodin/Caspr and contactin or contactin alone [6] were incubated with NF155-Fc (30 μg/ml) for 1 hr at room temperature, washed, then incubated with fluorescein-conjugated anti-human Fc (1:200) prior to fixation in 4% paraformaldehyde.

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