The Axonal Membrane Protein Caspr, a Homologue of Neurexin IV, Is a Component of the Septate-like Paranodal Junctions That Assemble during Myelination

Steven Einheber,* George Zanazzi,* William Ching,* Steven Scherer,† Teresa A. Milner,¶ Elior Peles,** and James L. Salzer*‡§

*Department of Cell Biology, †Department of Neurology, ‡The Kaplan Cancer Center, New York University Medical School, New York 10016; §Department of Neurology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; ¶Department of Neurology and Neuroscience, Division of Neurobiology, Cornell University Medical College, New York 10021; and **Sugen, Inc., Redwood City, California 94063

Abstract. We have investigated the potential role of contactin and contactin-associated protein (Caspr) in the axonal–glial interactions of myelination. In the nervous system, contactin is expressed by neurons, oligodendrocytes, and their progenitors, but not by Schwann cells. Expression of Caspr, a homologue of Neurexin IV, is restricted to neurons. Both contactin and Caspr are uniformly expressed at high levels on the surface of unensheathed neurites and are downregulated during myelination in vitro and in vivo. Contactin is downregulated along the entire myelinated nerve fiber. In contrast, Caspr expression initially remains elevated along segments of neurites associated with nascent myelin sheaths. With further maturation, Caspr is downregulated in the internode and becomes strikingly concentrated in the paranodal regions of the axon, suggesting that it redistributes from the internode to these sites.

Caspr expression is similarly restricted to the paranodes of mature myelinated axons in the peripheral and central nervous systems; it is more diffusely and persistently expressed in gray matter and on unmyelinated axons. Immunoelectron microscopy demonstrated that Caspr is localized to the septate-like junctions that form between axons and the paranodal loops of myelinating cells. Caspr is poorly extracted by non-ionic detergents, suggesting that it is associated with the axon cytoskeleton at these junctions. These results indicate that contactin and Caspr function independently during myelination and that their expression is regulated by glial ensheathment. They strongly implicate Caspr as a major transmembrane component of the paranodal junctions, whose molecular composition has previously been unknown, and suggest its role in the reciprocal signaling between axons and glia.

Myelinated nerve fibers play a critical role in the vertebrate nervous system by promoting the efficient and rapid propagation of action potentials via saltatory conduction (Huxley and Stämpfli, 1949). This mode of conduction requires the organization of myelinated fibers into longitudinal domains that are anatomically and functionally distinct. Three domains—the internode, the paranodal region, and the node of Ranvier—form as the result of, and can be distinguished by, specific interactions between axons and myelinating glial cells, i.e., Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system (Peters et al., 1991; Salzer, 1997). In the internode, the most stereotypic portion of myelinated fibers, the axon is separated from the inner glial membrane by a regular space of 12 nm or more and is surrounded by a compact myelin sheath. In the paranodal region, the compact myelin lamellae open up into a series of cytoplasmic (paranodal) loops that spiral around and physically invaginate the axon. These glial loops are closely apposed to the axon, being separated by a gap of only 2.5–3 nm, and form a series of septate-like junctions with the axon (shown schematically in Fig. 8). In electron micrographs of longitudinal sections through the paranodal region, these junctions appear as a series of ladder-like densities that arise from the outer leaflet of the axolemma and contact the glial membranes. At the nodes, which represent gaps between the myelin sheaths, the axon is relatively exposed to the extracellular environment. In larger myelinated fibers, Schwann cell microvilli and astrocytic processes are also closely associated with the nodal
axolemma in the peripheral and central nervous system (PNS and CNS), respectively.

Each of these regions of myelinated fibers are distinct with respect to their function in impulse conduction and organization of voltage-gated channels. Thus, voltage-gated sodium channels are strikingly concentrated (∼1,500/µm²) at the node of Ranvier, enabling regeneration of the action potential. Na⁺/K⁺ ATPase and Na⁺/Ca²⁺ exchangers are also enriched at the node (for a recent review see Waxman and Ritchie, 1993). Delayed rectifier potassium channels are enriched in the juxtaparanodal regions, where they may contribute to repolarization and tonic homeostasis (Wang et al., 1993; Mi et al., 1995). By contrast, the internode, which exhibits a reduced capacitance, has significantly lower concentrations of these voltage-gated channels. The mechanisms that regulate the distinct distributions of voltage-gated channels along the axon are not well understood. Paracrine and juxtacrine signals from glial cells appear to initiate clustering of sodium channels and other nodal components (Kaplan et al., 1997; for review see Salzer, 1997). The paranodal junctions may also contribute by providing a physical barrier that prevents lateral diffusion of ion channels and thereby separates these distinct domains (Rosenbluth, 1976; Gumbiner and Louvard, 1985).

The molecules that mediate the interactions between axons and myelinating glial cells at the paranodal junctions have been elusive. Several proteins of the glial cell are concentrated in the paranodal loops and mediate interactions between the glial processes rather than with the axon. Among these are E-cadherin, which is likely to mediate adherens-like junctions between these processes (Fannon et al., 1995), and connexin 32, which is a component of the gap junctions that form between these glial processes (Scherer et al., 1995). The myelin-associated glycoprotein (MAG), which is enriched in the inner glial membrane and appears to promote adhesion to the axon in the internode, is enriched in paranodal loops in the PNS but not the CNS (for review see Quarles et al., 1992). Its function in the paranodal loops of Schwann cells is presently unclear. Several Ig-related cell adhesion molecules (CAMs) on the axon, including specific isoforms of neurofascin and NrCAM, are concentrated at the node of Ranvier, rather than in the paranodes, and may have a role in sodium channel localization (Davis et al., 1996).

Recent studies suggested that contactin/F3/F11 might mediate interactions between axons and glial cells (Peles et al., 1995; Koch et al., 1997). Contactin/F3/F11 is a GPI-anchored neural Ig CAM that promotes nerve fiber outgrowth. It was recently shown to be expressed by Schwann cells in the chick (Willbold et al., 1997) and by rat oligodendrocytes (Koch et al., 1997), although characterization of its expression by these cells in vivo, including its distribution along myelinated fibers, is still incomplete.

Contactin is associated with the transmembrane protein Caspr (contactin-associated protein), which has been suggested to be a coreceptor with contactin (Peles et al., 1997b). Caspr is a type I integral membrane protein with a molecular mass of 190 kD that is highly expressed in the CNS; it is also present at reduced levels in several extraneuronal tissues (Peles et al., 1997b). It copurifies with contactin when the carbonic anhydrase domain of the receptor protein tyrosine phosphatase β is used as an affinity ligand. Contactin and Caspr also coimmunoprecipitate, suggesting that they are constitutively complexed. The extracellular domain of Caspr contains a series of laminin G-like and EGF-like domains characteristic of the neurexins; accordingly, Caspr is a member of the neurexin superfamily. The cytoplasmic segment of Caspr contains potential binding sites for SH3 domain–containing proteins and band 4.1 proteins but lacks the carboxy-terminal motif found in other neurexins that binds to PDZ domains. Of interest, the Drosophila homologue of Caspr, Neurexin IV (Baumgartner et al., 1996), has a similar extracellular domain organization, and a 30% amino acid identity (Peles et al., 1997a; Littleton et al., 1997), is expressed by glial cells and is a component of their septate junctions that compose the blood–nerve barrier (Baumgartner et al., 1996).

We now report that contactin and Caspr/Neurexin IV are differentially expressed and localized during myelination. Of particular note, Caspr, which is expressed exclusively by neurons, becomes concentrated in the paranodal junctions of mature myelinated fibers in both the CNS and PNS, whereas contactin is substantially downregulated. In unmyelinated fibers and in nascent myelinated fibers, Caspr has a more diffuse localization along the length of the axon, suggesting that it redistributes from the internode to the paranode. These results implicate Caspr as a major component of the septate junctions that form between axons and paranodal loops and suggest that it may mediate reciprocal signaling between axons and myelinating glial cells.

Materials and Methods

Antibodies

Monoclonal antibodies used in these studies included the anti-MAG monoclonal antibody MA513 (gift of M. Schachner, Swiss Federal Institute of Technology, Zürich, Switzerland) and anti-MBP monoclonal antibody SMI 94 (Stenberger Monoclonals, Baltimore, MD). Rabbit polyclonal antisera included an anti-contactin/F3 antiseraum (gift of C. Goridis, Institut de Biologie du Développement de Marseille, CNRS/INSERM/Université de la Méditerranée, Marseille, France); anti-Caspr antiseraum 60/61 generated against a bacterial fusion protein containing the Caspr cytoplasmic domain and the corresponding preimmune serum (Peles et al., 1997b).

Tissue Culture Methods

Primary rat Schwann cell and dorsal root ganglion (DRG) neuron cultures and myelinating Schwann cell/neuron cocultures were established as described previously (Einheber et al., 1993) with minor modifications. Neonatal rat Schwann cells were maintained in standard media consisting of DME (Whittaker Bioproducts, Inc., Walkersville, MD), 10% FBS (HyClone Laboratories, Inc., Logan, UT), and 2 mM glutamine (Life Technologies, Gaithersburg, MD) and were amplified in standard media supplemented with 5 ng/ml GGF2 (Cambridge Neurosciences, Cambridge, MA) and 4 µM forskolin (Sigma Chemical Co., St. Louis, MO). To examine Caspr and contactin expression by immunocytochemistry, 50,000 Schwann cells were plated onto poly-L-lysine–coated 12-mm coverslips and kept in standard media for a minimum of 3 d before fixation.

The dissociated neuronal cultures consisted of 4,000 rat embryonic day 16 or 17 DRG neurons plated onto 12-mm glass coverslips coated with amnoniated rat tail collagen (Biomedical Technologies, Inc., Stoughton, MA). These cultures were maintained in standard neuronal media, which consists of MEM (Life Technologies) supplemented with 10% FBS, 2 mM

1. Abbreviations used in this paper: CAM, cell adhesion molecule; Caspr, contactin-associated protein; CNS and PNS, central and peripheral nervous system; dPBS, Dulbecco’s PBS; DRG, dorsal root ganglia; MAG, myelin-associated glycoprotein; MBP, myelin basic protein.
glutamine, 0.4% glucose (Sigma Chemical Co.), and 50 ng/ml 2.5 S NGF (Bioproduction for Science, Inc., Indianapolis, IN). These cultures were treated for 2.5 wk with 5-fluorodeoxyuridine and uridine (both at 10-5 M) (Sigma Chemical Co.), which were added to the standard neuronal media in alternate feedings to eliminate nonneuronal cells. Myelinating Schwann cell/neuron cocultures were prepared by seeding purified DRG neuron cultures with 200,000 Schwann cells/coverslip in standard neuronal media. The media was replaced the next day with N2 media, which contained 5 mg/ml insulin (Sigma Chemical Co.), 10 mg/ml transferrin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 20 nM progesterone (Sigma Chemical Co.), 100 mM putrescine (Sigma Chemical Co.), 30 nM selenium (Sigma Chemical Co.), and 2 mM glutamine in a 1:1 mixture of DME and Ham’s F-12 (Life Technologies) supplemented with 2.5 S NGF. The cultures were maintained in N2 media for 3 d to allow the Schwann cells to populate the neurites. To initiate basal lamina formation and myelination, the cultures were fed the standard neuronal media supplemented with 50 µg/ml ascorbic acid (Sigma Chemical Co.). O-2A progenitors and oligodendrocytes were prepared as described previously (Canoll et al., 1996a). To examine Caspr and contactin expression on oligodendrocytes by immunocytochemistry, 50,000 O-2A cells were plated onto poly-L-lysine-coated Lab-Tek chamber slide wells (Fisher Scientific, Pittsburgh, PA) and kept in DMEM media for 3 d before fixation.

Immunoblotting

Cultures of DRG neurons and Schwann cells were lysed in a solution containing 95 mM NaCl, 25 mM Tris-Cl, pH 7.4, 10 mM EDTA, 2% SDS, 1 mM PMSE, 10 µg/ml aprotinin, and 20 µM leupeptin. O-2A and oligodendrocyte cultures were prepared as described (Canoll et al., 1996a). The nuclear lysates were prepared from nerves immersed in liquid nitrogen after their dissection (Einhheber et al., 1993). Protein concentrations of the lysates were determined by the Micro BCA method (Pierce Chemical Co., Rockford, IL). Lysates were subjected to SDS gel electrophoresis and blotted onto nitrocellulose. Blots were first probed with primary antibody against Caspr followed by 125I-labeled protein A (Amersham Corp., Arlington Heights, IL). Appropriate regions of the blots were cut out and re-incubated with antibodies to contactin/F3 and 125I-protein A. The blots were then scanned and quantitated on a Molecular Dynamics (Sunnyvale, CA) phosphorimagier.

For detergent extraction experiments, cultures grown in 35-mm dishes were rinsed with Dulbecco’s PBS (dpBS, Life Technologies) and extracted with 1% Triton X-100 in dpBS in the presence of protease inhibitors at 4°C for 30 min. The supernatants containing the Triton X-100-solubilized proteins were then collected, and the remaining insoluble material was further extracted in gel sample buffer containing 2.5% SDS for 10 min at room temperature. All samples were spun at 12,000 g for 30 min at room temperature, and equal volumes of the cleared supernatants were fractionated on 10% SDS-polyacrylamide gel and blotted to nitrocellulose. The blot was probed with Caspr polyclonal antisera and 125I-protein A and scanned on a phosphorimagier to quantitate the immunoblotted bands.

Light Microscopy and Immunofluorescence

Immunofluorescence microscopy was done as described previously (Einhheber et al., 1993) with minor modifications. Cultures grown on coverslips were washed with dpBS (Life Technologies), fixed with 4% paraformaldehyde in dpBS for 15 min, washed in dpBS, and then permeabilized with 100% methanol at –20°C for 15 min. The coverslips were then washed with dpBS and blocked for 30 min with Leibovitz’s L-15 media (Life Technologies) supplemented with 10% heat-inactivated FBS. The coverslips were incubated for 1 h at room temperature with primary antibodies diluted in the blocking solution, washed three times with blocking solution, and incubated for 1 h at room temperature with species-specific affinity-purified rhodamine-conjugated donkey anti-rabbit IgG or fluorescein-conjugated donkey anti-mouse IgG (Chemicon International, Inc., Temecula, CA) diluted 1:100 in the blocking solution. The coverslips were washed five times, mounted in Citifluor (Ted Pella, Inc., Redding, CA) on glass slides, and examined by epifluorescence microscopy.

For tissue immunocytochemistry, vibratome sections of brain (60 µm thick) and teased sciatic nerve fibers were prepared from acrolein perfused adult Sprague Dawley rats and incubated with primary antibodies as described previously (Einhheber et al., 1996). Primary antibodies bound to the tissue were detected using the avidin–biotin complex (ABC) peroxidase method and the chromagen 3,3-diaminobenzidine (Aldrich Chemical Co., Milwaukee, WI) (Einhheber et al., 1996).

Immunoelectron Microscopy

Tissue sections immunolabeled with Caspr polyclonal antibody were processed for silver-enhanced immunogold labeling as described (Chan et al., 1990). Briefly, immunolabeled tissue was incubated for 2 h in 1:50 dilution of 1-nm colloidal gold-conjugated goat anti–rabbit IgG (Amersham Corp.), postfixed in 2% glutaraldehyde in PBS for 10 min, and reacted with silver solution using the intesSEM kit (Amersham Corp.). For electron microscopy, the immunolabeled sections were then fixed for 1 h in 2% osmium tetroxide, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon 812 between two sheets of Aclar plastic. Ultrathin sections (50 nm) were cut from the Epon–tissue interface, collected on copper grids, counterstained with 5% uranyl acetate and Reynolds’s lead citrate, and examined with an electron microscope (model 201; Philips Electron Optics, Mahwah, NJ).

Northern Analysis

Total RNA was prepared from Schwann cells, O-2A progenitors, and oligodendroglia (consisting of a mixed population of O4+/O1– and O1+), rat brain, and postnatal sciatic nerves by CsCl gradient centrifugation (Chirgwin et al., 1979). Equal samples (10 µg) of total RNA were electrophoresed in 1% agarose, 2.2 M formaldehyde gels, transferred to nylon membranes (Duralon; Stratagene, La Jolla, CA) in 6× SSC, and UV cross-linked. Blots were prehybridized, hybridized, and washed using standard techniques; the final washing stringency of the wash was 0.2× SSC at 65°C for 30 min. cDNAs used as probes included an ~500-bp fragment to rat Caspr and an ~1.5-kb fragment to human contactin as described (Peles et al., 1997b). 32P-labeled cDNA probes with specific activities of 2–5 × 106 cpm/mg were prepared by primer extension with random hexamers using the Promega kit (Promega Corp., Madison, WI) according to the manufacturer’s instructions.

Results

Caspr and Contactin Are Distinctly Expressed by Neurons and Myelinating Glia

We first determined the expression patterns of contactin/F3/F11 (contactin) and Caspr using well-characterized antibodies that recognize the extracellular and the cytoplasmic domains of these proteins, respectively. Purified cultures of sensory neurons, Schwann cells, oligodendrocyte progenitors, and differentiated progenitors (consisting of both O4+/O1– prooligodendrocytes and O1+ oligodendrocytes) were prepared, and expression was analyzed by immunofluorescence (Fig. 1). Contactin is expressed robustly by neurons (Fig. 1A), by oligodendrocytes (Fig. 1 E), and by their progenitors but not by Schwann cells (Fig. 1 C). By contrast, Caspr expression is restricted to neurons and their processes (Fig. 1 B); no staining of Schwann cells (Fig. 1 D) or of cells in the oligodendrocyte lineage (Fig. 1 F) was observed. Both Caspr and contactin are diffusely distributed along the entire surface of neurons and their processes.

Meylination Regulates the Expression and Distribution of Caspr

We next examined contactin and Caspr expression in established cocultures of Schwann cells and DRG neurons (Fig. 2). These cultures contained significant numbers of myelinated fibers as visualized by staining for myelin basic protein (MBP), which is a component of compact myelin (Fig. 2, B and D). Contactin (Fig. 2 A) is significantly downregulated in the cocultures in both ensheathed and myelinated fibers compared with isolated primary neurons. Contactin continues to be expressed at relatively high levels on the occasional unensheathed neurite that persists in such cultures (Fig. 2 A, asterisks). These results indicate that di-
neurons expressed Caspr at robust levels, whereas it was undetectable in Schwann cell and oligodendrocyte lysates. Caspr expression was greatly reduced in the cocultures compared with the neurons (more than 10-fold in several experiments). A progressive reduction in Caspr levels was also noticeable between 1 and 3 wk of coculture.

In parallel, we examined whether Caspr might be part of a cytoskeleton-enriched, detergent-insoluble complex. We extracted neuron cultures and myelinating cocultures (at 4 wk) with 1% Triton X-100 (Fig. 3 A, lanes T), solubilized the remaining material with SDS (Fig. 3 A, lanes S), and analyzed both fractions by Western blotting (Fig. 3 A, top right). Only ~25% of the total Caspr in the neuron cultures was extracted by Triton (Fig. 3 A, compare lane DRG:T to DRG:S). In the myelinating cocultures, less than 10% was extracted by Triton (Fig. 3 A, compare lane 3 wk:T to 3 wk:S), suggesting that even more of the Caspr is part of a detergent-insoluble complex. In related studies, we have found that Caspr staining persists in the cocultures after Triton extraction, whereas MBP and MAG are completely removed (data not shown). Likewise, extraction of brain membrane fractions with a variety of nonionic detergents (e.g., Triton, Brij, digitonin, octylglucoside) released less than half of the total Caspr in each case (data not shown). These results indicate that Caspr is associated with a detergent-insoluble, cytoskeleton fraction in vitro and in vivo.

We also examined contactin expression by Western blotting. Contactin is expressed by neurons and oligodendrocytes, but not by Schwann cells, and is similarly progressively downregulated in the cocultures (Fig. 3 A, bottom). Of note, contactin is detected as a doublet on neurons and as a single band on oligodendrocytes. We had previously found that the upper band of this doublet is removed by phosphatidylinositol phospholipase C, whereas the lower band is phosphatidylinositol phospholipase C-resistant and is likely a preform of contactin (Rosen et al., 1992). As shown in Fig. 3 B, Caspr and contactin expression also significantly decrease during postnatal sciatic nerve development (day 1 through adulthood). At all times, they are present at substantially reduced levels in sciatic nerves when compared with hippocampus (Fig. 3 B, lane Hc) used as a CNS control.

A Northern blot for contactin (Fig. 3 C) confirms its high level expression in oligodendroglia; a major band of ~6.4 kb and minor bands between 3–4 kb were present, consistent with previous reports (Gennarini et al., 1989). No expression of contactin was detected in Schwann cells or at varying times of sciatic nerve development. By contrast, Caspr mRNA of the expected size (6.2 kb) was detected in the CNS control but not in glia or sciatic nerve samples (data not shown). These results are consistent with the protein studies described above and indicate that Caspr and contactin in sciatic nerve are exclusively of axonal origin.

**Redistribution of Caspr during Myelination**

To investigate further the mechanisms by which Caspr becomes concentrated at the paranodes, we have analyzed its distribution at different times of myelination in the coculture system (Fig. 4). Schwann cells were seeded onto pre-established networks of DRG neurites and maintained for several days in a defined media in which Schwann cells pro-

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**Figure 1.** Expression of contactin and Caspr by neurons, Schwann cells and oligodendrocytes. Primary cultures of sensory neurons (A and B), Schwann cells (C and D), and oligodendrocytes (E and F) were stained with antibodies to contactin (A, C, and E) and Caspr (B, D, and F). Bar, 50 μm.
liferate but do not ensheathe or myelinate nerve fibers. Ascorbic acid was then added (day 0) to initiate ensheathment and myelination. Cultures were fixed at various times thereafter and double stained for Caspr and MAG, a myelin-specific protein that is expressed at the onset of myelination (Owens and Bunge, 1989).

New myelin sheaths begin to form on days 3 to 4 in the cocultures and can be detected by their expression of MAG (Fig. 4B, asterisks). Caspr is initially diffusely expressed along the entire neurite at this time, although at somewhat reduced levels compared with pure populations of neurons (Fig. 4A). Interestingly, in some instances there appeared to be a slight increase in Caspr expression under some of the forming segments of myelin (data not shown). By 6–7 d, myelin sheaths have begun to compact, as indicated by the redistribution of MAG into the periaxonal glial membrane, Schmidt-Lanterman incisures and the paranodal loops. At the same time, Caspr began to accumulate into myelinating cultures extracted with Triton X-100 (T) and SDS (S) probed with the Caspr antisera. (B) Changes in Caspr and contactin expression during postnatal sciatic nerve development. 100 μg of protein lysate, prepared from sciatic nerves of rats at postnatal days 1, 3, 7, 11, 15, 21, 25, 30, 40, and adult (A) were separated by SDS-PAGE, blotted onto nitrocellulose, and probed with either Caspr (B, top) or contactin (B, bottom) antisera. Note that the amounts of Caspr and contactin detected in sciatic nerve were considerably smaller than that in 100 μg of hippocampal lysate (Hc). (C) Northern blot analysis of contactin expression. A Northern blot with 10 μg per lane of total RNA isolated from postnatal day 1, 6, 10, 15, 34, and adult sciatic nerves, total rat brain (CNS) and cultured O-2A progenitors, O4+ oligodendroglia, and Schwann cells (Sc) was probed with a 32P-labeled human contactin cDNA probe. Bars indicate the position of 28S and 18S rRNA.
the paranodes of a few myelinated segments. (Examples of nascent paranodes are indicated by the arrowheads in Fig. 4 C.) Of additional note, Caspr expression frequently appeared attenuated at the center of corresponding internodes.

By 11 d, there is a striking accumulation of Caspr into multiple paranodes (Fig. 4, E and F). Concentrations of Caspr were invariably associated with well-myelinated segments, particularly those associated with MAG-positive Schmidt-Lanterman incisures, and accumulated into both paranodes of a myelinated segment at approximately the same time. Expression in the internode was reduced in those segments containing concentrations of Caspr at the paranodes compared with other segments that were still in the process of myelination. Two examples are indicated by arrows in Fig. 4, E and F, which mark paranodal accumulations. It may be seen that the mature myelin internodes located above the arrows express less Caspr than the two nascent myelin internodes located below the arrows. Taken together, these results indicate that Caspr accumulation in the paranodal region is a late event that occurs with myelin compaction and maturation and is likely to reflect a redistribution from the internode.

**Caspr Is Concentrated in Paranodes in the CNS and PNS**

To determine whether Caspr is localized at the paranodes of myelinated fibers in the CNS and PNS in vivo, whole mounts of teased rat sciatic nerve fibers and sections through various regions of the rat brain were immunolabeled with anti-Caspr antibody and examined by light microscopy. Intense paranodal staining for Caspr was observed in sciatic nerve fibers (Fig. 5 A), and virtually all of the fiber tracts in the brain, including the large myelinated axons of the facial nerve (Fig. 5 B) and the small, thinly myelinated axons of the corpus callosum (Fig. 6 A). Minimal staining, if any at all, was evident in the internodes. Paranodal staining was not observed with the corresponding control preimmune serum (data not shown). Although the protein
appeared to be absent from the outer surfaces of the paranodal loops, it could not be determined at the light microscopic level whether Caspr immunoreactivity was associated with the inner surfaces of the paranodal loops adjacent to the axon, the axonal membrane, or with both. More precise localization of the protein was resolved by immunoelectron microscopy (see below).

In addition to staining of the fiber tracts, low to moderate levels of diffuse Caspr immunoreactivity were present throughout the neuropil of the brain. An example of such diffuse staining in the lateral septal nucleus is shown in Fig. 6A (SN). Considerable punctate staining, most likely representing paranodal regions of small myelinated fibers but possibly representing other neuronal structures, was found throughout the neuropil. In general, neuronal cell bodies in most regions of the brain examined were either unlabeled or only lightly labeled by the Caspr antibody.

The distribution of contactin immunoreactivity in brain sections was also examined by light microscopy (Fig. 6B). In contrast to Caspr, and consistent with the in vitro studies described above, contactin was not concentrated at paranodal regions of myelinated fibers in these sections. However, relatively strong contactin staining of cells, possibly interfascicular oligodendrocytes, was observed in fiber tracts such as the corpus callosum (Fig. 6B inset, arrows). Light to moderate contactin immunostaining was also prominent in many neurons distributed throughout the brain, such as those of the cerebral cortex (Fig. 6B).

Caspr Is Localized to the Septate-like Junctions of the Paranodes

To determine more precisely the localization of Caspr within the paranode and to distinguish whether it was associated with neuronal and/or glial cell membranes, the distribution of the protein in the corpus callosum and the facial nerve (within the pons) was examined by immunoelectron microscopy. The results of these studies confirmed the light microscopic observations that Caspr is primarily concentrated at the paranodal regions of myelinated fibers. Furthermore, they showed that the protein is a component of the axonal membrane and not that of the paranodal loops of the glial cell. Representative nodal regions of myelinated fibers in the corpus callosum showing the silver-enhanced immunogold particles denoting the distribution of Caspr are shown in Fig. 7A–C. Similar patterns of paranodal labeling were observed in sections of the facial nerve (data not shown). Most of the immunolabeling at nodal regions was restricted to the inner surface of the axonal membrane containing the septate-like junctions. An example of the characteristic septae of these junctions are shown at high magnification in the inset of Fig. 7A. This staining of the inner membrane surface is expected given the reactivity of the antibody with cytoplasmic determinants on Caspr. In favorable planes of section, such as that shown in Fig. 7B, strikingly intense immunolabeling was observed along the presumptive inner surface of the paranodal axonal membrane. Importantly, virtually no labeling was observed in the nodal gap itself and only occasional particles were detected in the axonal membrane or cytoplasm in the internodes of myelinated fibers. Taken together, these results indicate that Caspr is likely to be a component of the septate-like junctions at the paranodal region.

In addition to its concentration at the paranodal region of myelinated fibers, rather intense Caspr immunogold labeling was also observed around the inner surfaces of axonal membranes of some small-diameter nerve fibers that appeared to be unmyelinated. Examples of such fibers from the corpus callosum cut in cross section are shown in Fig. 7D (a). These labeled profiles are unlikely to represent cross sections through the paranodal regions of myelinated fibers.
highly concentrated in the paranodal region. These studies detectable on myelinated fibers, whereas Caspr becomes different fates during myelination. Contactin is nearly un-diffusely expressed on unensheathed nerve fibers, have very 

We have shown that contactin and Caspr, which are both significant levels of this protein.

Discussion

We have shown that contactin and Caspr, which are both diffusely expressed on unensheathed nerve fibers, have very different fates during myelination. Contactin is nearly undetectable on myelinated fibers, whereas Caspr becomes highly concentrated in the paranodal region. These studies indicate that, although contactin and Caspr interact laterally (i.e., exhibit cis interactions) when they are in the same membrane in other settings (Peles et al., 1997b), they have distinct roles during myelination and are not obligately associated. These findings also implicate Caspr as a major component of the septate-like paranodal junctions. The expression of contactin and the localization of Caspr are considered further below.

Contactin Expression during Myelination

In characterizing the expression of contactin, we have confirmed a recent report that oligodendrocytes express this protein in culture (Koch et al., 1997). This expression by oligodendrocytes and their progenitors could promote the initial interactions of axons and oligodendrocytes, possibly via a homophilic mechanism. Contactin also interacts with other ligands, notably receptor protein tyrosine phosphatase β (Peles et al., 1995), which is expressed at high levels by radial glial cells and oligodendrocyte progenitors (Canoll et al., 1996b). Expression of contactin may therefore promote homotypic interactions between progenitors or heterotypic interactions with radial glia on which they appear to migrate (Zerlin et al., 1995). Interestingly, although contactin is robustly expressed by oligodendrocytes and their progenitors in vitro and by what appear to be interfascicular oligodendrocytes in vivo (Fig. 6 B, inset), there is little expression in white matter tracts or compact myelin. Consistent with these findings, other studies have reported contactin expression by neurons and their processes, including occasional axons in white matter, but not in myelin itself (Gennarini et al., 1989; Faiivre-Sarrailh et al., 1992). Contactin was recently detected in myelin fractions by biochemical methods (Koch et al., 1997), potentially reflecting the presence of oligodendrocyte membranes in these myelin fractions or, alternatively, a greater sensitivity of the blotting techniques used.

In contrast, Schwann cells do not express contactin based on immunofluorescence, Western, and Northern analyses. These results differ from those of a recent report demonstrating expression of F11, the avian homologue of contactin/F3, in chick Schwann cells in vitro and in sciatic nerve (Willbold et al., 1997). This may reflect differences in contactin expression between avian and mammalian species. We have also found that contactin expression on nerve fibers dramatically declines during postnatal sciatic nerve development, a period of rapid myelination, and during myelination in vitro. This expression pattern resembles that of other adhesion molecules on axons, including NCAM and L1, that are also downregulated during the transition from ensheathment to myelination (Martini and Schachner, 1986; for review see Salzer, 1995). Contactin appears to be persistently expressed at low levels on unensheathed nerve fibers in the cocultures (see Fig. 2), indicating that downregulation depends on direct Schwann cell contact.

Caspr Is the First Marker Specific for Paranodal Junctions

The structure of the paranodal junctions that form between axons and the paranodal loops of oligodendrocytes and myelinating Schwann cells were described over thirty

Figure 6. Contactin/F3 is not concentrated in the paranodal region. Staining of the corpus callosum (cc) with antisera against Caspr and contactin/F3 is shown. Caspr staining (A) is concentrated in the paranodal regions of the small myelinated fibers of the corpus callosum (shown at higher magnification in the inset). Contactin/F3 immunoreactivity is concentrated in cells that may be interfascicular oligodendrocytes based on their morphology (arrows in the higher-magnification inset) and location. Minimal contactin/F3 immunoreactivity was apparent in the paranodal regions. Moderately intense but diffuse Caspr immunoreactivity in the lateral septal nucleus (SN in A) and light contactin/F3 staining of cells in the cortex (Ctx in B), some of which appear to be neurons, were also apparent. Bar, 100 μm.
years ago (Bargmann and Lindner, 1964; Andres, 1965), but their molecular composition has remained unknown. A major finding of this study is the identification of Caspr as the first known marker of these junctions (shown diagrammatically in Fig. 8). Caspr seems likely to be a major transmembrane component of these junctions as well. Previous ultrastructural studies demonstrated that septate junction components arise from the axon, extending ~3–5 nm to contact the glial membranes of the paranodal loops (Peters et al., 1991). Freeze fracture studies have revealed circumferential rows of intramembranous particles within the axolemma at these junctions (Wiley and Ellisman, 1980), potentially corresponding to Caspr. The time course of the focal accumulation of Caspr at these sites, i.e., at later stages of myelination, agrees well with earlier studies demonstrating that septate junctions develop with maturation of the myelinated fiber (Tao-Cheng and Rosenbluth, 1982).

Of particular note, *Drosophila* Neurexin IV, a homologue of Caspr, was recently shown to be an essential component of the septate junctions that form between specialized glial cells (Baumgartner et al., 1996); Neurexin IV mutants lack the transverse septae characteristic of these junctions. Taken together, these results strongly support the notion that Caspr is an integral membrane component of the septate-like junctions at the paranodes.

**Sequential Reorganization of the Axolemma during Myelination**

These studies emphasize that the organization of the axon into three distinct longitudinal domains, i.e., the node, the paranodal region, and the internode, occurs sequentially.
During myelination. Previous studies have demonstrated that initial events in the formation of the node, characterized by clustering of intramembranous particles and sodium channels, occurs at the onset of myelination before compact myelin formation (for review see Black et al., 1995; Salzer, 1997) and can even occur in its absence (Deerinck et al., 1997). In contrast, paranodal junctions form during later stages of myelination, after the node has begun to mature, and require the formation of appropriately compacted myelin (Rosenbluth, 1983). Consistent with the sequential development of these domains, we have found that ankyrin G, a marker of the nodal cytoskeleton (Kordeli and Bennett, 1991), accumulates at the node several days before the accumulation of Caspr in the paranodes (Zanazzi, G., V. Bennett, and J. Salzer, unpublished observations).

The dramatic changes in the distribution of Caspr also indicate that myelination is critical to this sequential reorganization. Caspr is uniformly expressed on the surface of unmyelinated DRG neurites. In the cocultures, Caspr remains diffusely expressed along the axon until later stages of myelination (see Fig. 4); it is persistently expressed on unmyelinated axons in the CNS in the adult (see Fig. 7 D).

As the myelin sheath matures, Caspr levels dramatically increase in the paranodal region, accumulating in the paranodes of the most mature myelin sheaths first (see Figs. 2 and 4), consistent with morphologic studies of paranodal junction development (Tao-Cheng and Rosenbluth, 1982). Caspr accumulation in the paranodes is accompanied by a simultaneous decrease in the internode (see Fig. 4, C and E). This decrease may reflect, in part, more rapid turnover of Caspr in the internode during myelination (see Fig. 3 A), as well as its redistribution to the paranodes. Interactions with glial ligands and/or elements of the axon cytoskeleton may target Caspr to the paranodal region. The association of Caspr with a detergent insoluble fraction in neurons, myelinating cocultures, and brain membrane fractions as well as specific sequence motifs of its cytoplasmic domain (discussed below) are consistent with the latter possibility.

### Potential Role of Paranodal Junctions in Channel Distribution and Signaling

It has been proposed that invertebrate septate junctions serve several functions, including a role analogous to that of tight junctions in providing a transcellular barrier to the passage of solute molecules and in promoting the generation of apical and basal lateral compartments (Lane, 1991). In strong support of this notion, the blood–nerve barrier that forms between the specialized glial cells is disrupted in a Drosophila Neurexin IV mutant (Baumgartner et al., 1996). The paranodal junctions of myelinated fibers probably have a similar role in forming a partial physical barrier. Studies in which electron-dense tracers were infused in peripheral nerves, for example, have demonstrated that these junctions provide a partial barrier to the diffusion of small ions and an absolute barrier for the passage of large molecular weight compounds into the internode (Hirano and Lena, 1995). By retarding ionic movements, these junctions are likely to promote more efficient saltatory conduction. Paranodal junctions may also regulate the distribution of voltage-gated channels. Indirect evidence, including freeze fracture studies, suggests they physically demarcate the boundaries of the node by limiting the lateral diffusion of Na+ channels, thereby confining them to the node (Rosenbluth, 1983). Thus, while the paranodal junctions may not be required for the initial clustering of Na+ channels, they may restrict the subsequent distribution of these channels. Less is known about factors that regulate the distribution of specific voltage-gated K+ channels (Kv1.1 and Kv1.2), which are concentrated in the juxtaparanodal region (Wang et al., 1993; Mi et al., 1995). The paranodal junctions are appropriately positioned to have a similar role in limiting the lateral diffusion of K+ channels but have not yet been directly implicated.

With this report, we have shown that paranodal junctions not only resemble invertebrate septate junctions in their morphology but also share homologous proteins. The molecular characterization of septate junctions has progressed rapidly in recent years, largely from analysis of Drosophila mutants. Invertebrate septate junctions have been found to be associated with actin filaments and associated proteins (Lane and Flores, 1988; Colombo et al., 1993). The localization of a second protein to Drosophila septate junctions, band 4.1–Coracle (Fehon et al., 1994), provides a mechanism for this linkage. Band 4.1 is a prototype of a large family of cytoskeletal proteins that includes the ERM (ezrin/radixin/moesin) family of cytoskeletal proteins and several phosphatases (for review see Tsukita et al., 1997). It functions as a link between transmembrane proteins and the underlying spectrin/actin cytoskeleton (Bennett and Gilligan, 1993). It is of interest that Drosophila Neurexin IV and Caspr both contain cytoplasmic sequences homologous to those in glycoporphin, a red blood cell protein, that binds to the erythrocyte band 4.1 protein. Of additional note, in Drosophila Neurexin IV mutants, band 4.1–Coracle is no longer restricted to the septate junctions (Baumgartner et al., 1996). These results provide strong support for a direct interaction of Neurexin IV with band 4.1–Coracle.

While it is not yet known whether Caspr is similarly linked to any band 4.1 superfamily members, current evidence supports its association with the axon cytoskeleton. The spacing between the septae at the paranodes is highly regular (25–30 nm and see Fig. 7 A, inset) (Peters et al., 1991), suggesting an organized structure that might be imposed by cytoskeletal associations. Indeed, previous ultrastructural studies have demonstrated a linkage between membrane proteins of the axon and the underlying cytoskeleton at the paranodes (Ichimura and Ellisman, 1991), although the nature of the proteins has not yet been determined. Finally, we have shown (Fig. 3 A) that Caspr is poorly extracted by nonionic detergents from neurons alone and from myelinating cocultures and brain membrane preparations. Taken together, these results indicate that Caspr is likely to be constitutively associated with the axon cytoskeleton, possibly through a band 4.1 superfamily member. Such a linkage could regulate its targeting to the paranodal junctions.

The structure of the cytoplasmic region of Caspr suggests it may also regulate intracellular signaling pathways in axons. Notably, this segment contains a proline-rich sequence with at least one canonical SH3 domain–binding site (Peles et al., 1997b). Consistent with this suggestion, fusion proteins containing the SH3 domains of the signaling proteins PLCγ, src, fyn, and the p85 subunit of PI 3-kinase bound
to the cytoplasmic domain of Caspr (Peles et al., 1997b). It is not yet known whether these proteins are associated with paranodal junctions in vivo. However, this would be an attractive possibility, as the paranodal region appears to be a site of local axon–glia signaling. For example, the diameter of the axon in the nodal region is dramatically reduced (up to 80% in large fibers). This reduced diameter reflects alterations in the level of phosphorylation and the packing density of axonal cytoskeletal proteins (Waegh et al., 1992). The rate of axonal transport is also significantly diminished in the paranodal region (Waegh et al., 1992; Fabricius et al., 1993), which is consistent with local signaling.

An important question is the identity of the glial ligand(s) for Caspr and whether Caspr regulates glial function. Freeze fracture studies have demonstrated that there are glial particles within the paranodal loops that are juxtaposed over axolemmal particles in the paranodal junctions (Wiley and Ellisman, 1980). These glial proteins are candidate ligands for Caspr. To date, they have not been identified nor are any glial proteins known that have a complementary distribution to Caspr. Like all neurexins, the extracellular segment of Caspr contains domains homologous to laminin G domains and EGF repeats (Littleton et al., 1997). Notably, Neurexin IV may interact with the discs-large tumor suppressor protein, a MAGUK protein that is found in septate junctions and has a role in cell signaling and differentiation (Woods and Bryant, 1991). This interaction may be unique to Drosophila Neurexin IV, as Caspr lacks a PDZ binding domain and several mammalian homologues of discs-large have not been localized to the paranodes (Cho et al., 1992; Müller et al., 1995; Hunt et al., 1996).

In summary, we have shown that the expression and distribution of Caspr and contactin are differentially regulated during myelination. Of particular interest is the localization of Caspr to the septate-like junctions of the paranode. This is the first component of these vertebrate junctions to be identified and this, together with its homology to Drosophila Neurexin IV, suggests that it is a major transmembrane component required for their integrity. These findings have important implications for the identity of other proteins that are associated with Caspr in these junctions and for the potential role of Caspr in reciprocal axon–glia signaling.

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Note Added in Proof. After this paper was submitted, the cloning and localization of paranodin, a protein identical in sequence to Caspr, was reported (Menegoz, M., P. Gaspar, M. Le Bert, T. Galvez, F. Burgaya, C. Palfrey, F. Ezan, F. Aruos, and J.-A. Girault. 1997. Neuron. 19:319–331).

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Caspr and Other Neurexins Are Novel Candidates to Mediate Axon–Glia Interactions

The neurexins comprise a large family of neuronal surface proteins that are candidate mediators of synaptic interactions and, potentially, target recognition (Ushkaryov et al., 1992). A recent study suggests that other neurexins may mediate axon–glia interactions. In particular, a pan-neurexin antibody, raised to a sequence present in neurexins I–III but not Caspr, stained the electromotor nerve of the electric fish at the axon interface with myelinating Schwann cells (Russell and Carlson, 1997). Whether or not other neurexins, in addition to Caspr, also mediate axon–glia interactions in mammals will require further study. This pan-neurexin antibody also stained the perineurial cells of the electromotor nerve, raising the possibility that neurexins may play a more general role in the tight association of cells in this layer. By Northern analysis, we have not detected Caspr mRNA in the sciatic nerve, suggesting it is not expressed in the vertebrate perineurium.
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