Retention of a cell adhesion complex at the paranodal junction requires the cytoplasmic region of Caspr

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An axonal complex of cell adhesion molecules consisting of Caspr and contactin has been found to be essential for the generation of the paranodal axo-glial junctions flanking the nodes of Ranvier. Here we report that although the extracellular region of Caspr was sufficient for directing it to the paranodes in transgenic mice, retention of the Caspr–contactin complex at the junction depended on the presence of an intact cytoplasmic domain of Caspr. Using immunoelectron microscopy, we found that a Caspr mutant lacking its intracellular domain was often found within the axon instead of the junctional axolemma. We further show that a short sequence in the cytoplasmic domain of Caspr mediated its binding to the cytoskeleton-associated protein 4.1B. Clustering of contactin on the cell surface induced coclustering of Caspr and immobilized protein 4.1B at the plasma membrane. Furthermore, deletion of the protein 4.1B binding site accelerated the internalization of a Caspr–contactin chimera from the cell surface. These results suggest that Caspr serves as a “transmembrane scaffold” that stabilizes the Caspr/contactin adhesion complex at the paranodal junction by connecting it to cytoskeletal components within the axon.

Introduction

The reciprocal interactions between neurons and glial cells are essential for the coordinated differentiation of axons and myelin-forming cells, which allow myelinating fibers to maximize their conduction velocity (Arroyo and Scherer, 2000; Peles and Salzer, 2000). The closest contact site between the axon and its myelinating Schwann cells or oligodendrocytes is found at the paranodal region that flanks the node of Ranvier. At this site, the cytoplasmic loops of the myelinating cell create a septate-like junction with the axon, which separates the electrical activity at the nodes of Ranvier from the internodal region that lies under the compact myelin sheath (Wiley and Ellisman, 1980; Rosenbluth, 1995). Three cell adhesion molecules have been identified thus far at the paranodes of both the peripheral nervous system (PNS)* and central nervous system (CNS). These include an axonal complex that consists of Caspr (contactin-associated protein, also known as Paranodin) and the GPI-linked protein contactin (Einheber et al., 1997; Menegoz et al., 1997; Peles et al., 1997; Rios et al., 2000), which binds to an isoform of neurofascin (NF155) present on the glial loops (Tait et al., 2000; Charles et al., 2002). Both Caspr and contactin are essential for the generation of the paranodal junction, and their absence results in the disappearance of the transverse bands, which are the hallmark of this axo-glial contact (Bhat et al., 2001; Boyle et al., 2001).

The generation and maintenance of neuronal polarity is achieved through specific sorting mechanisms that are followed by anchoring and clustering of various membrane proteins to distinct domains (Winckler and Mellman, 1999). In myelinated fibers, the location of such domains is controlled by the ensheathing glial cell and by intrinsic determinants within the axon (Ellisman, 1979; Deerinck et al., 1997; Kaplan et al., 1997; Waxman, 1997; Vabnick and Shrager, 1998; Bennett and Lambert, 1999; Peles and Salzer, 2000; Rasband and Shrager, 2000). The localization of Caspr during the generation of the paranodal junction may be mediated by its interaction with both a glial ligand and cytoplasmic components within the axon (Peles and Salzer, 2000; Rios et al., 2000; Pedraza et al., 2001). The presence of Caspr at the paranodes and the juxtamesaxon (Menegoz et al., 1997; Arroyo et al., 1999), as well as its appearance in a spiral below the overlying turn of the paranodal loops that forms during development (Pedraza et al., 2001), strongly suggests that its localization in the axon is regulated by the overlying myelin sheath. The extracellular region of Caspr binds laterally to contactin when both proteins

*Abbreviations used in this paper: CNS, central nervous system; GST, glutathione-S-transferase; HA, hemagglutinin; PNS, peripheral nervous system.

Key words: axon; node of Ranvier; myelin; axo-glia junction; protein 4.1.
are expressed in the same cell (i.e., cis interactions; Peles et al., 1997), generating a receptor complex that binds neurofascin (Volkmer et al., 1998; Charles et al., 2002). The interaction with contactin is required for an efficient export of Caspr from the endoplasmic reticulum and its transport to the plasma membrane in transfected cells (Faivre-Sarrailh et al., 2000). In agreement, in contactin-null mice, Caspr is retained within neuronal cell bodies, demonstrating that contactin is essential for axonal sorting of Caspr (Boyle et al., 2001). Furthermore, contactin is absent from the paranodal junction in mice lacking Caspr (Bhat et al., 2001), suggesting that the localization of both proteins at this site is interdependent. Similarly, disruption of the paranodal junction in galactolipid-deficient mice results in the disappearance of both proteins from the paranodes (Dupree et al., 1999; Poliak et al., 2001). The absence of contactin from the paranodal junction in mice lacking Caspr may suggest that the latter is required to anchor the Caspr–contactin complex to the axonal cytoskeleton at this site. A candidate protein that may connect Caspr to the axonal cytoskeleton at the paranodes is 4.1B, a member of the protein 4.1 family, which links membrane proteins with the actin/spectrin cytoskeleton (Baumgartner et al., 1996; Menegoz et al., 1997; Hoover and Bryant, 2000) and is found at the axonal paranodes and juxtaparanodal region (Ohara et al., 2000; Poliak et al., 2001).

In this study we used transgenic mice expressing a deletion mutant of Caspr lacking its cytoplasmic region to examine the mechanisms involved in the localization of the Caspr–contactin complex at the paranodal junction. Our results suggest that although the extracellular region of Caspr is sufficient to direct it to the paranodal junction, retention of the Caspr–contactin complex at this site requires the intracellular domain of Caspr, which may link it to the axonal cytoskeleton through protein 4.1B.

Results

Generation of transgenic mice expressing a deletion mutant of Caspr in neurons

During myelination, Caspr is targeted to the paranodal junctions, located at both sides of the node of Ranvier. To determine whether the intracellular region of Caspr contains signals involved in its localization at the paranodal junctions, we have constructed a deletion mutant in which this domain was replaced with a hemagglutinin tag (CSPdCT–HA; Fig. 1). As a control, we have generated a tagged version of Caspr (CSP–HA) by fusing the HA tag to the carboxy terminus of the molecule. The presence of the HA tag in these constructs enables a clear distinction between the endogenous and the expressed proteins. The expression of these constructs in transfected HEK-293 cells could be detected using an HA tag antibody as well as an antibody directed against the extracellular domain (i.e., CSP–HA and CSPdCT–HA); an antibody against Caspr’s intracellular region recognized CSP–HA but not CSPdCT–HA (Fig. 1). We next examined the ability of the tagged proteins to associate with contactin, as this interaction was previously shown to be required for surface expression and axonal transport of Caspr (Faivre-Sarrailh et al., 2000; Boyle et al., 2001). As shown in Fig. 1 C, when coexpressed in HEK-293 cells, both CSP–HA and CSPdCT–HA formed a stable complex with contactin, which could be immunoprecipitated by an HA tag antibody. Furthermore, both transgenes were efficiently expressed on the cell surface, as was evident by staining the cells with a monoclonal antibody that recognized the extracellular domain of Caspr in intact cells (Fig. 1 D), as well as by surface biotinylation experiments (unpublished data).

To direct the expression of these Caspr constructs in neurons, they were placed under the transcription regulatory elements of Thy1.2, a GPI-linked cell surface glycoprotein expressed in the CNS in peripheral long-projecting neurons,
thymus, and fibroblasts (Gordon et al., 1987). The expression cassette used in the present study contained a deletion in the third intron of the Thy1.2 gene, restricting the expression of the transgenes to the nervous system (Caroni, 1997; Feng et al., 2000). An important advantage of this system is that the expression using the Thy1 promoter is well documented (Caroni, 1997) and results from stochastic expression of the trans-genes with contactin in vivo as the endogenous Caspr protein (Fig. 2 D).

Retention of Caspr–contactin complex at the paranodes requires the cytoplasmic tail of Caspr

To further determine whether the transgenes were properly located at the paranodal septate-like junctions at the ultrastructural level, we examined their localization in optic nerves using immunoelectron microscopy. In cross sections at the level of the paranodes of wild-type and CSPdCT–HA mice, staining with an antibody against the intracellular region of Caspr.
which only recognizes the endogenous protein, resulted in a circumferential labeling surrounding the axon (Fig. 5, A and B). A similar staining pattern was obtained in optic nerves from CSP–HA mice labeled with an HA tag antibody (Fig. 5 C). In contrast, specific labeling of optic nerves from CSPdCT–HA animals using an antibody to HA tag was frequently detected within the axon (Fig. 5 D). At these sites, the gold particles were occasionally surrounded by a membrane configuration that was better detected by carefully tilting the sample. In favorable planes of the section, the staining was clearly observed within a vesicular structure (Fig. 5 F, inset). We then repeated the same analysis using longitudinal sections of optic nerves from the different transgenic animals. As expected, the endogenous protein was clearly detected at the axonal membrane in optic nerve sections from wild-type mouse (Fig. 5 E). Similar staining was also revealed using an HA tag antibody on optic nerve sections from CSP–HA (unpublished data). In contrast, in CSPdCT–HA mice, HA tag labeling was not confined to the axolemma, and was also located within the axon (Fig. 5, F and G). As summarized in Table I, internal distribution of the gold particles of HA-labeled nerves was found in 76.9% of the sections examined in CSPdCT–HA, compared with only 5.3% in CSP–HA. The latter was comparable to the 6.6% of internal labeling of Caspr detected in wild-type nerves. Importantly, staining of CSPdCT–HA at the axonal circumference was observed in 66% of the sections, demonstrating that this mutant was still residually found at the paranodal junction. The presence of CSPdCT–HA molecules within the axon may result from its reduced insertion into the axolemma, or alternatively from its destabilization and internalization due to the inability of this mutant to interact with cytoskeletal components at the paranodal junction.

Because CSPdCT–HA was associated with contactin similarly to a wild-type Caspr (Fig. 1 C and Fig. 2 D), we examined whether the mislocalization of this mutant within the axon may also affect the subcellular localization of contactin. Whereas contactin was detected at the axonal circumference in optic nerve from wild-type and CSP–HA mice (Fig. 5, H and I), in optic nerves derived from CSPdCT–HA animals, it was less confined to the axonal circumference and was occasionally seen within the axon (Fig. 5, J and K). Altogether, these results suggest that although the extracellular region of Caspr is sufficient to direct it to the paranodes, its cytoplasmic domain is required for retention of the Caspr–contactin complex at the paranodal junction.

The cytoplasmic region of Caspr is required for binding and immobilization of protein 4.1B

Our results so far suggest that the maintenance of the Caspr–contactin complex at the paranodal junction may require its linkage to the axonal cytoskeleton through the cytoplasmic do-
Interaction of Caspr with protein 4.1B. (A) Pulldown of protein 4.1B by the cytoplasmic domain of Caspr. Lysates of HEK-293 cells expressing protein 4.1B were mixed with agarose-bound GST or GST fusion protein containing the cytoplasmic domain of Caspr as indicated. Bound proteins were immunoblotted with an antibody to protein 4.1B. Immunoprecipitation with an antibody to protein 4.1B (4.1B) was used as a control. (B) Coimmunoprecipitation of protein 4.1B with Caspr from rat brain. Adult rat brain lysates were subjected to immunoprecipitation with antibodies to protein 4.1B (4.1B) or the cytoplasmic (Caspr/CT) or extracellular (Caspr/ECD) domains of Caspr as indicated. Preimmune serum (CS) or protein A beads (beads) were used as controls. (C) Association of protein 4.1B with CSP–HA, but not with CSPdCT–HA. HEK-293 cells were transfected with CSP–HA or CSPdCT–HA, with (+m4.1B) or without myc-tagged protein 4.1B as indicated on the top. Cell lysates were subjected to immunoprecipitation with an antibody against the extracellular domain of Caspr. Washed immune complexes were separated on SDS gel and immunoblotted with an antibody to myc (right) or HA tag (left). The sizes of mol wt markers are shown on the right in kD. (D) Binding of βC–Fc fusion protein under nonclustering conditions. Hela cells expressing CSPdCT–HA, contactin, and protein 4.1B were stained with the indicated antibodies or with βC–Fc fusion protein. The second and fourth panels show the same cell stained for contactin and the βC–Fc fusion protein. The second and fourth panels show the same cell stained for contactin and the βC–Fc fusion protein. (E) Clustering of CSP–HA, but not CSPdCT–HA, induces aggregation of protein 4.1B. Hela cells expressing contactin, protein 4.1B, and CSP–HA were mixed with agarose-bound GST or GST fusion protein containing the carbonic anhydrase domain of RPTPβ (βC–Fc), previously shown to bind contactin (Peles et al., 1995). As shown in Fig. 6 D, expression of CSPdCT–HA, contactin, and protein 4.1B at the plasma membrane was detected in HeLa cells coexpressing these proteins. As expected, binding of βC–Fc to these cells, without allowing clustering to occur, resulted in an identical pattern to that obtained using a contactin antibody (Fig. 6 D, compare the second and fourth panels) or an antibody against Caspr (unpublished data). Clustering of βC–Fc fusion protein on cells expressing protein 4.1B, contactin, and either CSP–HA or CSPdCT–HA resulted in aggregation of contactin and the tagged Caspr molecules (Fig. 6 E). In contrast, protein 4.1B was incorporated into these clusters when coexpressed with CSP–HA but not with CSPdCT–HA.

To further analyze whether the interaction of Caspr with protein 4.1B affected the stabilization of Caspr on the cell
surface, we constructed two chimeric proteins in which the transmembrane and cytoplasmic domain of Caspr (ConCT) or a deletion mutant lacking nine amino acids in its juxtamembrane region (ConJXCT) were fused to the extracellular domain of contactin (Fig. 7 A). The latter was constructed based on the observation that the juxtamembrane region of Caspr contains a putative binding site for members of the protein 4.1 family (Menegoz et al., 1997; Peles et al., 1997; Hoover and Bryant, 2000). These chimeras were efficiently expressed on the cell surface, thus resulting in the targeting of the cytoplasmic domain of Caspr to the plasma membrane (Fig. 7 B). However, only ConCT, not ConJXCT, formed a complex with protein 4.1B (Fig. 7 C), and induced its aggregation at the plasma membrane upon clustering with GC–Fc (Fig. 7 D), demonstrating that ConJXCT lacks the binding site for protein 4.1B.

Surface biotinylation experiments were then used to examine the internalization of ConCT or ConJXCT from the cell surface. HEK-293 cells expressing equal amounts of protein 4.1B and ConCT (top) or ConJXCT (bottom) were biotinylated using sulfo-NHS-S-S-biotin. Cells were then incubated with GC–Fc-containing medium at 37°C for the indicated times to allow internalization of biotinylated surface proteins. Cells were placed on ice to stop trafficking and, subsequently, were either treated with glutathione to remove remaining labeled proteins on the cell surface (internalized) or were left untreated (total). Biotinylated proteins were precipitated from cell lysates using agarose–streptavidin followed by immunoblotting with anti-HA tag antibody.

The ability of Caspr to mobilize protein 4.1B at the plasma membrane raises the possibility that Caspr may recruit 4.1B at the paranodal junction. Thus, to determine whether the paranodal localization of protein 4.1B depends on the presence of Caspr, we examined its distribution in
myelinated nerves derived from contactin-deficient mice, which lack the Caspr–contactin complex at their paranodes (Boyle et al., 2001). Staining of teased sciatic nerves of 8-d-old wild-type mice revealed that protein 4.1B was concentrated at the paranodal junction (Fig. 8). In contrast, in age-matched nerves derived from contactin-null mice, protein 4.1B was distributed along the axon. It should be noted that in peripheral nerves at this age, Caspr is already localized at all paranodes, whereas Caspr2 is only starting to appear (Poliak et al., 2001). At older ages, paranodal accumulation of protein 4.1B was evident when Caspr2 was abnormally present at the paranodes instead of the juxtaparanodal region (unpublished data). Taken together, these results demonstrate that Caspr binds protein 4.1B through a short juxtamembrane sequence in its cytoplasmic tail, an association that could immobilize protein 4.1B to Caspr–contactin sites at the cell membrane. This in turn serves to stabilize Caspr at the cell surface.

Discussion

The localization of ion channels and cell adhesion molecules to distinct domains at and around the nodes of Ranvier is thought to be regulated by signals provided by the overlying myelinating glial cells, as well as by cytoplasmic proteins within the axon (Bennett and Lambert, 1999; Peles and Salzer, 2000). Caspr and contactin form an adhesion complex that is essential for the generation of the paranodal junction and, subsequently, for the organization of the nodal area (Bhat et al., 2001; Boyle et al., 2001). Here we report that although the extracellular region of Caspr was sufficient to direct it to the paranodal region, its cytoplasmic domain was necessary for retention of the Caspr–contactin complex at the junction. Ultrastructural analysis revealed that a Caspr mutant lacking its intracellular domain (CSPdCT–HA) was often found within the axon instead of the junctional axolemma. Notably, the CSPdCT–HA mutant was residually found at the paranodal junction, indicating that the intracellular region of Caspr is not required for its insertion into the plasma membrane. This conclusion was also supported by the observation that, similarly to the wild-type protein, this mutant efficiently reached the cell surface when coexpressed with contactin. Furthermore, deletion of a short juxtamembrane sequence that serves as a protein 4.1B binding site resulted in faster internalization of a chimeric protein in which the extracellular region of contactin was fused to the transmembrane and cytoplasmic domain of Caspr. Taken together, the presence of CSPdCT–HA within the axon most likely results from its instability at the paranodal junction and its internalization, rather than from its inability to be inserted into the axonal membrane. At present, it is not clear whether CSPdCT–HA is trafficked back to the cell body for degradation or is being recycled back to the plasma membrane.

Caspr and contactin form a lateral complex that is found at the paranodal junction (Rios et al., 2000). Expression of the CSPdCT–HA mutant, but not of a full-length Caspr in transgenic mice, resulted in mislocalization of contactin. This result suggests that contactin, which is a GPI-linked protein anchored only to the outer leaflet of the membrane, depends on lateral association with Caspr to be maintained at specific sites along the axon. We propose that, after an initial targeting phase, which requires the extracellular domain of Caspr and depends on its interaction with contactin, a second process of stabilization takes place once the complex is deposited at the paranodes. This process is mediated by the intracellular domain of Caspr that links the complex to the axonal cytoskeleton (Fig. 9). This mechanism may well explain the notable lack of contactin from the paranodal domain in Caspr-null mice (Bhat et al., 2001); in the absence of Caspr, the linkage between contactin and the underlying axonal cytoskeleton at the paranodal junction is disrupted, resulting in the disappearance of contactin from this site.

The localization of cell adhesion molecules at cell junctions depends on their interaction with cytoskeleton-associated proteins (Knust, 2000; Muller, 2000). Candidate axonal proteins that may be involved in the localization of Caspr at the paranodal junction are members of the protein 4.1 family, which link membrane proteins with the actin/spectrin cytoskeleton (Hoover and Bryant, 2000; Bennett and Baines, 2001). Four different 4.1 proteins are expressed in the ner-
ous system at different subcellular locations (Walensky et al., 1999; Yamakawa et al., 1999; Parra et al., 2000; Yamakawa and Ohara, 2000); one of which (protein 4.1B) is concentrated at the axonal paranodes and juxtaparanodal region (Ohara et al., 2000; Poliak et al., 2001). A previous study showed that the cytoplasmic domain of Caspr could precipitate protein 4.1R from red blood cells and brain lysates (Menegoz et al., 1997); however, a direct interaction between these two proteins was not demonstrated. Here, we extend these observations and show that Caspr physically associates with protein 4.1B. This association is mediated by a short juxtamembrane sequence (nine amino acids) present in the cytoplasmic domain of Caspr, which shows strong similarity to the protein 4.1R binding site found in erythrocyte glycoporphin C (Marfatia et al., 1995). Furthermore, we show that clustering of the Caspr–contactin complex on the cell surface immobilized protein 4.1B into these clusters, demonstrating that it is recruited to Caspr-containing sites on the plasma membrane. Consistent with this notion, we found that protein 4.1B was abnormally distributed along peripheral myelinated axons of contactin-null mice, which entirely lack the Caspr–contactin complex in their paranodes. Mislocalization of protein 4.1B was also observed in galactolipid-deficient mice, in which Caspr and contactin are displaced from the paranodes and are occasionally detected along the axon (Poliak et al., 2001). In these two paranodal mutants, the position of protein 4.1B was strongly correlated with the appearance of Caspr and Caspr2, suggesting that both Caspr family members may regulate its localization in myelinated axons. It was previously shown that neurexin IV, a Drosophila homologue of Caspr and Caspr2, associates with and recruits the protein 4.1 homologue Coracle to the septate junction (Baumgartner et al., 1996). However, in contrast to the complete absence of Coracle from the septate junction in neurexin IV mutants, in coracle mutants, neurexin IV still reached the lateral membrane but was not subsequently confined at the septate junction (Ward et al., 1998). These results indicate that although Coracle does not play a role in the targeting of neurexin IV to the plasma membrane, it is required for its maintenance at the junction. In analogy, we found that a deletion mutant of Caspr that lacks its intracellular domain and is unable to bind protein 4.1B was targeted to the paranodes, but was not maintained properly at the junction. Because of the geometry of the myelinating cell, the generation of the paranodal junction occurs gradually and continues as additional loops are attached to the axon (Rosenbluth, 1995). As a result, paranodal accumulation of Caspr is composed of a number of rings that represent each turn of the myelin wall and, thus, does not constitute a uniform domain. During myelination of dorsal root ganglion neurons by Schwann cells in vitro, Caspr is detected in a spiral corresponding to the overlying turn of the forming paranodal loop, which is later consolidated into a tight helical coil (Pedraza et al., 2001). We have found no evidence for the accumulation of 4.1B with Caspr during this process, suggesting that it may be recruited at a later stage when Caspr is already found at the paranodal junction. Taken together, it is reasonable to suggest that during the generation of the paranodal junction, protein 4.1B is immobilized at Caspr-containing sites on the axolemma. This in turn may bridge the Caspr–contactin complex to the rich cytoskeletal core present at the axonal paranodes (Ichimura and Ellisman, 1991).

An important question is what determined the localization of Caspr and Caspr2 in myelinating axons. Our observation that a Caspr mutant lacking the cytoplasmic domain reaches the paranodal region argues against the possibility that the cytoplasmic domains of Caspr and Caspr2 are responsible for their differential targeting and localization. Instead, these are more likely to be controlled by specific interactions mediated by the distinct extracellular domains of Caspr and Caspr2 (Poliak et al., 1999). Although Caspr binds to contactin and indirectly to neurofascin 155 (Charles et al., 2002; unpublished data), found at the paranodal junction, Caspr2 does not interact with these molecules, but may bind to TAG-1, a contactin family member found at the juxtaparanodes (Traka et al., 2002). While uncovering a role for the cytoplasmic domain of Caspr in maintenance of the Caspr–contactin complex at the paranodes, our results do not exclude the possibility of an additional contribution of a glial ligand that binds the Caspr–contactin complex in this process. Nevertheless, our results raise the intriguing possibility that the chief function of Caspr is to provide a “transmembrane scaffold” that stabilizes the Caspr–contactin adhesion complex at the paranodal junction by connecting it to cytoskeletal components within the axon. This illustrates one mechanism by which the axonal cytoskeleton cooperates with glial cues to organize functional domains along myelinated axons.

**Materials and methods**

**Constructs and transgenic mice**

HA-tagged constructs were all generated from human Caspr cDNA using PCR and standard cloning procedures. In CSP-HA, the HA tag (amino acids YPYDVPDYAS) was inserted at position 1385 after the carboxy-terminal glutamic acid (E1384), whereas in CSPdCT–HA, it replaced the cytoplasmic sequence from the lysine at position 1312. These genes were cloned into a Thy1.2 expression cassette (Caroni, 1997), linearized, and introduced by pronuclear injection into fertilized eggs derived from CB6F1 mice. Pseudopregnant CD-1 outbred albino females were used as foster mothers for embryo transfer. Founder mice were genotyped by Southern blot hybridization with a DNA fragment containing the first 710 bp of human Caspr. Founders were further crossed with CB6F1 mice and interbred to generate lines. Transgenic mice were routinely identified by PCR of tail genomic DNA, using the appropriate primers derived from human Caspr cDNA and the HA tag. The same primers were also used for RT-PCR analyses on RNAs prepared from mice brains. Myc tag protein 4.1B was generated by cloning the open reading of KIAA0987 downstream of a myc tag–containing pCDNA3 vector (Invitrogen). For the generation of ConCT and ConJXCT, the transmembrane and cytoplasmic domain of Caspr or a deletion mutant lacking nine amino acids in the juxtaparanode region (Fig. 7) were generated by PCR and attached after amino acid 1020 of human contactin (Pele et al., 1995), replacing its GPI-linkage sequence.

**Immunoprecipitation and immunoblot analysis**

For preparation of mouse brain membranes, adult brains were homogenized in a glass homogenizer in a buffer containing 20 mM Hepes, pH 7.4, 0.32 M sucrose, 1 mM EGTA, 1.5 mM MgSO4, 10 μg/ml aprotinin and leupeptin, and 1 mM PMSF. Nuclei and heavy cell debris were removed by low speed centrifugation (3,000 g for 10 min at 4°C), and the supernatants were centrifuged at 40,000 g for 60 min. Membrane pellets were solubilized in Triton X-100 lysis buffer (Poliak et al., 1999) for 1 h on ice and then the detergent-insoluble material was removed by centrifugation. Immunoprecipitation of Caspr and 4.1B was done using 14-wk-old rat brain homogenized in Triton X-100 lysis buffer (Poliak et al., 1999). For immunoprecipitation, solubilized membrane supernatants were incubated with antibodies coupled to Sepharose–protein A beads (Amersham Pharmacia Biotech) or to agarose anti–mouse IgG beads (Sigma-Aldrich), followed by...
Western blotting analyses using the ECL detection system (Amersham Pharmacia Biotech) as previously described (Poliak et al., 1999). The antibodies used, polyclonal antibody P6061 directed to the intracellular region of Caspr and monoclonal antibody M275 directed to its extracellular domain, were previously described (Peles et al., 1997; Poliak et al., 1999). Monoclonal antibody for Na$^+$ channels was previously described (Rasband et al., 1999). Antibodies against HA tag and myc tag were purchased from Boehringer and Santa Cruz Biotechnology, Inc. Polyclonal antibodies against contactin were generated by immunization of rabbits with a purified human contactin–Ig fusion protein as described previously (Rios et al., 2000). Polyclonal antibodies against protein 4.1B were generated by immunizing rabbits with a GST fusion protein containing amino acids 7-179 of human protein 4.1B (GenBank/EMBL/DBJ accession no. AB023204) according to Yamakawa et al., 2000. Removal of antibodies against GST and affinity purification of the antibodies were performed as described previously (Poliak et al., 1999).

Internalization assay

The internalization of ConCT and ConJXCT was analyzed by cell surface biotinylation as previously described (Cao et al., 1998). HEK-293 cells stably expressing protein 4.1B were transfected with ConCT or ConJXCT. 48 h later, the cells were biotinylated with 0.5 mg/ml sulfo-NHS-S-biotin in PBS for 30 min at 4°C. Excess biotin was removed by three washes with TBS and the cells were then incubated in 37°C with DME containing 1% FCS for various times to allow for endocytosis. Biotin attached to proteins still remaining on the cell surface was stripped by washing the cells twice with 0.5 mM glutathione in a buffer containing 75 mM NaCl, 75 mM NaOH, and 10% FCS on ice. For each time point, a sample of cells not treated with glutathione was used as a control. The cells were then incubated twice for 15 min in buffer containing 50 mM iodoacetamide, 1% BSA in PBS and further lysed with 1% Triton X-100 solubilization buffer (Poliak et al., 1999). Biotinylated antibodies were isolated from the supernatant using UltraLink immobilized NeutrAvidin beads (Pierce Chemical Co.). Beads were washed three times with HNTG buffer (Poliak et al., 1999), eluted with DTT-containing SDS-PAGE buffer, and subjected to Western blotting using an anti–HA tag antibody.

Immunofluorescence

Tissues. Mouse optic nerves were isolated and directly frozen in O.C.T.

mounting medium (Tissue-Tek) and 10-μm sections were cut with a cryo-

stat. For sciatic nerve labeling experiments, nerves were isolated and cooled CCD camera.

ously (Poliak et al., 1999). Immunofluorescence slides were viewed and

PBS for 30 min at 4°C. After extensive washing with cacodylate buffer, the samples were cryo-

ected by immersion for 5 min in 15% sucrose and 5% glycerol and 30 min in 30% sucrose and 10% glycerol and then frozen and thawed twice in isopentane cooled in liquid nitrogen. Tissues were embedded in 7% agar mixed and cut at 50-μm thickness using a vibrotome. Sections were blocked for 1 h in 0.1% saponin (Sigma-Aldrich), 3% normal goat serum, 0.5% BSA, and 0.1% glycine in PBS. First antibodies were incubated overnight in PBS containing 1% normal goat serum, 0.1% glycine, and 0.5% BSA, extensively washed in PBS, and incubated with Nanogold 1:40 secondary antibodies (Nanoprobe). The sections were then washed and fixed for 2 h with 2% OsO4 and 5% tert-butanol in distilled water before silver intensifica-

tion using HQ Silver (Nanoprobe). Samples were fixed in 3% glutaralde-

hyde, 3% paraformaldehyde, and 0.2% picric acid in cacodylate buffer and then with 1% osmium tetroxide, 0.5% potassium dichromate, and 0.5% potassium hexacyanoferrate. The tissue was postfixed with 2% aqueous uranyl acetate followed by ethanol dehydration and embedded in EM-

BED 812 (EMS). Sections were cut using a diamond knife (Diatome) and examined using a Philips CM-12 transmission electron microscope at ac-

celerating voltage of 100 kV.

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