Molecular Composition of the Node of Ranvier: Identification of Ankyrin- binding Cell Adhesion Molecules Neurofascin (Mucin+/Third FNIII Domain−) and NrCAM at Nodal Axon Segments

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Abstract. Neurofascin, NrCAM, L1, and NgCAM are a family of Ig/FNIII cell adhesion molecules that share ankyrin-binding activity in their cytoplasmic domains, and are candidates to form membrane-spanning complexes with members of the ankyrin family of spectrin-binding proteins in a variety of cellular contexts in the nervous system. Specialized forms of ankyrin, 270 kD and/or 480 kD ankyrin$_G$ are components of the membrane undercoat of axons at the node of Ranvier. This paper focuses on definition of the isoforms of ankyrin-binding cell adhesion molecules localized with ankyrin$_G$ at the nodal axon segment. The exon usage of two major forms of neurofascin was determined by isolation of full-length cDNAs and used to prepare isoform-specific antibodies. An isoform of neurofascin containing a mucin-like domain and lacking the third FNIII domain was concentrated at axon initial segments and colocalized at nodes of Ranvier with ankyrin$_G$ and the voltage-dependent sodium channel. An alternative form of neurofascin lacking the mucin-like domain and containing the third FNIII domain was present in unmyelinated axons. The antibody initially raised against neurofascin was used to screen a rat brain cDNA expression library. In addition to neurofascin, this screen yielded a clone with 80% sequence identity to NrCAM from chicken. The sequences of two full-length cDNAs are presented. NrCAM is most closely related to neurofascin among the other members of the L1/neurofascin/NgCAM family, with over 70% identity between cytoplasmic domains. NrCAM, visualized with antibodies specific for the ecto-domain, also was found to be coexpressed with neurofascin at nodes of Ranvier and at axon initial segments. This is the first characterization of defined neuronal cell adhesion molecules localized to axonal membranes at the node of Ranvier of myelinated axons.

MYELINATION of axons is an important evolutionary advance of vertebrates that permits rapid, saltatory propagation of action potentials without an increase in axonal diameter. This physiological achievement is based on adaptations at a molecular level resulting in an elegant cooperation between glial cells and nerve axons. Myelinated axons are enveloped by multiple insulating layers of glial cell membrane which are interrupted at specialized regions first discovered by Ranvier (1874) by light microscopy and now known as nodes of Ranvier. Nodes of Ranvier are the sites where ion fluxes occur in propagation of action potentials, and are comprised of two adjacent specializations of the axonal plasma membrane characterized by high local concentrations of voltage-regulated ion channels: a paranodal region underlying the paranodal processes of glial cells which contains fast 4-AP-sensitive K^+ channels, and a nodal axon segment between paranodes which is enriched in voltage-dependent sodium channels (Waxman and Ritchie, 1993). The paranodal domain of axons is tightly opposed to paranodal loops of either Schwann cells in the peripheral nervous system or oligodendrocytes in the central nervous system. The nodal axon segment is in contact with specialized microvilli of either Schwann cells (Berthold and Rydmark, 1995) or astrocytes (Black et al., 1995). Nodes of Ranvier are of considerable clinical interest due to their involvement in pathological conditions including peripheral neuropathies (Griffin et al., 1996; Sima et al., 1993), axonal ischemic injury (Waxman et al., 1992), and trauma (Maxwell et al., 1991). Nodes also are the sites of regeneration of damaged peripheral nerve axons (Fawcett and Keynes, 1990). Myelinated axons and nodes of Ranvier also exemplify basic issues for cell biologists: formation of polarized cell domains, assembly of integral proteins into lateral membrane domains, and formation of morphologi-

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cal structures that require cooperation between distinct types of cells.

An essential step in understanding assembly and function of nodes of Ranvier is to define the molecular organization of this structure. Proteins localized in paranodal loops of glial cells include E-cadherin and associated proteins (Fannon et al., 1995), the gap junction protein connexin 32 (Scherer et al., 1995), myelin-associated glycoprotein (Guarles and Trapp, 1984; Trapp et al., 1989), as well as cytoskeletal proteins actin and spectrin (Trapp et al., 1989). However, little is currently known of the composition of the nodal axon segment. One strategy to achieve this goal is to begin with identification of unique components of this specialized membrane domain and then to determine their nearest neighbors. Voltage-dependent sodium channels are the defining membrane protein of the nodal axon and are concentrated ~40-fold relative to internodal membranes. The voltage-dependent sodium channel copurifies with the membrane skeletal protein ankyrin, and associates with ankyrin in in vitro binding assays (Srinivasan et al., 1988). Ankyrin is highly concentrated at the node of Ranvier (Kordeli et al., 1990) and is a component of the distinctive dense plaque of material on the cytoplasmic surface of the nodal axonal membrane resolved by early electron microscopists (Robertson, 1959; Peters, 1966). Ankyrin at the node of Ranvier is a distinct isoform (Kordeli and Bennett, 1992) of a family of spectrin-binding proteins that associate via their membrane-binding domains with diverse integral proteins, including ion channels, calcium release channels, and cell adhesion molecules (Bennett and Gilligan, 1993; Lambert and Bennett, 1993). Ankyrins are localized in specialized membrane domains in addition to nodes of Ranvier including sites of cell–cell contact in epithelial tissues (Drenckhahn and Bennett, 1987; Nelson and Veshnock, 1987), the neuromuscular junction (Flucher and Daniels, 1989), and transverse-tubule-related structures in striated muscle (Li et al., 1993). Ankyrin at nodes of Ranvier is a candidate to participate in a complex involving the voltage-dependent sodium channel and the spectrin/actin network.

The isoform of ankyrin localized at the node of Ranvier has recently been identified as 480 and 270 kD alternatively spliced variants of the ankyrin$_G$ gene (Kordeli et al., 1995). 480 and 270 kD ankyrin$_G$ contain a membrane-binding domain comprised of ANK repeats, a spectrin-binding domain, a 46-kD serine/threonine-rich domain glycosylated with O-GlucNAc residues, which distinguishes nodal isoforms of ankyrins from other ankyrins (Zhang and Bennett, 1996), a stretch of up to 2,100 amino acids for 480-kD isoforms predicted to be configured as a random coil (Chan et al., 1993; Kordeli et al., 1995) and a COOH-terminal domain. Two distinct membrane protein-binding sites have been identified in the membrane-binding domain of ankyrin (Michaely and Bennett, 1995*a*,*b*), suggesting the possibility that two voltage-gated sodium channels or perhaps a channel and an additional ankyrin-binding protein could simultaneously bind to the same ankyrin molecule.

Neurofascin, NrCAM, L1, and NgCAM in vertebrates and neuroglian in Drosophila are a family of Ig/FNIII[1] cell adhesion molecules that share ankyrin-binding activity in their cytoplasmic domains, and are candidates to form membrane-spanning complexes with members of the ankyrin family of spectrin-binding proteins in a variety of cellular contexts in the nervous system (Davis et al., 1993; Davis and Bennett, 1994; Dubreuil et al., 1996). These molecules are a subgroup of the immunoglobulin super family of cell adhesion molecules and are characterized by extracellular domains comprised of 6 Ig domains and 3–5 FNIII domains and cytoplasmic domains containing an ankyrin-binding site localized to a highly conserved stretch of amino acids (Davis and Bennett, 1994). This family has been credited with a wide range of functions, both adhesive and signaling in nature, during the development of the nervous system. These functions include neurite extension (Morales et al., 1993, Williams et al., 1992), axonal guidance (Stoeckli and Landmesser, 1995; Hortsch and Goodman, 1991), synaptogenesis (Itoh et al., 1995), an involvement in neuron-glial cell interactions (Suter et al., 1995) and myelination (Wood et al., 1990). Many of these activities are highly localized to defined areas of the neuronal and glial cell plasma membrane illustrating the potential importance of the ability of these molecules to interact with ankyrin and the membrane skeleton. Indeed, mutations in the cytoplasmic domain of L1, have been associated with mental retardation in humans (Jouet et al., 1995), although whether these mutations affect ankyrin-binding activity, or the ability of L1 to activate second messenger signaling pathways remains to be established.

As to be expected from the range of functions associated with the ankyrin-binding cell adhesion molecules, members of this family exhibit considerable diversity in their extracellular domains. The primary sequences of these domains are quite diverse between different family members, and within individual genes a second degree of diversity is generated by alternative mRNA splicing (Grumet, 1991; Sonderreger and Rathgen, 1992). Neurofascin, for example, has six potential sites for alternative exons (Davis et al., 1993; Volkmer et al., 1992), with a potential for 64 variants if all possible combinations are expressed.

Members of this family of ankyrin-binding cell adhesion molecules have been localized at the node of Ranvier (Mirsky et al., 1986; Rieger et al., 1986; Davis et al., 1993), and are candidates to interact with 480 and 270 kD ankyrin$_G$. These cell adhesion molecules at the node of Ranvier could potentially be important in mediating axonal-glial contacts resulting in signaling as well as simple adhesion, and in establishing connections between cytoplasmic and extracellular proteins. However, more information is required with regard to gene product and exon usage in order to evaluate possible functions at the node of Ranvier, as well as in other contexts in the nervous system. This paper focuses on definition of the isoforms of ankyrin-binding cell adhesion molecules localized to the nodal axon segment. The exon usage of two major forms of neurofascin were determined by isolation of full-length cDNAs, and this information was used to prepare isoform-specific antibodies. An isoform of neurofascin containing a mucin-like domain and lacking the third FNIII domain was localized to nodes of Ranvier as well as axon initial segments, while an alternative form of neurofascin lacking the mucin-like domain and containing the third FNIII domain

1. *Abbreviation used in this paper*: FNIII, fibronectin type 3.

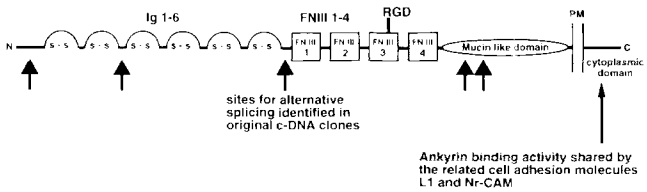


Figure 1. Model of the domain organization of rat neurofascin derived from the composite sequence showing regions for potential alternative splicing (*arrows*).

was present in unmyelinated axons. In addition, NrCAM, which is encoded by a distinct gene closely related to neurofascin, was found to be coexpressed with neurofascin at nodes of Ranvier and axon initial segments.

Materials and Methods

Methods

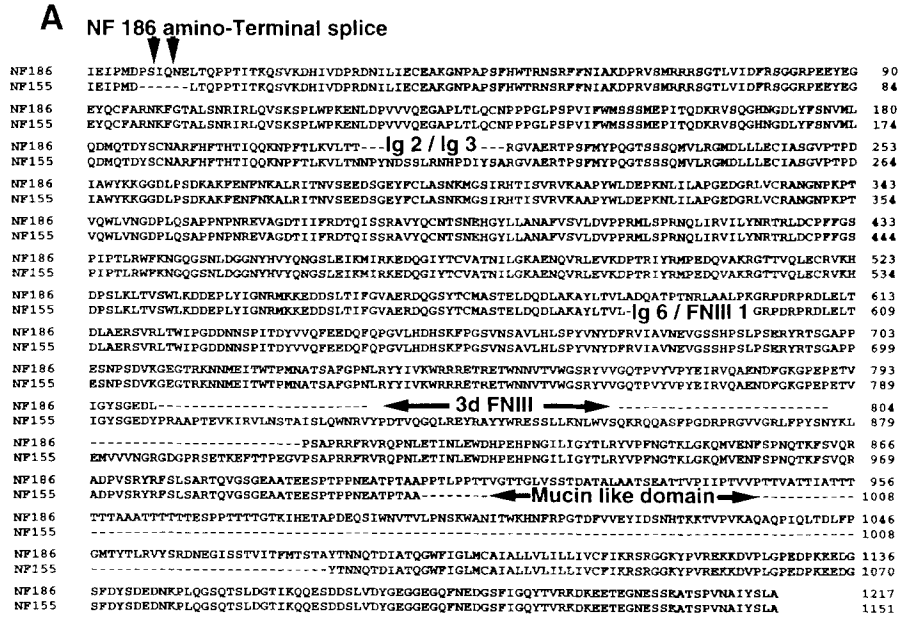
SDS-PAGE, immunoblot analysis following transfer of polypeptides to nitrocellulose paper, and preparation of affinity-purified rabbit antibodies were performed as described for ankyrin and other proteins (Davis and Bennett, 1984). Isolation of neurofascin polypeptides, L1, and NrCAM from detergent extracts of adult rat brain membranes were performed as described (Davis et al., 1993; Davis and Bennett, 1994). Molecular biology methods were performed essentially as described (Sambrook et al., 1989). cDNA clones were subcloned from λ phage into Bluescript (KS+) plasmids. DNA sequencing of plasmids was performed by the dideoxy chain termination method using oligonucleotide primers to initiate the reactions. Immunocytochemistry with frozen sections of rat tissue from animals perfused with 2% paraformaldehyde was performed as described (Kordeli et al., 1990; Kordeli and Bennett, 1991). Antibodies were visualized with rhodamine-labeled goat anti-rabbit Ig from Cappel (Malvern, PA) alone, or together with fluorescein-labeled mouse monoclonal anti-chicken Ig (clone CH31 from Sigma Chemical Co., St. Louis, MO) in double-labeling experiments.

Isolation of Full-Length cDNAs Encoding Alternatively Spliced Forms of Neurofascin and NrCAM

Nucleic acid probes 0.2–0.5 kb in length corresponding to the 5′ and 3′ end of clones encoding neurofascin (Davis et al., 1993) and NrCAM (identified during initial screening for neurofascin) were radiolabeled and used to screen a 5′ stretch oligo-dT and random-primed λ GT11 expression library prepared from adult rat brain mRNA with an average insert size of 1.6 kb (Clontech, Palo Alto, CA).

Expression and Purification of Recombinant Proteins

cDNAs encoding extracellular domains of rat neurofascin (aa 581-1020 of nf155) and NrCAM (aa 583-1018 of nr22) were ligated into a pET bacterial expression vector, which contains a T7 promoter (Studier et al., 1990). A unique NheI restriction site was generated by PCR. Constructs were inserted into the NheI site immediately 3′ to the AUG codon so that ex-



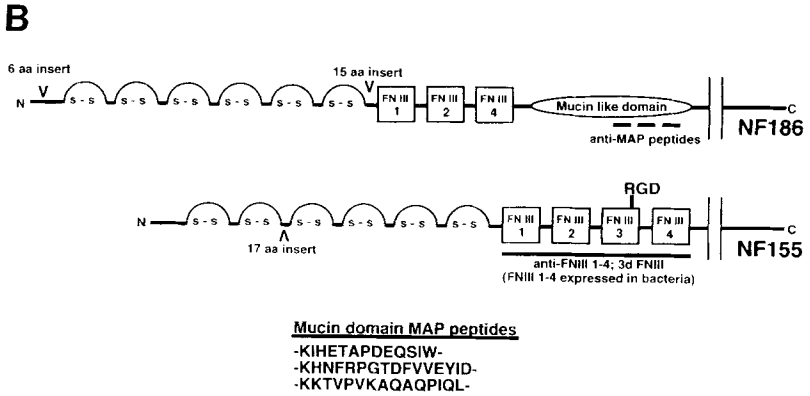


Figure 2. Mutually exclusive patterns of exon usage in isoforms of rat neurofascin. (*A*) Alignment of the deduced amino-acid sequences of two neurofascin full-length clones. Full-length c-DNA clones were isolated from a rat brain λ gt11 expression library using both 5′ and 3′ c-DNA probes on double lifts of the library (Materials and Methods). Two clones with alternative splicing at the amino terminus corresponding to isolated protein products were fully sequenced. (*B*) Models of the domain organization and alternative splicing of neurofascin full-length clones. Regions selected for production of clone-specific antibodies are underlined (Materials and Methods).

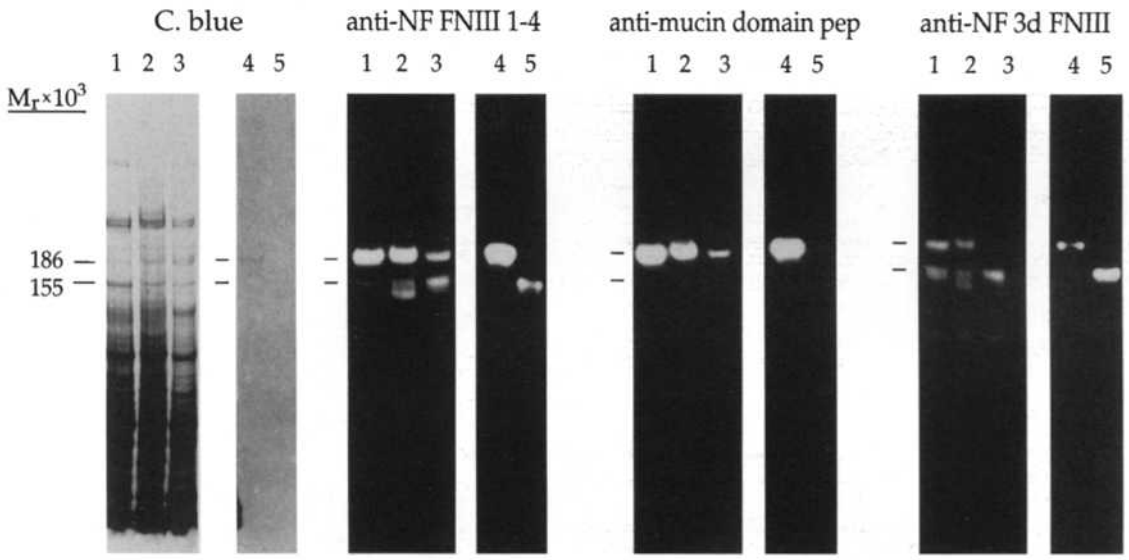


Figure 3. Characterization of antibodies raised against neurofascin mucin-like and FNIII domains. Immunoblots of various regions of rat brain were prepared using affinity-purified Ig against total neurofascin FNIII 1-4 domain, the mucin-like domain, and the third FNIII domain (Materials and Methods). (Lane *1*) Forebrain; (lane *2*) cerebellum; (lane *3*) brain stem; (lane *4*) isolated 186-kD neurofascin; (lane *5*) isolated 155/140-kD neurofascin.

pressed polypeptides have the additional sequence MAS on the amino terminus (Davis et al., 1991). The recombinant plasmids were used to transform a nonexpressor JM109 strain. Plasmids purified from the JM109 strain were later transformed into an expressor strain of *E. coli* (BL21 (DE3). Expression of recombinant polypeptides was induced by addition of IPTG. The bacteria were harvested at 4 h postinduction. Expressed proteins were isolated from inclusion bodies solubilized in 8 M urea, 1 M NaBr, 10 mM sodium phosphate, 1 mM dithiothreitol, 10 mM glycine, 1 mM Na EDTA, pH 7.4, using gel filtration on Superose 12 and ion exchange chromatography on Mono S and Mono Q resins.

Antibodies

Mucin-like domain specific antibodies to neurofascin were generated in rabbits using three multiple antigen peptides (Maps) as an antigen (KIHETAPDEQSIW; KHNFRPGTDFVVEYID; KKIVPVKAQAQPIQL) and were affinity purified on a 186-kD neurofascin-agarose column. Neurofascin and NrCam FNIII-domain antibodies were generated in rabbits using purified FNIII domains(1-4) which were expressed in bacteria (neurofascin residues 581-1020; NrCam residues 583-1018). The antibody was then affinity purified from sera on native 186-kD neurofascin and NrCam ecto-domain Sepharose columns, respectively. One of three rabbits immunized with the neurofascin FNIII domain produced a serum which preferentially reacted with one of the lower neurofascin protein products. This serum was affinity purified by first passing it through a precolumn of native 186-kD neurofascin-agarose followed by collection and elution from a 155/140-kD neurofascin-agarose column. Affinity-purified rabbit antibody against the α subunit of the voltage-dependent sodium channel and chicken antibody against the tail domain of 480/270-kD ankyrin$_G$ were prepared as described (Lambert, S., J.Q. Davis, and V. Bennett, manuscript in preparation).

Results

Reciprocal Expression of the Mucin-like Domain and Third FNIII Domain in Rat Neurofascin Transcripts

Neurofascin in adult rat brain includes polypeptides of 186 and 155 kD and a minor form of 140 kD confined to the cerebellum that represent products of alternatively spliced mRNAs, based on a six–amino acid insertion present in the 186-kD polypeptide but not the 155- and 140-kD polypeptides (Davis et al., 1993). Potential diversity due to splicing also was suggested by alternative exons at multiple sites located in the extracellular domain in partial cDNAs isolated during cloning of neurofascin from rat (Davis et al., 1993) and chicken (Volkmer et al., 1992) (Fig. 1). Antibody that recognized 186, 155, and 140 kD neurofascin cross-reacted strongly with nodes of Ranvier, as well as other structures in peripheral nerve (Davis et al., 1993). Immunoblots of sciatic nerve revealed the 155-kD polypeptide as the major form of neurofascin, and thus a candidate for the isoform of neurofascin at the node of Ranvier (Davis et al., 1993). However, nodes of Ranvier represent only a small fraction of the total protein, and immunoblot results could reflect contributions of other structures. The question of which of these alternatively spliced forms of neurofascin were localized at nodes of Ranvier was answered by first determining the pattern of exon usage for the major forms of neurofascin, and then using this information to develop isoform-specific antibodies.

The strategy to determine actual combinations of exons present in the major neurofascin transcripts was to isolate and sequence full-length cDNAs. A cDNA library prepared from adult rat brain was screened for clones producing DNA reacting with probes derived from both 5′ and 3′ ends of neurofascin composite cDNA (Materials and Methods). This screen resulted in two clones with sequences and predicted domain organization presented in Fig. 2. One sequence contains a 173 residue domain enriched in serine, threonine, and proline residues with sequence similarity to mucin, lacks the third FNIII domain, and contains insertions at the NH_2 terminus (PSIQNE)

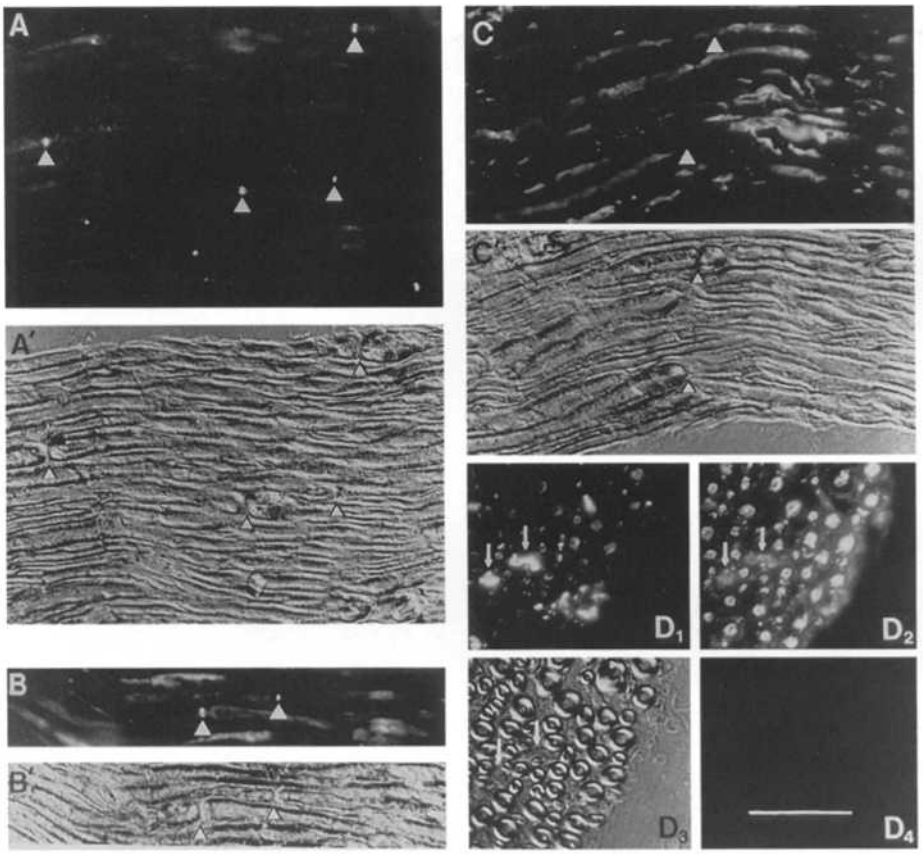


Figure 4. Localization of neurofascin (mucin+/FNIII−) and (mucin−/FNIII+) in rat sciatic nerve by indirect immunofluorescence. 5 μM frozen sections of rat sciatic nerve (*A–D*) were examined by immunofluorescence using affinity-purified rabbit Ig against the mucin-like domain of neurofascin (*A*); 480/270-kD ankyrin$_G$ (*B*), the third FNIII domain of neurofascin (*C* and D_1). D_2 is double-labeled with a monoclonal antibody to neurofilament as a marker for axons. *A–C* are longitudinal sections. *D1–4* are cross-sections of sciatic nerve showing both myelinated neurons and bundles of unmyelinated axons. *A'–C'* and D_3 are corresponding DIC images of the stained sections. Arrowheads denote nodes of Ranvier. Bar, 100 μm.

and between the Ig and FNIII domains (ADQATPTNRLAALPK). The other sequence contains a third FNIII domain, and lacks a mucin-like domain as well as inserted sequences at the NH_2-terminal site and at the Ig/FNIII domain boundary, but does have an inserted sequence (NNPYNDSSLRNHPDIYSA) between the second and third Ig domains. We infer that these transcripts result from alternative splicing of the neurofascin gene. A formal proof that these particular combinations of exons exist as RNA transcripts would require additional work beyond the scope of this paper. An unanticipated feature of the sequences was the completely reciprocal pattern of exon usage resulting in substantial differences between the extracellular domains of these forms of neurofascin. This result implies mutually exclusive alternate splicing pathways and will be of interest to explore further.

Antibody raised against peptides from the mucin-like domain cross-reacted with the 186-kD neurofascin polypeptide and not with 155 or 140 kD forms of neurofascin (Fig. 3). Several attempts to prepare antibodies against peptides derived from the third FNIII domain were not successful (part of this study). However, antibodies were raised against a polypeptide containing all four FNIII domains, and reacted, as expected, with 186, 155, and 140 kD neurofascin polypeptides (Fig. 3). Antibodies from one rabbit reacted preferentially with the 155-kD neurofascin polypeptide compared to 186 kD and 140 kD forms of neurofascin, and were preadsorbed with immobilized native 186 kD neurofascin and affinity-purified using 155 and 140 kD polypeptides coupled to agarose (Fig. 3) (Materials and Methods). A weak reaction with 186 kD and 140 kD forms of neurofascin on immunoblots remained even after two passages through the 186-kD neurofascin-column (not shown). These antibodies may recognize epitopes not available on the native protein and expressed only after denaturation. Antibodies reacting preferentially with 155 kD neurofascin are most likely directed against epitopes in the third FNIII domain, since they were raised against all four FNIII domains but react only weakly with protein expressed in cultured cells transfected with the transcript, NF186 (Fig. 2) which lacks the third FNIII domain (not shown).

These results demonstrate that a 186-kD neurofascin contains the mucin-like domain, and lacks the third FNIII domain, while a 155-kD neurofascin lacks the mucin-like domain and contains the third FNIII domain. In addition, 186 kD neurofascin is known, based on amino acid sequence analysis, to contain a 6–amino acid insertion at the NH_2 terminus, while 155 kD neurofascin lacks this insertion (Davis et al., 1993). 186 kD neurofascin therefore is likely to be encoded by the first full-length sequence and 155 kD neurofascin by the second full-length sequence. Biochemical evidence that 186 kD neurofascin contains a mucin-like domain is that this polypeptide is degraded by *O*-sialoglycoprotease, an enzyme from *Pasturella hemolytica* that selectively degrades only polypeptides containing clustered, sialic acid-modified O-linked saccharides (Sutherland et al., 1992; Norgard et al., 1993). 155 kD neurofascin is resistant to *O*-sialoglycoprotease under the same conditions where 186 kD neurofascin is degraded (not shown).

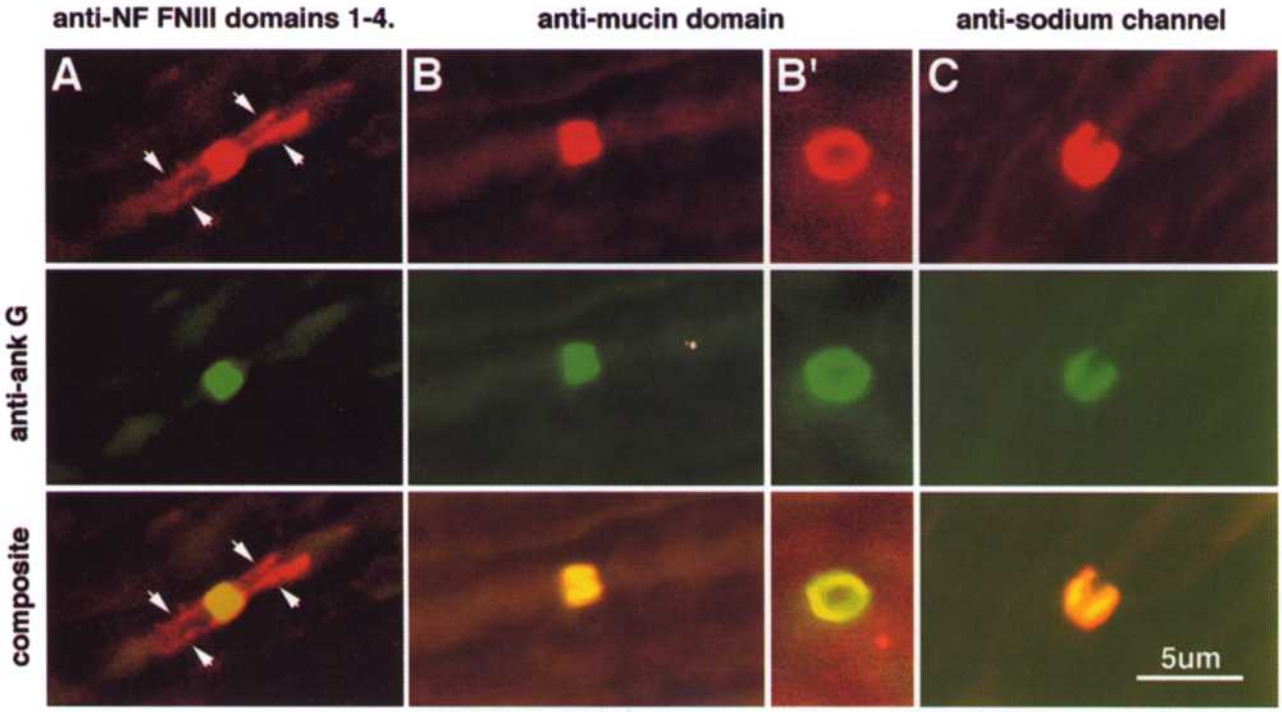


Figure 5. Neurofascin (mucin+/third FNIII−) colocalizes with ankyrin$_G$ and the voltage-dependent sodium channel at the nodal axon segments. 5 μm frozen sections of rat sciatic nerve were double-labeled with affinity-purified Ig against 480/270-kD ankyrin$_G$ and (*A*) anti-neurofascin FNIII domains 1-4. (*B*) Anti-neurofascin mucin-like domain and (*C*) the voltage-dependent sodium channel (Materials and Methods). Double-labeled images were collected using a confocal microscope and these composite images are shown in the bottom row. *B'* shows double-labeling for neurofascin mucin-like domain and ankyrin$_G$ on the axonal plasma membrane in transverse nodal sections. Arrowheads (*A*) indicate paranodal staining with neurofascin FNIII domains not seen with antibodies against ankyrin$_G$ or the neurofascin mucin-like domain.

140 kD neurofascin apparently lacks both mucin and the 3rd FNIII domain, suggesting additional diversity not encompassed by available full-length cDNAs. The complex pattern of expression of neurofascin polypeptides observed by Volkmer and colleagues (Volkmer et al., 1992) provides further evidence for additional alternatively spliced forms of neurofascin in adult as well as developing brain. Therefore, in the remainder of this paper neurofascin resolved by immunofluorescence will be referred to as neurofascin (mucin+/3rd FNIII−) and neurofascin (mucin−/3rd FNIII+) without further specification of exon usage.

Neurofascin (Mucin+/3rd FNIII−) Is Localized at the Node of Ranvier While Neurofascin (Mucin−/3rd FNIII+) Is Limited to Unmyelinated Axons

Frozen sections of sciatic nerve and cerebellum were stained with antibodies against either the mucin-like domain or third FNIII domain of neurofascin (Fig. 4). Antibody against the mucin domain stained myelinated axons at highly focused sites that coincided with nodes of Ranvier in DIC micrographs (Fig. 4, *A* and *A'*). In contrast, antibody against the third FNIII domain did not react with nodes of Ranvier, but did label structures that we interpret as bundles of unmyelinated axons (Fig. 4). This interpretation is based on transverse sections where label by antibody is excluded from myelinated axons, visualized in DIC micrographs, but is colocalized with neurofilaments, which are a marker for axons (Fig. 4, *D1–3*).

Nodes of Ranvier include nodal and paranodal regions of the axonal membrane, which are distinguished by localization of voltage-dependent sodium channels (Waxman and Ritchie, 1993) and ankyrin (Kordeli et al., 1990) in the nodal membrane and rapidly activating potassium channels in the paranodal domain (Wang et al., 1993). Colocalization of neurofascin with ankyrin$_G$ and the voltage-dependent sodium channel in the nodal region of axons was evaluated by double-labeling with a chicken antibody against the tail domain of ankyrin together with rabbit antibodies against either the mucin-like domain of neurofascin or against the voltage-dependent sodium channel (Fig. 5 *C*). All of the nodes visualized with antibody against the neurofascin mucin-domain also were labeled with antibody against ankyrin$_G$, and, at the level of the confocal microscope, the staining for these antibodies colocalized at nodes. Transverse sections through nodes of Ranvier also revealed colocalization of neurofascin and ankyrin$_G$ on the axonal plasma membrane (Fig. 5 *B'*). All of the nodes observed with antibody against the voltage-dependent sodium channel also were labeled with antibody to ankyrin$_G$ (Fig. 5). These results imply that antibodies against ankyrin$_G$, neurofascin mucin domain, and the voltage-dependent sodium channel label the same site of the axonal plasma membrane of all nodes of Ranvier in sciatic nerve.

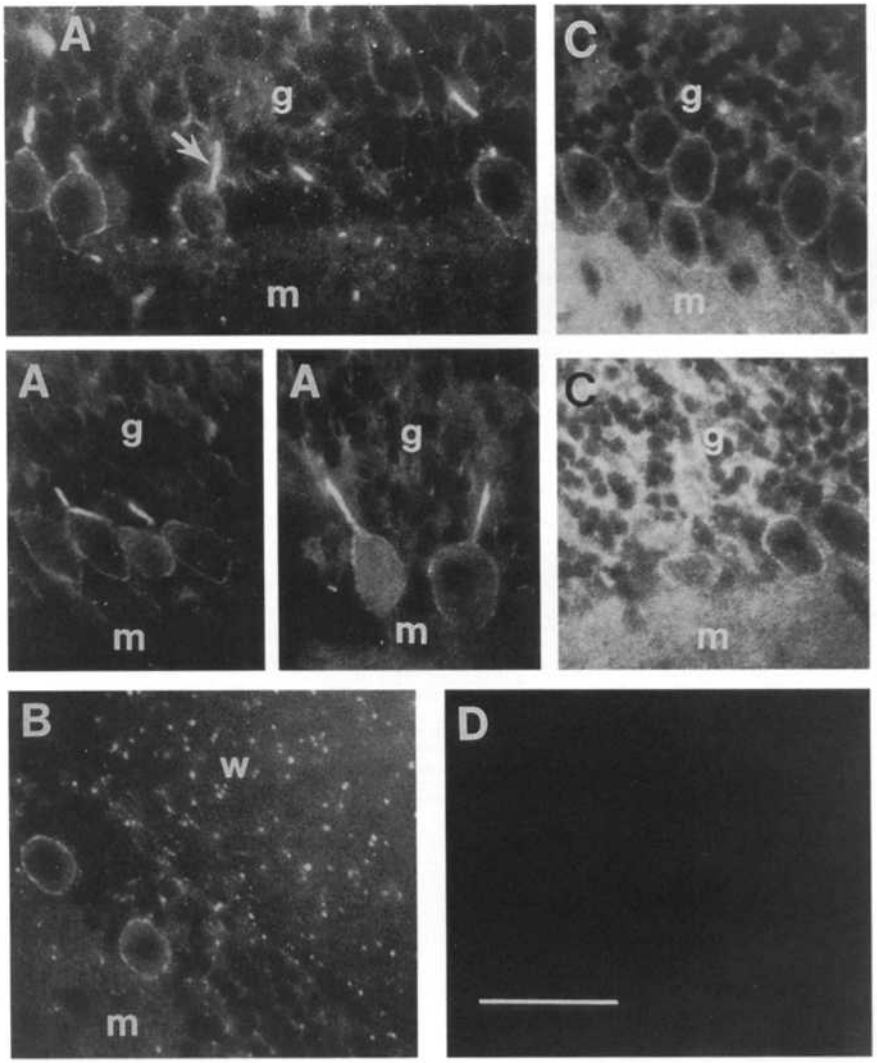


Figure 6. Neurofascin (mucin+/third FNIII−) is concentrated on Purkinje cell initial segments in the central nervous system, while neurofascin (mucin-/third FNIII+) is not concentrated at these regions, but is found in a more general distribution on the Purkinje and granule cell membranes. 5-μM frozen sections of rat cerebellum were examined by indirect immunofluorescence with affinity-purified Ig against neurofascin mucin-like domain (*A* and *B*) and neurofascin third FNIII domain (*C*) (Materials and Methods). Arrow denotes axon initial segments of Purkinje cells, *m* marks the molecular layer; *g*, the granule cell layer; and *w*, myelinated tracts. Note numerous small dots in the myelinated area which are interpreted as CNS nodes of Ranvier. Bar, 100 μm.

Antibody against neurofascin FNIII domains 1–4 labeled paranodal areas of the axonal membrane in addition to the nodal region (Fig. 5). Paranodal staining does not coincide with ankyrin$_G$. This finding emphasizes the specificity of the localization of forms of neurofascin containing mucin domain to the nodal membrane, and raises the issue of the exon usage of the paranodal neurofascin. Paranodal neurofascin apparently lacks both a mucin domain as well as the third FNIII domain, and may be related to the 140-kD neurofascin polypeptide.

Axon initial segments of Purkinje cells were labeled by antibody against the mucin-like domain of neurofascin in sections of the cerebellum (Fig. 6). Purkinje cell bodies also were labeled, but at a lower intensity. The molecular layer, containing dendrites of Purkinje cells and unmyelinated axons, was not labeled significantly by antibody against the mucin-like domain. Granule cell neurons exhibited some labeling, which could not be definitively assigned at a light microscope level. However, at least some of the staining of granule cell neurons could be at axon initial segments. Antibody reacting preferentially with the third FNIII domain exhibited a complementary pattern of staining to that observed with antibody against the mucin domain. Purkinje cell bodies were stained, while axon initial segments were not. The molecular layer was intensely stained in a pattern consistent with labeling of parallel fiber axons. The granule cell layer also was stained at higher intensity than observed with antibody against the mucin domain.

NrCAM Is Localized at the Node of Ranvier and in Unmyelinated Axons

The antibody initially raised against intact neurofascin that labeled nodes of Ranvier was used to screen a cDNA expression library. In addition to neurofascin, this screen resulted in a clone with 80% sequence identity to NrCAM from chicken (Grumet et al., 1991; Kayyem et al., 1992). NrCAM is most closely related to neurofascin among the other members of the L1/neurofascin/NgCAM family, with over 70% identity between cytoplasmic domains. The sequence of two full-length cDNA clones isolated by library screening with 5′ and 3′ probes as described for neurofascin is presented in Fig. 7. A partial sequence of rat NrCAM has been reported (Mocosco and Sanes, 1995), but this is the first full-length mammalian NrCAM sequence. Each clone has six Ig domains, four FNIII domains, a membrane-spanning domain, and a cytoplasmic

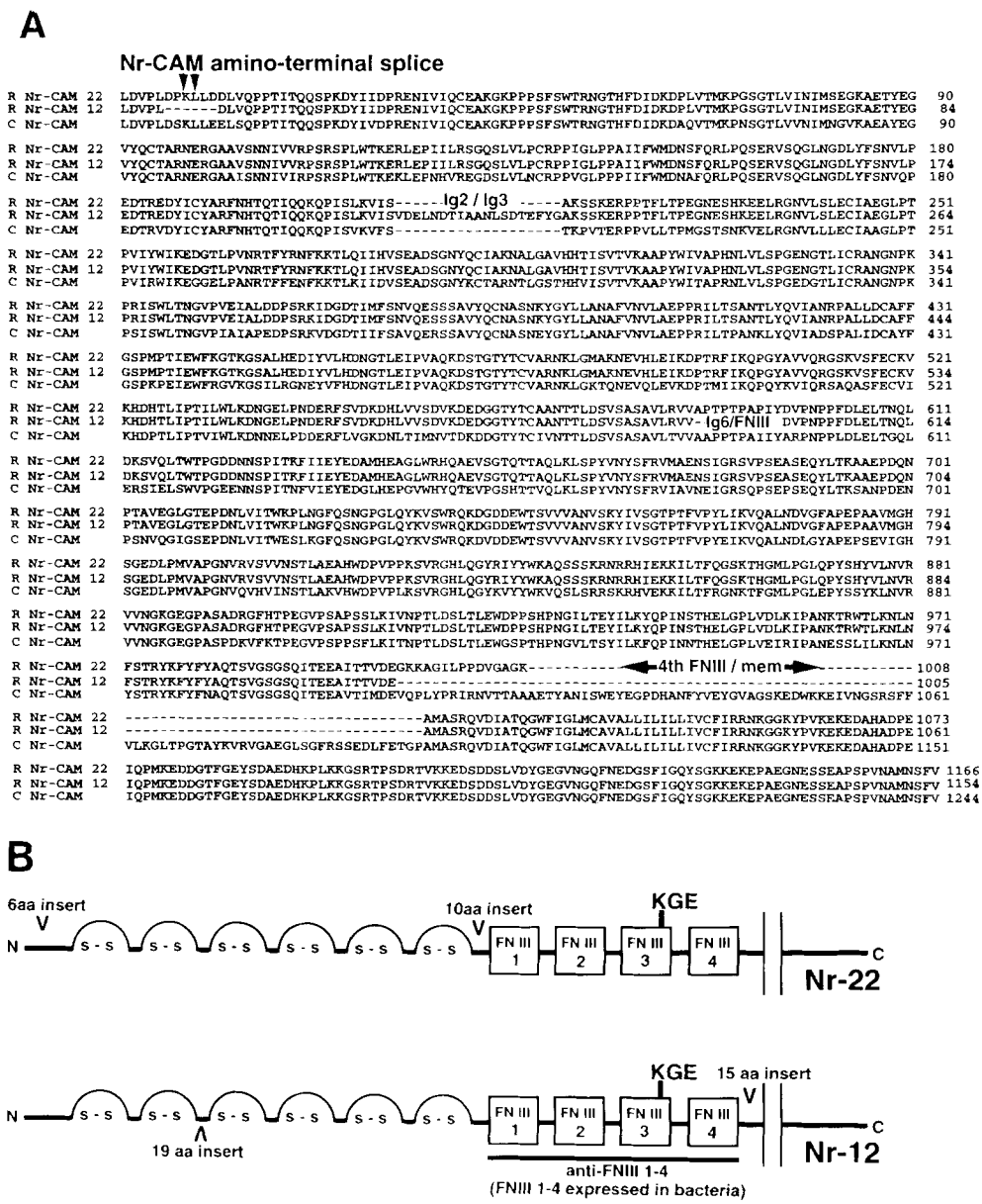


Figure 7. Characterization of two rat NrCAM full-length clones. (*A*) Alignment of the deduced amino acid sequences. (*B*) Schematic models of the domain organization and alternative splicing of NrCAM full-length clones. The underlined region represents the region used to generate NrCAM specific antibodies (Materials and Methods). Full-length cDNA clones were isolated from a rat brain λ gt11 library (Clontech) using both 5′ and 3′ cDNA probes on double-lifts of the library. The probes were generated from a composite full-length sequence of rat NrCAM initially isolated by expression cloning in λ GT11 using affinity-purified antibodies against native neurofascin (part of this study).

domain. They differ in a reciprocal pattern of expression of inserted sequences: one clone has a six–amino acid insertion at the NH_2 terminus, a 10–residue insertion between Ig and FNIII domains, and a 15–residue insertion between FNIII and membrane-spanning domains, while the other lacks these insertions and has a 19–residue insertion between the second and third Ig domains. The pattern of exon usage of NrCAM transcripts is similar to neurofascin: both are fully reciprocal and transcripts encoding the NH_2-terminal six residue insertion also have an insertion between Ig and FNIII domains. NrCAM from chicken contains an external cleavage site resulting in a 140-kD fragment comprised entirely of extracellular sequence noncovalently associated with a membrane-spanning fragment (Kayyem et al., 1992). Rat NrCAM has the same property and was isolated as a soluble 140-kD polypeptide (Fig. 8).

Antibodies were raised against NrCAM FNIII domains 1-4 which cross-react with both full-length cDNAs encoding NrCAM, but not neurofascin or L1 (Fig. 8). These antibodies labeled nodes of Ranvier of myelinated axons as well as unmyelinated axons in frozen sections of rat sciatic nerve (Figs. 9 and 10). $Ankyrin_G$ and NrCAM were colocalized at nodes of Ranvier at all nodes observed using confocal microscopy (Fig. 10). A transverse section through a node revealed axonal staining similar to that of neurofascin (mucin+/third FNIII−) (Fig. 10). These results, together with findings with neurofascin indicate that NrCAM, neurofascin (mucin+/third FNIII−), and the voltage-dependent sodium channel are general components of nodes of Ranvier, at least in peripheral nerve. NrCAM also is localized in axon initial segments of Purkinje cell neurons in the cerebellum (Fig. 10 *C*).

Discussion

This report presents evidence that NrCAM and alterna-

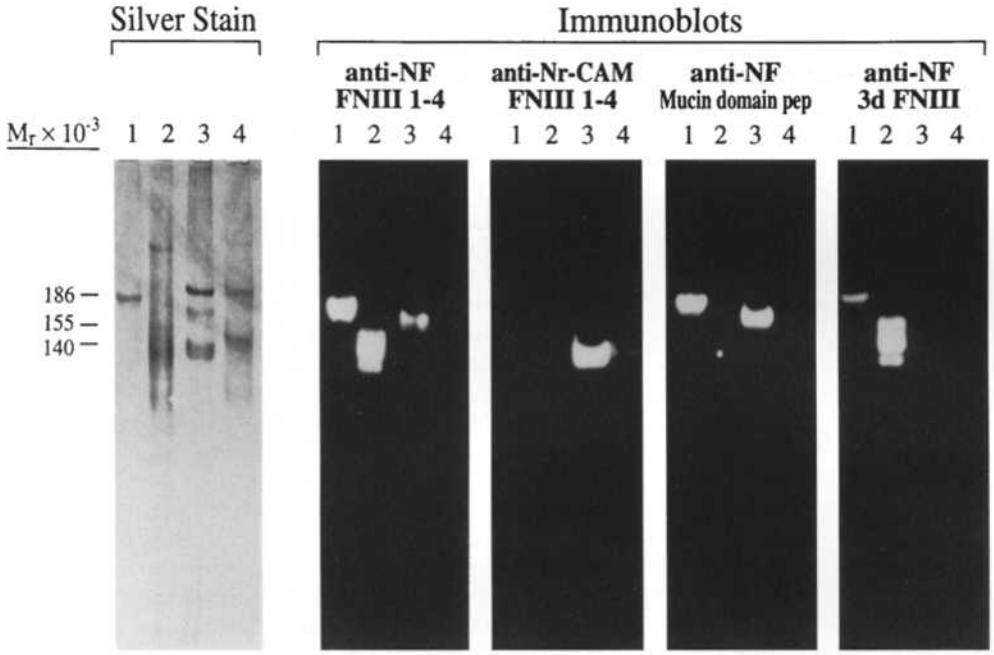


Figure 8. Antibodies to rat neurofascin and NrCAM only recognize their respective protein product and not closely related genes. Immunoblots of isolated neurofascin, NrCam, and L1 were prepared and probed with affinity-purified Ig against neurofascin FNIII 1-4, neurofascin mucin-like domain, neurofascin third FNIII domain, FNIII domains of NrCam. (Lane *1*) 186-kD neurofascin; (lane *2*) partially purified 155/140-kD neurofascin; (lane *3*) partially purified NrCAM ectodomain and a 180-kD form of neurofascin; (lane *4*) L1.

tively spliced forms of neurofascin, containing a mucin-like domain and lacking the third FNIII domain, are colocalized with ankyrin$_G$ and the voltage-dependent sodium channel at nodal axon segments of nodes of Ranvier at the level of light microscopy. This is the first characterization of defined neuronal cell adhesion molecules localized to axonal membranes at the node of Ranvier of myelinated axons. Earlier reports have suggested that NCAM (Rieger et al., 1986), L1 (Mirsky et al., 1986), and NgCAM (Rieger et al., 1986) are present at nodes of Ranvier. However, other investigators have found that L1 and NCAM, although present in unmyelinated axon bundles, actually are downregulated during myelination and are absent from nodes of Ranvier of mature myelinated axons (Bartsch et al., 1989; Martini and Schachner, 1986; Martini, 1994). Antibodies against NgCAM which labeled nodes of Ranvier (Rieger et al., 1986) were prepared before molecular cloning of L1, neurofascin, NrCAM and NgCAM, and were not characterized with respect to specificity for products of these genes or their alternatively spliced variants. More recently, antibodies were prepared by our laboratory against pure neurofascin which labeled nodes of Ranvier (Davis et al., 1993), but subsequently were found to also cross-react with NrCAM, possibly through shared epitopes in the highly conserved cytoplasmic domains of these proteins.

Nodal forms of neurofascin and NrCAM are candidates to form a molecular complex with the specialized isoform of ankyrin$_G$ which also is localized at the node of Ranvier (Kordeli et al., 1990, 1995). The proposed ankyrin-neurofascin/NrCAM complex would be predicted to be configured as an extended rod-shaped structure extending from the extracellular space through the plasma membrane and up to 200 nm into the axoplasm (Fig. 11). The rationale for this structure is based on direct visualization of neurofascin as a 40–60 nm rod by electron microscopy (Davis et al., 1993), and a conceptual model for ankyrin$_G$ as a ball and chain based on homology between the COOH-terminal portion of 480 kD ankyrin$_G$ and the random-coil sequence present in 440 kD ankyrin$_B$ (Chan et al., 1993; Kordeli et al., 1995). NrCAM is likely to have the same general shape as neurofascin, since both proteins contain the same number of copies of independently folded Ig and FNIII domains. Ichimura and Ellisman (1991) have reported visualization of transmembrane filaments at the node of Ranvier that are strikingly similar in dimensions to those predicted for a neurofascin/NrCAM-480-kD ankyrin$_G$ complex (Ichimura and Ellisman, 1991). The observed filaments extend 40–80 nm from Schwann cell microvillar processes in the extracellular space to the axonal membrane and continue into the axoplasm and appear to contact cytoskeletal structures. Ankyrin has been demonstrated to bind to microtubules (Bennett and Davis, 1981) and intermediate filaments (Georgatos and Marchesi, 1985), and, in principle, could be responsible for the cytoskeletal contacts resolved by electron microscopy. It will be important to directly evaluate by immunogold labeling the relationship of the transcellular filaments of Ichimura and Ellisman (1991) to ankyrin and ankyrin-binding adhesion molecules.

The function of cell–cell linkages in combination with an ankyrin-mediated colinear attachment to cytoskeletal structures is an interesting question. One possibility is a mechanical role in stabilizing the node of Ranvier to shear stresses, by analogy to the interconnected network of intermediate filaments in keratinocytes. It is pertinent in this regard that the node of Ranvier is vulnerable to trauma and is the primary site of damage during stretch-induced injury to optic nerves (Maxwell et al., 1991). Another potential activity for extended ankyrin-adhesion molecule complexes is direct delivery of axonally transported membrane organelles to membrane sites defined by extracellular contacts with glial cells.

In addition to cell adhesion, members of the ankyrin-binding family of cell adhesion molecules have been impli-

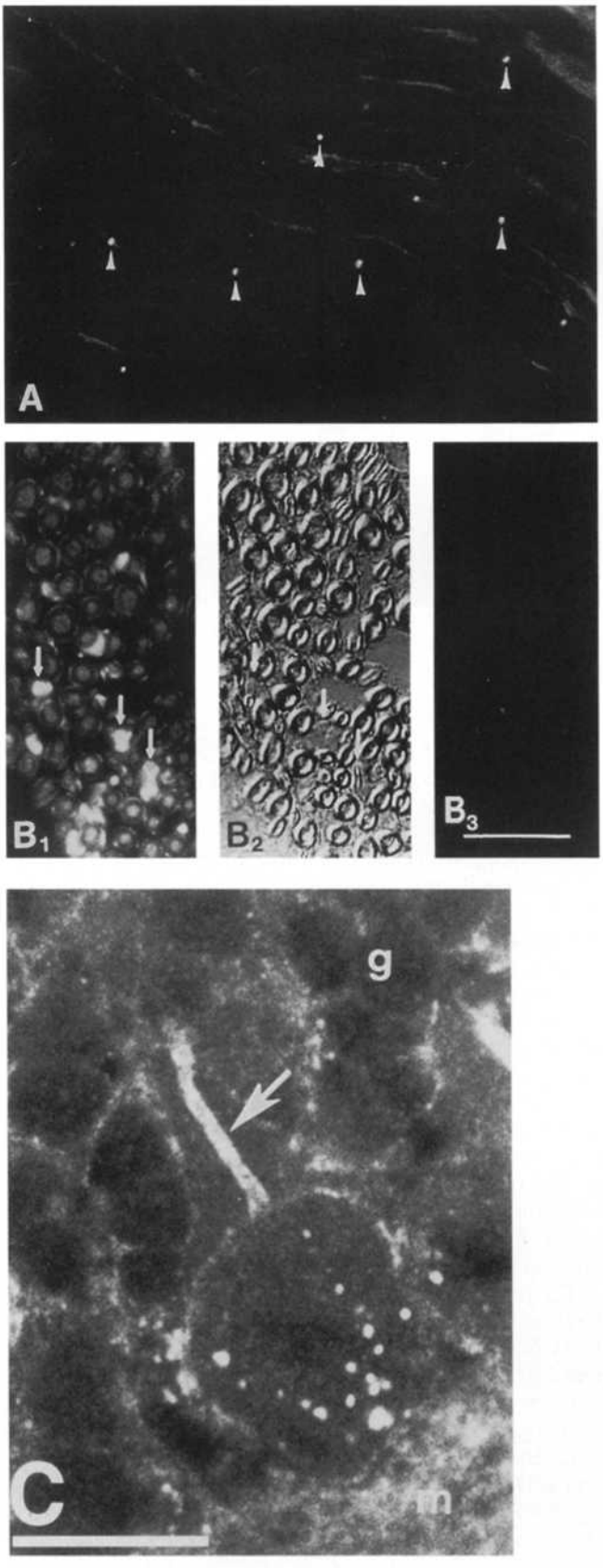


Figure 9. Localization of NrCAM at nodes of Ranvier and unmyelinated axons in rat sciatic nerve (*A*, B_{1-3}) and the Purkinje neuron axon initial segment in cerebellum (*C*). 5-μM frozen sections of rat sciatic nerve and cerebellum were examined by immunofluorescence using affinity purified Ig against NrCAM FNIII domains 1-4 (Materials and Methods). *A* is a longitudinal section where arrows mark nodes of Ranvier and B_{1-3} are transverse sections where arrows mark bundles of unmyelinated axons (see Fig. 4) B_3 is an image from sections stained with nonimmune Ig. Bars: (*C*) 20 μm; (B_3) 100 μm.

cated in the activation of intracellular second messenger signaling pathways. Changes in the organization of the axonal cytoskeleton, axonal caliber in the PNS, and rates of axonal transport have been observed at the node of Ranvier. Based on studies with dysmyelinating mutant mice, de Waegh et al. (1992) have hypothesized that these changes are due to a kinase-phosphatase system activated by localized signaling molecules at the node that respond to axonal-glial contacts. Nr-CAM and neurofascin are excellent candidates for these localized signaling molecules and could be instrumental in axonal differentiation at the node. A signaling mechanism between axons and Schwann cells also presumably directs individual myelination of some axons and communal ensheathement of others (Aguayo et al., 1976; Weinberg and Spencer, 1976). The alternative forms of neurofascin present in myelinated and unmyelinated axons are candidates to participate at some level in axon-Schwann cell interactions. Interesting questions for future work are whether alternatively splicing of neurofascin is an intrinsic property of neurons independent of Schwann cell contact, and if the alternative forms of neurofascin direct distinct Schwann cell behaviors. Answers to these questions could be of practical value in understanding and promoting regeneration of peripheral nerves.

The membrane-binding domain of ankyrin contains at least two distinct binding sites, and can bind to both neurofascin and the anion exchanger in in vitro assays (Michaely and Bennett, 1995*a*, *b*). The potential of ankyrin to form multivalent complexes with membrane proteins suggests another function for ankyrin-binding cell adhesion molecules at the node of Ranvier. Neurofascin and NrCAM could be involved in ankyrin-mediated lateral complexes with other ankyrin-binding proteins such as the voltage-dependent sodium channel and certain isoforms of the Na/K ATPase which are localized at the node of Ranvier (Waxman and Ritchie,1993; Ariyasu et al., 1985). Linkage of ion channels to cell adhesion molecules could play a role in targeting or stabilizing these molecules in the nodal portion of myelinated axons defined by extracellular signals provided by Schwann cells or astrocytes.

Cytoplasmic domains of neurofascin and NrCAM are likely to participate in protein interactions in addition to ankyrin. NrCAM, for example, contains a COOH-terminal motif, NAMNSFV, which is predicted to associate with PDZ domains of the PSD-95/discs-large/ZO-1 family of membrane skeletal proteins (Kornau et al., 1995; Gomperts, 1996). PSD-95 is concentrated at synaptic regions and associates with the NMDA receptor (Kornau et al., 1995) and Shaker-type K^+ channels (Kim et al., 1995). It will be of interest to determine if nodes of Ranvier include protein(s) containing PDZ domains.

Neurofascin at the nodal axon segment contains a mucin-like domain, while neurofascin lacking a mucin-like domain is evident in the paranodal region of the axon as well as unmyelinated axons (Fig. 4). The paranodal form

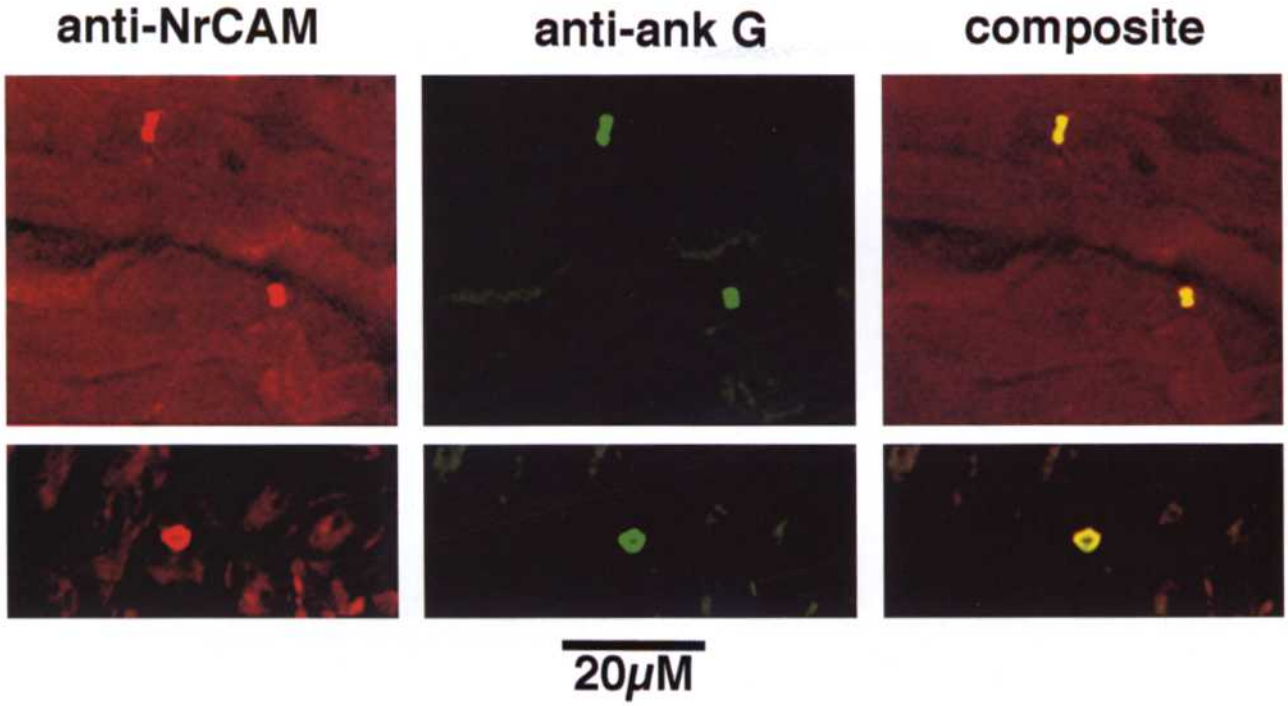


Figure 10. NrCAM is colocalized with 480/270 kD ankyrin$_G$ at nodal axon segments in rat sciatic nerve. 5-μM frozen sections of rat sciatic nerve were examined by immunofluorescence using affinity-purified rabbit Ig against NrCAM FNIII domains 1–4, and chicken antibody against 480/270 ankyrin$_G$ (Materials and Methods). Double-labeled images were collected using a confocal microscope. Bottom panels show a transverse section through a node of Ranvier while upper panels are longitudinal sections.

of neurofascin is not localized with ankyrin$_G$, suggesting that this form of neurofascin may participate in cytoskeletal interactions in addition to ankyrin and/or be inhibited in ability to bind to ankyrin. It is of interest that the 186-kD (mucin+/third FNIII−) form of neurofascin binds to ankyrin with a 10-fold higher affinity than the 155-kD (mucin−/third FNIII+) neurofascin (Davis et al., 1993). Differences in ankyrin-affinity could be responsible for segregation of alternative forms of neurofascin between paranodal and nodal areas of axons. These observations raise questions of whether the mucin-like domain is responsible for enhancement of affinity for ankyrin and if the mucin domain has specialized functions in the nodal axon segment. Mucin-related domains are present in a number of cell surface proteins including alternatively spliced forms of NCAM (Walsh et al., 1989), proteins in the immune system (Varki, 1994; Shimizu and Shaw, 1993), and α-dystroglycan (Brancaccio et al., 1995; Smalheiser and Kim, 1995). O-glycosylated polypeptides are extended (Jentroft, 1991; Brancaccio et al., 1995) and have been proposed to elevate protein domains above the cell surface. Mucin domains also are ligands for proteins including selectins (Varki, 1994; Shimizu and Shaw, 1993) and could mediate specific protein interactions for neurofascin at the node of Ranvier.

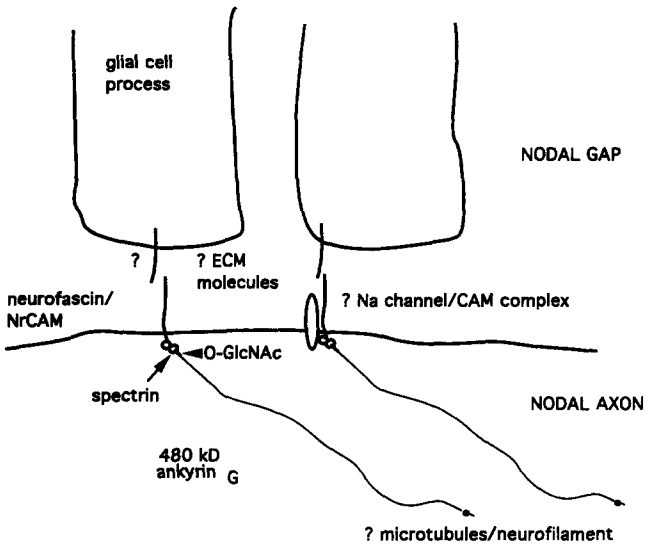


Figure 11. Schematic model of cell adhesion molecule/ankyrin complexes at the nodal axon segment.

The mucin-like domain of neurofascin could be involved in pathological conditions as well as play a role in the normal function of the node of Ranvier. Some patients with Guillain-Barre syndrome, an immune-mediated peripheral neuropathy, exhibit morphological changes at the node of Ranvier followed by axonal degeneration (Griffin et al., 1996). These symptoms are accompanied by elevated production of an antibody reacting with carbohydrate epitopes present in GM1 monosialoganglioside, which are located at nodes of Ranvier (Pestronk, 1991; Corbo et al., 1993). The mucin-like domain of neurofascin may also be a target for pathological antibodies in this syndrome as well as other neuropathies including forms of multiple sclerosis.

The nodal gap of peripheral nerves contains Schwann cell microvilli which approaches within 40–80 nm of the external surface of the axon (Berhold and Rydmark, 1995), while in the central nervous system astrocyte processes perform an analogous role (Black et al., 1995). The nodal gap also contains extracellular matrix proteins visualized by staining with cations (Zagoren, 1984). Neurofascin and NrCAM are candidates to participate in linkages between the axon and glial cell processes as well as components of the extracellular matrix at the node of Ranvier. Candidates for the counter receptors for neurofascin and NrCAM in Schwann cell microvilli or astrocyte processes include hetero- as well as homophilic interactions with membrane-spanning or GPI-linked adhesion molecules. NrCAM is capable of homophilic interactions (Mauro et al., 1994) and binds to the GPI-linked Ig/FNIII proteins F11/contactin (Morales et al., 1993) and axonin/TAG1 (Suter et al., 1995). The β-2 subunit of the voltage-dependent sodium channel has homology to F11 (Isom et al., 1995) and potentially could also associate laterally with NrCAM. Localization of F11 or axonin-1 at nodes of Ranvier has not been evaluated. However, F11 does interact with the extracellular matrix protein tenascin$_R$ (Brummendorf et al., 1993; Rathgen et al., 1991) which is localized at nodes of Ranvier (Rieger et al., 1986; Martini et al., 1990; Jung et al., 1993). It will be of interest to evaluate whether F11 or axonin-1 are expressed by glial cells at nodes of Ranvier and if so to evaluate possible selective expression of alternatively spliced variants.

Axon initial segments and nodal segments share features of a high density of voltage-dependent sodium channels, a dense membrane undercoating, and are enriched in 480/270-kD isoforms of ankyrin$_G$ (Kordeli et al., 1995). This report presents evidence that axon initial segments also contain high concentrations of the same ankyrin-binding cell adhesion molecules as the nodal axon segment (Figs. 6 and 9 *C*). These findings suggest that domains of axons specialized for initiation or propagation of action potentials share a common molecular organization and may have evolved from a common precursor. It is of interest to know that postsynaptic crypts of neuromuscular junctions also contain voltage-dependent sodium channels and an isoform of ankyrin (Flucher and Daniels, 1989). It will be of interest to determine the ankyrin isoform at neuromuscular junctions and whether ankyrin-binding cell adhesion molecules also are present at these sites.

Neurofascin and NrCAM are relatively abundant membrane proteins and together account for about 1% of the total membrane protein in brain tissue (Davis et al., 1993; Davis and Bennett, 1994). The localization of such constitutively expressed proteins at specialized areas of axons was unanticipated. However, the node of Ranvier is a relatively recent adaptation of vertebrates, and de novo evolution of novel proteins dedicated to this structure does not seem likely. A corollary of a recent evolutionary origin for the node of Ranvier is that the molecular organization and mechanisms involved in assembly of this domain will apply in a more general way the understanding of other types of membrane domains in the nervous system and perhaps other tissues.

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