# Structure of the Axonal Surface Recognition Molecule Neurofascin and Its Relationship to a Neural Subgroup of the Immunoglobulin Superfamily

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***Abstract.*** The chick axon-associated surface glycoprotein neurofascin is implicated in axonal growth and fasciculation as revealed by antibody perturbation experiments. Here we report the complete cDNA sequence of neurofascin. It is composed of four structural elements: At the $NH_2$ terminus neurofascin contains six Ig-like motifs of the C2 subcategory followed by four fibronectin type III (FNIII)-related repeats. Between the FNIII-like repeats and the plasma membrane spanning region neurofascin contains a domain 75-amino acid residues–long rich in proline, alanine and threonine which might be the target of extensive O-linked glycosylation. A transmembrane segment is followed by a 113-amino acid residues–long cytoplasmic domain. Sequence comparisons indicate that neurofascin is most closely related to chick Nr-CAM and forms with L1 (Ng-CAM) and Nr-CAM a subgroup within the vertebrate Ig superfamily.

Sequencing of several overlapping cDNA probes reveals interesting heterogeneities throughout the neurofascin polypeptide. Genomic Southern blots analyzed with neurofascin cDNA clones suggest that neurofascin is encoded by a single gene and its pre-mRNA might be therefore alternatively spliced. Northern blot analysis with domain specific probes showed that neurofascin mRNAs of about 8.5 kb are expressed throughout development in embryonic brain but not in liver.

Isolation of neurofascin by immunoaffinity chromatography results in several molecular mass components. To analyze their origin the amino-terminal sequences of several neurofascin components were determined. The $NH_2$-terminal sequences of the 185, 160, and 110–135 kD components are all the same as the $NH_2$ termini predicted by the cDNA sequence, whereas the other neurofascin components start with a sequence found in a putative alternatively spliced segment between the Ig- and FNIII-like part indicating that they are derived by proteolytic cleavage. A combination of enzymatic and chemical deglycosylation procedures and the analysis of peanut lectin binding reveals O- and N-linked carbohydrates on neurofascin components which might generate additional heterogeneity.

The extension of axons to their target regions during development depends on specific pathway choices. Growth cones of extending axons explore their local environment suggesting that they recognize specific signals present in their environment (Dodd and Jessell, 1988; Bixby and Harris, 1991). These signals include diffusible molecules which might act as chemoattractants and might be released by intermediate or final cellular targets (Placzek et al., 1990). Extracellular matrix and cell surface glycoproteins expressed by neuronal or non-neuronal cells represent other factors important to regulate axonal growth. A variety of axon-associated surface proteins have been described in the past which can be categorized into three major structural groups: the cadherins (Takeichi, 1991), the integrins (Reichardt and Tomaselli, 1991), and the Ig superfamily (Rathjen and Jessell, 1991; Hortsch and Goodman, 1991; Walsh and Doherty, 1991). The neural members of the Ig superfamily implicated in axonal growth can be further grouped according to the occurrence of Ig-related repeats alone or of both Ig- and fibronectin type III (FNIII)[1]-like domains. Axon-associated proteins with only Ig-like domains include DM-GRASP/SC1, MAG, and fasciclin III (Burns et al., 1991; Tanaka et al., 1991; Hortsch and Goodman, 1991). In vertebrates the subfamily containing both Ig- and FNIII-related domains can be provisionally further subdivided into two subgroups (Rathjen, 1991; Grumet et al., 1991): L1(Ng-CAM) and Nr-CAM are members of one subgroup, while TAG-1(axonin-1) and F11(F3) form the second

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[1] *Abbreviations used in this paper*: DAF, decay accelerating factor; endoH, endoglycosidase; FNIII, fibronectin type III; LDL, low density lipoprotein; TFMS, trifluoromethane sulfonic acid.

group. These last two axon-associated glycoproteins share an overall amino acid identity of ~50% and are composed of six Ig-like domains of the C2 subcategory and four repeats similar to FNIII-related domains. In contrast to L1 and Nr-CAM which are transmembrane proteins they are attached to the plasma membrane via GPI (Brümmendorf et al., 1989; Gennarini et al., 1989; Wolff et al., 1989; Furley et al., 1990; Zuellig et al., 1992).

In our previously reported series of mAb screens conducted to identify high molecular mass glycoproteins that are primarily expressed on axons in developing fiber tracts of the chick nervous system, we initially characterized three different neurite-associated surface glycoproteins, namely F11, neurofascin, and G4. By classical in vitro antibody perturbation experiments, we demonstrated that these proteins are involved in the fasciculation of temporal retinal axons and growth of sympathetic axons on other axons (Rathjen et al., 1987*a*,*b*; Chang et al., 1987). The F11 protein undergoes heterophilic interactions in parts of the developing nervous system with restrictin, a neural extracellular matrix glycoprotein composed of structural elements also found in tenascin (cytotactin) (Rathjen et al., 1991; Nörenberg et al., 1992). In contrast, the G4 protein which was found to be related to mouse L1 and identical to chick Ng-CAM and 8D9 (Rathjen and Schachner, 1984; Grumet and Edelman, 1984; Lemmon and McLoon, 1986; Rathjen et al., 1987*b*; Wolff et al., 1987; Moos et al., 1988; Burgoon, et al., 1991) functions in vitro both as a homophilic as well as a heterophilic axon outgrowth promoting molecule (Kuhn et al., 1991; Lemmon et al., 1989; Kadmon et al., 1990, Chang et al., 1990).

In vivo neurofascin, like L1, TAG-1 or F11, is confined to layers bearing axons and is expressed at stages that correlate with axon outgrowth supporting the in vitro antibody perturbation experiments (Rathjen et al., 1987*a*). In many regions of the developing nervous system, it appears to be colocalized on long-projecting axons with L1 but shows a more transient distribution pattern and is considerably weaker expressed (Rathjen et al., 1987*a*). In contrast to L1, in some axon tracts including the tectobulbar fascicles neurofascin appears nonhomogenously localized (Kröger and Schwarz, 1990).

Proper understanding of the role neurofascin plays during the process of axon outgrowth certainly requires a detailed description of its molecular structure. As a first step we have therefore established the primary structure of neurofascin by cDNA cloning and determined its relationship to other proteins implicated in axon–axon interactions. The deduced amino acid sequence indicates that it is a new member of the Ig superfamily composed of six Ig-like domains of the C2 set, four fibronectin type III-like repeats, a segment rich in proline, alanine, and threonine and a transmembrane and cytoplasmic region. The comparison of these sequence data with that of other neural members of the Ig superfamily groups neurofascin into the L1 subfamily of neural Ig-like proteins. Furthermore, analysis of several cDNA clones reveals that multiple variants of neurofascin exist and genomic Southern blots suggest that neurofascin is encoded by a single gene. The different neurofascin forms might therefore arise through the process of alternative pre-mRNA splicing. Additional heterogeneity of neurofascin polypeptides is generated by O- and N-linked glycosylation.

# *Materials and Methods*

## *Antibodies and Purification of Neurofascin*

Production and specificity of mouse monoclonal and rabbit polyclonal antibodies to neurofascin have been detailed elsewhere (Rathjen et al., 1987*a*). Affinity purified polyclonal antibodies of the rabbit Ig fraction were isolated on neurofascin coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Neurofascin was purified by immunoaffinity chromatography from detergent extracts of plasma membrane preparations obtained from adult chicken brain (Rathjen et al., 1987*a*).

## *Deglycosylation Experiments and Protein Analytical Procedures*

Enzymatic deglycosylations of immunoaffinity purified neurofascin were performed for 4–16 h at 37°C by N-glycosidase F, endoglycosidase H, neuraminidase (Arthrobacter), O-glycosidase (all four enzymes were from Boehringer Mannheim GmbH, Mannheim, Germany) or combinations of these enzymes essentially according to the instructions of the supplier and as detailed elsewhere (Rathjen et al., 1991; Wolff et al., 1987). Chemical deglycosylation of neurofascin components with trifluoromethanesulfonic acid was performed according to Edge et al. (1981) with slight modifications (Wolff et al., 1987). Protein samples were analyzed by SDS-PAGE on 7% acrylamide gels according to Laemmli (1970) and protein bands were visualized by silver staining as described by Ansorge (1985). Immunotransfer analysis of neurofascin and deglycosylated neurofascin was carried out using mAb F6 to neurofascin and biotinylated peanut lectin (Boehringer Mannheim Biochemicals) as described (Rathjen et al., 1987*a*; Wolff et al., 1987). Protein was quantified according to Peterson (1977). Neurofascin 185- and 160-kD components were prepared for $NH_2$-terminal sequence analysis by subjecting immunoaffinity isolates to preparative SDS-PAGE (Laemmli, 1970) and electroelution (Hunkapiller et al., 1983). $NH_2$-terminal sequences of other neurofascin components were obtained from bands blotted on a Problott membrane (Applied Biosystems Inc., Foster City, CA) according to the instructions of the manufacturer. To obtain internal amino acid sequences, peptides were generated from the carboxamidomethylated neurofascin 110–135-kD component by tryptic digestion and separated by reverse-phase HPLC using a trifluoroacetic acid–acetonitrile buffer gradient. Tryptic peptides were analyzed on a gas-phase sequenator constructed and operated as detailed elsewhere (Gausepohl et al., 1986).

## *cDNA Libraries, Screening, and DNA Sequencing*

A λgt11 cDNA library prepared from adult chicken brain (Clontech, Palo Alto, CA) was screened using affinity-purified polyclonal antibodies or a mixture of eight mAbs to neurofascin followed by alkaline phosphatase–conjugated second antibodies as described (Huynh et al., 1985). Positive phages were isolated and inserts were subcloned into the plasmid Bluescript KS+ (Stratagene, La Jolla, CA) for restriction enzyme mapping and sequencing. Additional cDNA clones were obtained by hybridization screening of the same library with cDNA fragments labeled by the method detailed by Feinberg and Vogelstein (1986) using $^{32}$P-dCTP (Amersham International, Amersham, UK). To cover sequences located in 5′-direction of the neurofascin cDNA clones obtained above, an additional λgt10 library was constructed using 2.5 μg adult brain poly(A)$^+$RNA supplied by Clontech (Palo Alto, CA). First strand synthesis was specifically primed by 0.5 μg of each of two primers, a 22-mer 5′-GTACTCCTGATGCAATGCACTC-3′ and a 17-mer 5′-TTCTGCTGGATGGTGTG-3′ corresponding to two sequences located at the 5′-end of clone NF-192 as indicated in Fig. 1 *A*. The RNAse H method was used for the cDNA synthesis by a commercially available kit (Pharmacia Fine Chemicals). The cDNA was ligated into λgt10 arms and packaged using Gigapack Gold (Stratagene). Primary plaques were screened with the 5′-end *SacI* subfragment of clone NF-192. Nucleotide sequences were analyzed on both strands by the dideoxy chain-termination method of Sanger et al. (1977) using a kit supplied by Pharmacia Fine Chemicals. Generation of nested deletions by the Exonuclease III method to produce overlapping sequences was performed as described elsewhere (Sambrook et al., 1989). Nucleotide and protein sequences were analyzed using the DNASTAR program package for microcomputer systems (DNASTAR Inc., Madison, WI). Sequence alignments and evaluation of their significance by quality ratios of compared proteins and individual domains were obtained using the Gap and PileUp of the GCG program (University of Wisconsin, Madison, WI).

## Southern and Northern Blots

10 $\mu$g of chicken genomic DNA was digested with *Eco*RI, *Bam*HI, or both and resolved on a 0.8% agarose gel. After transfer to Hybond N membranes (Amersham International) bands were detected with the insert of cDNA clone NF-105 using stringent washing conditions. For Northern hybridization samples of 2 $\mu$g poly(A)$^+$RNA prepared from different tissues using an extraction kit supplied by Invitrogen (San Diego, CA) were applied to each lane of a 0.8% denaturing formaldehyde agarose gel, run and transferred to nylon membranes (Amersham International) according to published protocols (Sambrook et al., 1989). Hybridization was performed after addition of $^{32}$P-dCTP-labeled DNA fragments to yield 2 × $10^6$ cpm/ml and membranes were subjected to stringent washing conditions after overnight hybridization prior to autoradiography. Northern probes detecting common neurofascin sequences were excised from cDNA clone NF-192 and probes specific for the third fibronectin type III repeat and the PAT domain were amplified from cDNA clones NF-105 and NF-82 by PCR. Amplification of the third fibronectin type III domain was performed with oligonucleotides 5'-GGGAATTCTACAGATGTTAGGATA-3' and 5'-GGGGATCCAACTCCTTGACTTCGCT-3' and for the PAT domain using 5'-GGGAATTCACCTACAACCGAGCTA-3' and 5'-GGGGATCCGCCAGCTCCTGTTTTGT-3'. The PCR primers introduced *Eco*RI and *Bam*HI restriction sites at the ends of the respective amplification products which were used for subcloning into the plasmid Bluescript KS+ for sequence analysis.

## DNA Transfection into Eucaryotic Cells

Using standard cloning procedures, cDNA clone NF-192 was combined with cDNA clone NF-S533 to obtain a continuous neurofascin open reading frame that was then cloned into the eucaryotic expression vector pSG5 (Stratagene). NIH 3T3 cells were seeded into 60-mm dishes to obtain a subconfluent monolayer, and 10 $\mu$g of super coiled plasmid DNA were transfected by the calcium precipitate technique as described elsewhere (Gorman et al., 1982). Cells were further grown overnight and exposed to mAb F6 directed to neurofascin for cell surface staining, washed, fixed, and treated with a FITC-coupled rabbit anti-mouse antibody (Dianova) for fluorescence microscopic detection of neurofascin.

# Results

## Isolation of cDNA Clones Encoding Neurofascin

Affinity-purified polyclonal and a mixture of eight mAbs specific for neurofascin were used to screen a λgt11 cDNA library constructed from adult chick brain mRNA. Approximately 2.5 × $10^6$ phages were screened, and three cDNA clones immunoreactive with both antibody preparations were isolated (designated NF-82, NF-105, and NF-155) and subjected to further analysis (see Fig. 1 *A*). cDNA clones NF-180 and NF-192 were yielded by rescreening 2.5 × $10^6$ phage from the same library with a radiolabeled insert from NF-105. To obtain cDNA clones covering the $NH_2$-terminal region of neurofascin a λgt10 cDNA library from adult chicken brain mRNA was constructed using two different oligonucleotides corresponding to 5' located sequences of cDNA clone NF-192. Screening of 1 × $10^6$ phages from this library with the 5' fragment generated by the restriction enzyme *Sac*I yielded cDNA clones NF-S199, NF-S465, NF-S527, and NF-S533.

In total nine overlapping cDNA clones were selected and subjected to sequence analysis (Fig. 1 *A*). A composite sequence of these cDNA clones of 4,041 bp including the deduced protein sequence is shown in Fig. 2 and a schematic representation of the domain organization in Fig. 7. A start codon at nucleotide position 109 and a stop codon at site 3,925 delineate an open reading frame coding for a polypeptide of 1,272 amino acids with a molecular mass of 142,255 D. Other reading frames contain multiple stop codons throughout the sequence. The DNA sequences flanking the putative start codon match with a conventional translation initiation consensus sequence (Kozak, 1984). A potential polyadenylation signal and a poly(A) tail were not found indicating that the 3' non-coding sequences of the neurofascin mRNA were not present on the cDNA clones isolated. Hydrophobicity analysis of the predicted amino acid sequence reveal two major and two minor hydrophobic stretches (Fig. 1 *B*; Kyte and Doolittle, 1982). One major hydrophobic sequence of 24 amino acid residues is located adjacent to the initiation codon and the other comprising amino acid residues 1,112 to 1,134 is located at the COOH-terminal portion of the polypeptide. The first may constitute a signal

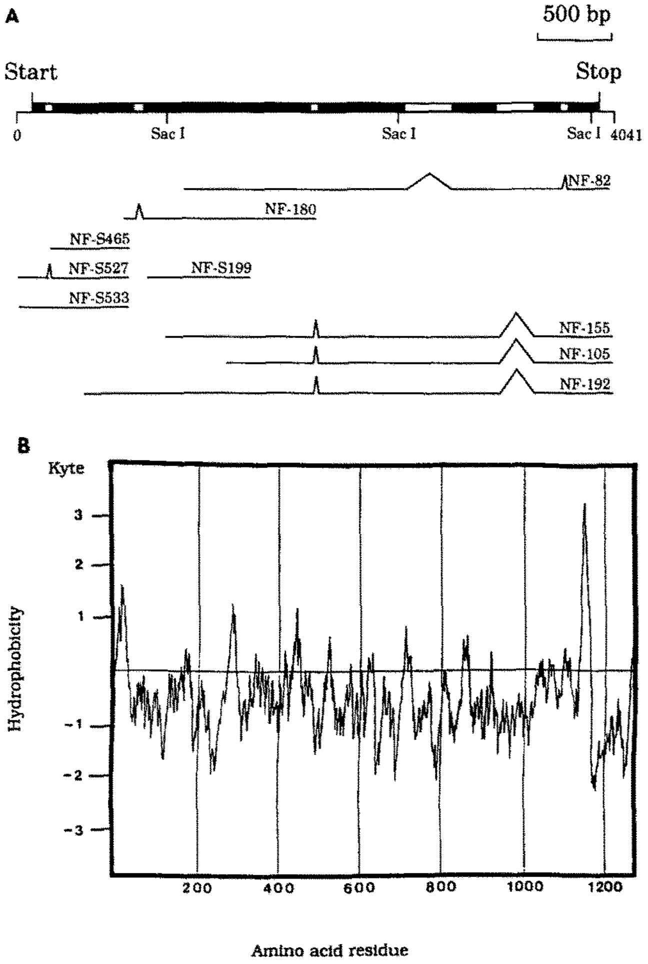

*Figure 1.* Schematic representation of the neurofascin cDNA clones (*A*) and hydrophobicity analysis of neurofascin amino acid sequence (*B*). (*A*) All nine sequenced cDNA clones are shown to scale. Sac I in the upper bar depicts *Sac*I restriction sites, boxes indicate the open reading frame of neurofascin cDNA and thin lines correspond to 5' and 3' untranslated segments. Black boxes represent sequences common to all clones and open boxes sequences of only certain clones. cDNA clones marked with S were obtained from a specifically primed cDNA library constructed from adult chicken brain mRNA. (*B*) Hydrophobicity plot of the predicted neurofascin amino acid sequence according to Kyte and Doolittle (1982) starting at the translation initiation site (amino acid residue -25). The positive peak at the left corresponds to the $NH_2$-terminal located signal peptide, while the major peak on the right represents the putative plasma membrane spanning segment.

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                                                                                                                        M   V   L   H   S   H   Q      -19
gatccccggc tgcggcgatc ccggcgctcc ggtgtggctg aggacccaac agccatcagc cctccttggg ggagcaggtg aggtcacagc attgttttga tatccaaa atg gtc ctg cac agc cac cag  129

 L   T   Y   A   G   I   A   F   A   L   C   L   H   H   L   I   S   A   I   E   V   P   L   D   S   N   I   Q   S   E   L   P   Q   P   P   T   I    19
ctc acc tac gcg ggg atc gca ttc gct ctg tgc ctc cac cac ctc atc agc gcc att gaa gtc cct ctg gat tca aat att cag agt gaa ttg cct cag ccc ccc acc atc  242

 T   K   Q   S   V   K   D   Y   I   V   D   P   R   D   N   I   F   I   E   C   E   A   K   G   N   P   V   P   T   F   S   W   T   R   N   G   K    56
acc aag cag tct gtg aag gac tac atc gtt gac ccc cgg gac aac atc ttc att gaa tgt gaa gcc aaa ggg aat cct gtt ccc acc ttt tcc tgg aca cgg aat ggg aag  351

 F   F   N   V   A   K   D   P   K   V   S   M   R   R   R   S   G   T   L   V   I   D   F   H   G   G   G   R   P   D   D   Y   E   G   E   Y   Q    93
ttc ttc aac gtg gca aag gac ccc aaa gtg tcc atg cgg agg cgg tcg ggg aca ttg gtc atc gat ttc cat ggg ggt ggg cgg ccg gat gac tac gag ggc gag tac caa  462

 C   F   A   R   N   D   Y   G   T   A   L   S   S   K   I   H   L   Q   V   S   R   S   P   L   W   P   K   E   K   V   D   V   I   E   V   D   E   130
tgc ttc gcc cgc aat gat tat ggc act gca ctg tcc agc aaa atc cac ctc cag gtg tcc aga tct ccc ctg tgg ccc aag gag aag gtg gat gtc att gag gtt gac gaa  573

 G   A   P   L   S   L   Q   C   N   P   P   P   G   L   P   P   P   V   I   F   W   M   S   S   S   M   E   P   I   H   Q   D   K   R   V   S   Q   167
ggt gct ccg ctc agc ctg cag tgc aac ccg cct cct ggt ctg cct cct cct gtc atc ttc tgg atg agc agc tcc atg gag ccc atc cac cag gac aag cgt gtc tcc cag  684

 G   Q   N   G   D   L   Y   F   S   N   V   M   L   Q   D   A   Q   T   D   Y   S   C   N   A   R   F   H   F   T   H   T   I   Q   Q   K   N   P   204
ggc cag aat ggt gac ctg tac ttc tcc aat gtc atg ctg cag gat gcc cag act gac tac agc tgc aat gca cgc ttc cac ttc acc cac acc atc cag cag aaa aat ccc  795
                                             *                       *
 Y   T   L   K   V   K   T   K   K   P   H   N   E   T   S   L   R   N   H   T   D   M   Y   S   A   R   G   V   T   E   T   T   P   S   F   M   Y   241
tac acc ctc aag gtg aaa acc aag aaa ccc cat aat gaa acg tcc tta cga aat cac act gac atg tac agt gcc cga ggg gta acg gaa aca aca ccc agc ttc atg tac  906

 P   Y   G   T   S   S   S   Q   M   V   L   R   G   V   D   L   L   L   E   C   I   A   S   G   V   P   A   P   D   I   M   W   Y   K   K   G   G   278
cca tat ggg acc tcc agc agc cag atg gtg ctc cga ggg gtg gac ctc ttg ctg gag tgc att gca tca gga gta cca gca cca gac atc atg tgg tac aag aaa gga ggt  1017
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 E   L   P   A   G   K   T   K   L   E   N   F   N   K   A   L   R   I   S   N   V   S   E   E   D   S   G   E   Y   F   C   L   A   S   N   K   M   315
gag ctc cca gca ggc aaa acc aag ctg gaa aac ttt aac aag gcc ctt cgt atc tcc aac gtc tca gag gaa gac tct ggg gag tat ttc tgc ctg gca tcc aac aag atg  1128

 G   S   I   R   H   T   I   S   V   R   V   K   A   A   P   Y   W   L   D   E   P   Q   N   L   I   L   A   P   G   E   D   G   R   L   V   C   R   352
ggc agc atc cgc cac acg atc tcg gtg aga gtg aag gct gcc ccg tat tgg ctg gat gag cca cag aat ctc att ttg gcc cct ggt gag gac ggc agg ttg gtg tgt cga  1239

 A   N   G   N   P   K   P   S   I   Q   W   L   V   N   G   E   P   I   E   G   S   P   P   N   P   S   R   E   V   A   G   D   T   I   V   F   R   389
gcc aat ggg aac ccc aag cct tca atc cag tgg ttg gtg aat gga gag ccc att gaa ggt tct cca ccc aac cca agc aga gag gtg gct gga gat acc att gtg ttt cga  1350
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 D   T   Q   I   G   S   S   A   V   Y   Q   C   N   A   S   N   E   H   G   Y   L   L   A   N   A   F   V   S   V   L   D   V   P   P   R   I   L   426
gac acg cag atc ggc agc agc gct gtg tac caa tgc aat gct tcc aac gag cac ggc tac ctc ctt gcc aat gcc ttt gtc agt gtc ctg gat gtg cca cca cgg ata ctg  1461
                                                 *
 A   P   R   N   Q   L   I   K   V   I   Q   Y   N   R   T   R   L   D   C   P   F   F   G   S   P   I   P   T   L   R   W   F   K   N   G   Q   G   463
gcc cca cgc aac cag ctc atc aaa gtc atc cag tac aac agg acc cgg ctg gac tgc cct ttt ttc ggc tca ccc atc ccc acc ctg cga tgg ttt aag aac ggc cag ggg  1572
                                                 *
 N   M   L   D   G   G   N   Y   K   A   H   E   N   G   S   L   E   M   S   M   A   R   K   E   D   Q   G   I   Y   T   C   V   A   T   N   I   L   500
aac atg ctg gat gga ggg aac tac aag gcg cat gag aac ggg agc ttg gag atg agc atg gct cgg aag gag gat cag ggc atc tac acc tgt gtt gcc acc aac atc ctg  1683

 G   K   V   E   A   Q   V   R   L   E   V   K   D   P   T   R   I   V   R   G   P   E   D   Q   V   V   K   R   G   S   M   P   R   L   H   C   R   537
ggc aaa gtg gag gcc cag gtt cgc ctg gaa gtc aaa gac cct acc agg att gtg aga ggc ccc gaa gat cag gtg gtg aag agg ggc tcc atg cct cgc ctg cac tgc cgg  1794

 V   K   H   D   P   T   L   K   L   T   V   T   W   L   K   D   D   A   P   L   Y   I   G   N   R   M   K   K   E   D   D   G   L   T   I   Y   G   574
gtg aag cac gac cca aca ctg aag ctc acg gtc acc tgg ctg aag gac gac gct cct ctc tac att ggg aac agg atg aaa aaa gaa gac gac ggt ctg aca ata tat ggc  1905

 V   A   E   K   D   Q   G   D   Y   T   C   V   A   S   T   E   L   D   K   D   S   A   K   A   Y   L   T   V   L   A   I   P   A   N   R   L   R   611
gtg gct gag aag gac cag ggt gac tac acc tgc gtg gcc agc aca gag ctg gac aag gac tca gct aaa gcc tac ctc acc gtg cta gca atc cct gct aac cgt ttg aga  2016
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 D   L   P   K   E   R   P   D   R   P   R   D   L   E   L   S   D   L   A   E   R   S   V   K   L   T   W   I   P   G   D   D   N   N   S   P   I   648
gac tta cct aaa gag cga ccc gac cgg ccc cgg gac ctg gag ctg tca gac ctg gct gag agg agc gtg aag ctg aca tgg att cct ggc gat gac aac aac agc ccc atc  2127
                                                                         *
 T   D   Y   I   V   Q   F   E   E   D   R   F   Q   P   G   T   W   H   N   H   S   R   Y   P   G   N   V   N   S   A   L   L   S   L   S   P   Y   685
aca gac tac atc gtc cag ttt gag gag gac cgc ttc cag cct ggc acg tgg cac aac cac tcc agg tat cct ggg aat gtc aac tcg gcc ctc ctg agc ctc tct cct tac  2238
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 V   N   Y   Q   F   R   V   I   A   V   N   D   V   G   S   S   L   P   S   M   P   S   E   R   Y   Q   T   S   G   A   R   P   E   I   N   P   T   722
gtc aac tac caa ttt cgg gtg att gca gtg aac gac gtg ggc agc agc ctg ccc agc atg ccc tca gaa cga tac cag acc agc ggg gca cga cct gaa att aac cca aca  2349
                                                                             *
 G   V   Q   G   A   G   T   Q   K   N   N   M   E   I   T   W   T   P   L   N   A   T   Q   A   Y   G   P   N   L   R   Y   I   V   R   W   R   R   759
gga gtt caa gga gca ggg acc caa aaa aac aac atg gag ata acc tgg acg cct ctg aat gca act caa gcc tat ggg ccc aac ctc cgt tac atc gtg cgg tgg agg cga  2460
                                 *
 R   D   P   R   G   S   W   Y   N   E   T   V   K   A   P   R   H   V   V   W   N   T   P   I   Y   V   P   Y   E   I   K   V   Q   A   E   N   D   796
agg gac cca cgt ggg agt tgg tac aac gag acg gtg aag gca cca cga cac gtc gtc tgg aac aca ccc atc tac gtc ccc tac gag atc aaa gtg cag gca gag aat gac  2571
                                                                                                                             *
 F   G   R   A   P   E   P   E   T   Y   I   G   Y   S   G   E   D   Y   P   K   A   A   P   T   D   V   R   I   R   V   L   N   S   T   A   I   A   833
ttt ggt aga gct cca gag cct gag acc tac atc ggc tac tca ggg gaa gat tat ccc aag gct gca cct aca gat gtt agg ata aga gtt tta aac agc act gcc att gct  2682

 L   T   W   T   R   V   H   L   D   T   I   Q   G   Q   L   K   E   Y   R   A   Y   F   W   R   D   S   S   L   L   K   N   L   W   V   S   K   K   870
ctg aca tgg acc cgc gtg cac ctg gac acc atc cag gga cag ctt aag gag tac aga gcc tat ttc tgg aga gac agt agt ttg ctg aag aac ctg tgg gtc tcc aaa aaa  2793

 R   Q   Y   V   S   F   P   G   D   R   N   R   G   I   V   S   R   L   F   P   Y   S   N   Y   K   L   E   M   V   V   T   N   G   R   G   D   G   907
cgg cag tat gtg agt ttt cct gga gac cga aac cgg ggc ata gtg tcc cgg ctg ttc cct tac agc aac tac aag ctt gag atg gtt gta acc aac ggg aga ggc gat ggg  2904

 P   R   S   E   V   K   E   F   P   T   P   E   G   V   P   S   S   P   R   Y   L   R   I   R   Q   P   N   L   E   S   I   N   L   E   W   D   H   944
ccc cgc agc gaa gtc aag gag ttc ccc aca cct gaa gga gtg ccc agc tcc ccc agg tac tta aga atc cga cag cca aat ctg gaa agc atc aat ttg gag tgg gat cac  3015
                                                                         *                                           *               *
 P   E   H   P   N   G   V   L   T   G   Y   N   L   R   Y   Q   A   F   N   G   S   K   T   G   R   T   L   V   E   N   F   S   P   N   Q   T   R   981
cca gaa cat ccc aat gga gtc ctc acg gga tac aac ctt aga tat caa gcc ttt aac gga tcc aaa acg ggc aga acc ctg gta gag aac ttc tct ccc aac cag aca agg  3126

 F   T   V   Q   R   T   D   P   I   S   R   Y   R   F   F   L   R   A   R   T   Q   V   G   D   G   E   V   I   V   E   E   S   P   A   L   L   N   1018
ttc act gtg cag agg aca gac ccc atc tcg cgc tat cga ttc ttc ctg cgt gct cgg aca cag gtg gga gat gga gaa gtc ata gtg gaa gag tca cca gcg ctc ctg aat  3237

 E   A   T   P   T   P   A   S   T   W   L   P   P   P   T   T   E   L   T   P   A   A   T   I   A   T   T   T   T   T   A   T   P   T   T   E   T   1055
gaa gcc acg cca acc cca gcc tcc acc tgg ttg cct cca cct aca acc gag cta act cca gca gcc acc att gcc acc aca acc acc act gcc acc cct act act gaa acc  3348

 P   P   T   E   I   P   T   T   A   I   P   T   T   T   T   T   T   T   A   T   A   A   S   T   V   A   S   T   T   T   T   A   E   R   A   A   A   1092
cct cct act gaa atc cct act act gcc atc cct acc acc acc act act aca acc gcc aca gct gcc agc acc gtc gca agc act aca aca act gca gag agg gct gcg gca  3459

 A   T   T   K   Q   E   L   A   Y   T   K   N   H   V   D   I   A   T   Q   G   W   F   I   G   L   M   C   A   I   A   L   L   V   L   I   L   L   1129
gcc acc aca aaa cag gag ctg gct tac acc aag aac cac gtg gac atc gcg acc cag ggc tgg ttc atc ggg ctg atg tgt gcc atc gcc ctc ctg gtc ctc att ctg ctg  3570

 I   V   C   F   I   K   R   S   R   G   G   K   Y   P   V   R   D   N   K   D   E   H   L   N   P   E   D   K   N   V   E   D   G   S   F   D   Y   1166
att gtt tgc ttc att aag agg agc aga gga ggg aaa tac cca gtg cgt gac aac aaa gat gag cac ctg aat cct gaa gac aag aac gta gaa gat ggc tca ttc gac tac  3681

 R   S   L   E   S   D   E   D   N   K   P   L   P   N   S   Q   T   S   L   D   G   T   I   K   Q   Q   E   S   D   D   S   L   V   D   Y   G   E   1203
agg tct ctt gaa agc gat gaa gac aac aaa cca ctg ccc aac agc cag acc tcc ctg gat ggc acg ata aag caa cag gag agt gac gac agc ttg gtg gac tac gga gag  3792

 G   G   E   G   Q   F   N   E   D   G   S   F   I   G   Q   Y   T   V   K   K   D   K   E   E   T   E   G   N   E   S   S   E   A   T   S   P   V   1240
ggt ggg gaa ggg cag ttc aac gag gac ggc tcc ttc att ggc cag tac aca gtg aaa aag gac aag gag gaa acg gaa ggc aac gag agc tcg gaa gcc acg tcc cca gtc  3903

 N   A   I   Y   S   L   A                                                                                                                           1247
aat gct atc tac tca tta gcg tagcgc aatgcaatgg gaccacgaac agcctatggg gcttgtagtg gctgggggtt aaacagccac caccgccact gcctccaact caacatacga atgaaaacca  4032

accaatgaca c                                                                                                                                            4041
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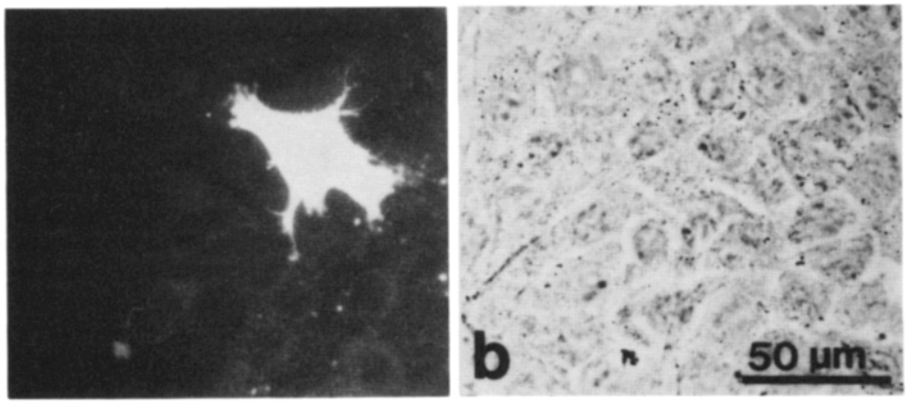

*Figure 3.* NIH 3T3 cells transiently transfected with pSG5-NF1. 24 h after transfection unfixed cell cultures were labeled indirectly with mAb F6 to demonstrate neurofascin cell surface expression (*A*). *B* represents the phase-contrast micrograph of the same field shown in *A*.

sequence for membrane translocation of an extracellular region of 1,111 amino acids. The second may serve as a plasma membrane spanning domain composed of 23 amino acid residues which is followed by a putative cytoplasmic domain of 113 amino acid residues. The signal peptide also meets the criteria for a typical signal peptide cleavage site (von Heijne, 1986). The function of the minor hydrophobic sequences in the extracellular portion of the polypeptide at amino acid residue 250 to 268 and 408 to 427 remains unknown. To further confirm the predicted amino acid sequence, the $NH_2$-termini of the 185- and 160-kD polypeptides from immunoaffinity isolates and of several tryptic peptides derived from the 110–135-kD component of neurofascin were subjected to Edmann degradation. All seven peptide sequences match with the predicted polypeptide (Fig. 2). To show conclusively that the characterized cDNA clones represent neurofascin and that the $NH_2$-terminal hydrophobic sequence functions as a signal peptide, the cDNA of neurofascin was subcloned into the eukaryotic expression vector pSG5 and transiently transfected into NIH 3T3 cells. Expression of neurofascin on the surface of NIH 3T3 cells was detected by mAbs to neurofascin (Fig. 3 *A*). Non-transfected NIH 3T3 cells were not labeled by antibodies to neurofascin.

### *Neurofascin Contains Structural Elements of Proteins Implicated in Axonal Growth*

Analysis of the predicted amino acid sequence of neurofascin reveals that the polypeptide contains four major structural characteristics: six Ig-like repeats at the $NH_2$-terminal half (amino acid residues 1 to 620), four domains similar to the FNIII motifs (amino acid residues 621 to 1,025), a 75 amino acid residues long segment rich in proline, alanine, and threonine (amino acid residues 1,026 to 1,100) and a transmembrane plus a cytoplasmic domain of 135 amino acid residues (amino acid residues 1,112 to 1,247).

The immunoglobulin-like domains are about 100-amino acid residues long and the distances between the conserved cysteine residues and the presence of typical conserved amino acid residues in the vicinity of the carboxy-proximal cysteine places these domains into the C2 subcategory of Ig-related domains (Williams and Barclay, 1988). Sequencing of several overlapping neurofascin encoding cDNA clones reveals two interesting heterogeneities in the Ig-like part which might arise by differential pre-mRNA splicing events. Close to the $NH_2$ terminus there is a six-amino acid–long sequence (SNIQSE, amino acid residue 7 to 12) encoded by cDNA clone NF-S533 but not by NF-S527. The existence of both $NH_2$ termini in neurofascin polypeptides is confirmed by Edmann degradation of the 185- and 160-kD band (see Fig. 5). Another variation in the $NH_2$-terminal half of neurofascin is found between the second and third Ig-like domain (amino acid residues 212 to 229): NF-192 contains a 18-amino acid residues–long segment (KKPHNETSLRNH-TDMYSA) that introduces two additional potential N-linked glycosylation sites and that is replaced by a single threonine in NF-180.

The Ig-like domains are followed by four fibronectin type III-like repeats of 97 to 103 amino acid residues including highly conserved tryptophan and tyrosine residues in their $NH_2$- and COOH-terminal regions, respectively. Between the sixth Ig- and the first FNIII-like domain there is a 12-amino acid residues–long stretch (AIPANRLRDLPK, amino acid residues 604 to 615) that is encoded by cDNA clone NF-82 but not by clones NF-192, NF-105, and NF-155. This segment seems to be accessible to proteolytic cleavage as several neurofascin components contain the peptide sequence

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*Figure 2.* (*A*) Nucleotide sequence and deduced amino acid sequence of neurofascin. The longest open reading frame contains 1,272 amino acids starting at the methionine residue at base number 109. Thick bars mark two hydrophobic sequences adjacent to the translation initiation site and at amino acid residues 1,112 to 1,134 which may represent the signal peptide and a plasma membrane spanning domain, respectively. Broken lines indicate peptide sequences within the neurofascin open reading frame which were determined by Edman degradation analysis of purified neurofascin components. An additional peptide sequence (APEPETYIGYSGEDLPSSPR, in the FNIII-like region) only continuously represented in NF-82 is not underlined in the figure. For the two sequences at the $NH_2$ terminus see also Fig. 5. Arrows indicate the borders of sequences which are found only in certain cDNA clones and which might be alternatively spliced in the neurofascin pre-mRNA. Putative asparagine linked N-glycosyation sites are marked by asterisks. Cysteine, tryptophan, and tyrosine residues characteristic for Ig- and FNIII-related domains, respectively, are printed enlarged. These sequence data are available from EMBL/GenBank/DDBJ under accession number X65224.

DLPKE at their $NH_2$ termini (see Fig. 5). It might therefore generate a flexible region between the Ig- and FNIII-like part in neurofascin polypeptides. The third FNIII-like repeat contains the tripeptide RGD appropriately spaced to a tyrosine residue and in a highly charged region as it has been reported for the cell attachment site in the 10th type III repeat of fibronectin (Pierschbacher and Ruoslahti, 1984). A RGD sequence in a similar environment has also been reported for Ng-CAM and TAG-1 but not for Nr-CAM, L1, F11, or axonin-1 (Grumet et al., 1991; Furley et al., 1990; Burgoon et al., 1991; Brümmendorf et al., 1989; Ranscht, 1988; Moos et al., 1988; Zuellig et al., 1992). Diversity in the FNIII-like part of the neurofascin polypeptide is created by this third FNIII-like repeat which is encoded by cDNA clones NF-192, NF-105, NF-155, and that is replaced by a single leucine in NF-82 suggesting that this region might represent another alternatively spliced segment of the neurofascin pre-mRNA. Furthermore, a peptide sequence (see legend of Fig. 2) obtained by Edmann degradation containing this leucine residue confirms the non-existence of the third FNIII-related domain in certain neurofascin polypeptides.

Between the FNIII-like repeats and the plasma membrane–spanning segment a sequence of 75 amino acid residues rich in proline, threonine, and alanine was found in cDNA clone NF-82 but not in clones NF-192, NF-105, and NF-155 suggesting that this domain might be alternatively spliced like the third FNIII-like repeat. 42% of all residues in this domain, designated PAT (Pro-Ala-Thr), are of threonine which might be targets of extensive O-linked glycosylation. The high proline content in this region (12% of all amino acid residues) might generate additional flexibility in the neurofascin polypeptide but might also be the reason for the fast and frequent fragmentation of purified neurofascin polypeptides (see Fig. 5). A similar structure of 37 amino acid residues has been detected in the so-called MSD region of NCAM between the two FNIII-like domains (Dickson et al., 1987; Walsh et al., 1989).

The cytoplasmic domain of neurofascin, of 113 amino acid residues in length, contains several potential serine and threonine phosphorylation sites (Kemp and Pearson, 1990). Diversity in the cytoplasmic segment is revealed by cDNA clone NF-82 that lacks four amino acid residues (RSLE; amino acid residues 1,167 to 1,170) which, however, are expressed by cDNA clones NF-105, NF-155, and NF-192.

### *Neurofascin mRNA Expression and Neurofascin Gene*

To analyze the expression of neurofascin mRNAs during embryonic development two DNA fragments comprising the third FNIII-like repeat (nucleotides 2,640 to 2,927) and the PAT domain (nucleotides 3,275 to 3,482) were amplified by PCR from cDNA clones NF-105 and NF-82, subcloned, checked by sequencing and labeled for Northern hybridization. Both probes as well as a probe derived from the 5′ end 579-bp *SacI* fragment of NF-192 which encodes sequences of the Ig-like domains detect a single mRNA species of ~8.5 kb in brain but not in liver (Fig. 4, *A–C*). Expression of mRNAs specifying the third FNIII-like or the PAT domain are hardly detected at E6, E8, and E12 but become clearly visible at E16 and retain a slightly higher level in the adult brain (Fig. 4, *B* and *C*, respectively). A probe directed to the Ig-like domains detects a mRNA at E8, its signal is reduced at E12 and is increased at E16 to a level higher than that observed at E8 (Fig. 4 *A*). Comparison of the signals obtained indicate that the decrease and increase of Ig-like domain specific mRNAs do not coincide with a similar behavior of mRNAs specific for the PAT domain or the third FNIII-like repeat. This indicates that both domains are not represented in the major mRNA species of neurofascin at early embryonic stages. Hence, neurofascin mRNAs show different, developmentally regulated expression patterns and underline the high degree of neurofascin expression complexity which might be obtained by alternative splicing events. However, no mRNA species of different length were detected which might be due to a mutual exchange of mRNA segments resulting in mRNAs of similar length or to insensitivity of Northern blot analysis to detect low abundance mRNAs of different length.

Genomic Southern analyses with the insert from cDNA clone NF-105 yielded a single band at 15 kb after digestion with *Eco*RI or two bands at 15 and 4.0 kb in *Bam*Hl digests (Fig. 4 *D*, lane *1* and *2*, respectively). Treatment of DNA with both enzymes results in bands at 6.0, 3.8, and in a very weakly labeled band at 1.1 kb (Fig. 4 *D*, lane 3). These data are compatible with the assumption that neurofascin is encoded by a single gene in the chicken genome and that the different variants detected by cDNA cloning might arise by alternative pre-mRNA splicing.

### *Neurofascin Components: Origin and Carbohydrate Type*

In comparison to other axon-associated glycoproteins an unusual feature of neurofascin is that multiple molecular mass components are obtained when it is purified from detergent extracts of plasma membrane preparations by immunoaffinity chromatography. The following molecular mass bands are resolved on a 7% acrylamide gel: a weakly stained and diffuse migrating band at 250 to 300 kD, bands at 185 and 160 kD, a doublet at 150 kD, a diffuse migrating band at 110 to 135 kD, a doublet at 92 kD, and several minor bands ranging from 80 to 40 kD (Fig. 5, lane *1*). In immunotransfers, these molecular mass components are all recognized by the mAb used to purify neurofascin (not shown) indicating that they are isolated through the binding to the mAb affinity column and not by co-isolation with neurofascin as it has been observed for F11 and restrictin (Rathjen et al., 1991). To characterize the origin and relationship of the individual bands to the cDNA sequence, $NH_2$-terminal amino acid sequences of several neurofascin components were determined by Edmann degradation (Fig. 5). The 185-, the 160-, and the 110–135-kD bands contain all the $NH_2$ termini predicted by the cDNA sequence whereas the other components sequenced including the 250–300-kD band begin within a segment lying between the Ig- and FNIII-like domains that might be alternatively spliced (see also Fig. 2). These data indicate that the multiple molecular mass components obtained are authentic neurofascin components and which might be generated by proteolytic cleavage. This notion is in line with the analysis of tryptic finger prints demonstrating that the 160-, 110–135-, and 92-kD components are related to the 185-kD component (Rathjen et al., 1987*a*). The finding that the bands running at 250–300 kD are breakdown products suggests that they contain an unusual posttranslational modification, possibly an extensive glycosylation of the PAT domain. This would imply that intact neurofascin

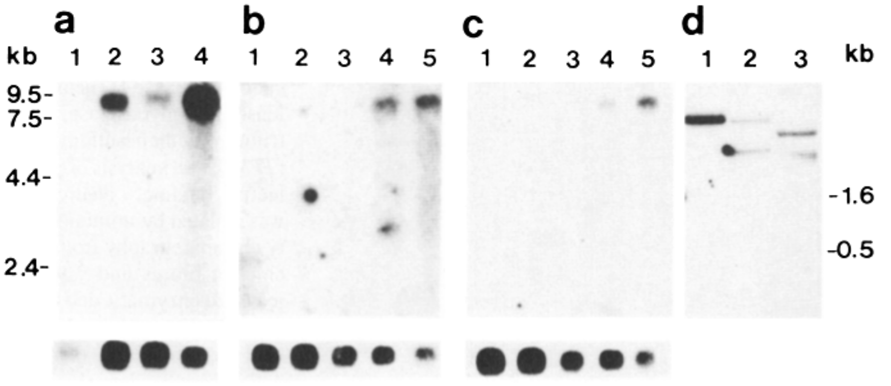


*Figure 4.* Expression of neurofascin mRNA in chicken brain during development and hybridization analysis of genomic DNA. (*A*, *B*, and *C*) Poly(*A*)$^+$ RNA, 2 $\mu$g per lane, from liver, embryonic brain, and adult brain were resolved on a 0.8% agarose/formaldehyde gel, transferred to a nylon membrane and hybridized with a probe covering the $NH_2$-terminal Ig-like domains (*Sac*I fragment of the 5′ end of NF-192) to detect mRNAs encoding all forms of neurofascin (*A*), the third FNIII-related repeat (*B*) and the segment rich in proline, alanine and threonine (*C*). *A*, lane *1* contains RNA from liver, lane *2* from embryonic brain day eight, lane *3* from embryonic brain day 12, and lane *4* from embryonic brain day 16. *B* and *C*, lane *1* contains RNA from brain of embryonic day 6, lane *2* from brain of embryonic day 8, lane *3* from embryonic brain of day 12, lane *4* from embryonic brain of day 16 and lane *5* from adult brain. A transcript of 8.5 kb is revealed in embryonic and adult brain but not in liver tissue. Positions of size markers are given at the left of *A* in kb. The blot was re-analyzed with a probe for $\beta$-actin to determine the amount of mRNA from embryonic brain tissues loaded in each lane. The mRNA encoding $\beta$-actin is downregulated in adult brain and liver tissue resulting in a significant lower hybridization signal (lane *1* in *A*, lane *5* in *B* and *D*; McHugh et al., 1991). (*D*) Chicken genomic DNA, 10 $\mu$g per lane, digested with *Eco*RI (lane *1*), BamH1 (lane *2*) or both (lane *3*) was resolved on a 0.8% agarose gel. After transfer to a nylon membrane, the blot was analyzed with the insert of NF-105. Digestion with *Eco*RI reveals one single band at 15 kb, while digestion with *Bam*H1, which cleaves the insert used as probe, results in two bands at 5 and 4.0 kb. Three bands at 6.0, 3.8, and 1.1 kb are obtained when genomic DNA is digested with both enzymes. These results are compatible with the assumption that neurofascin is encoded by single gene. Relative migration of size markers is indicated at the right and at the left of the panel in kb.

expressing a glycosylated PAT domain does not enter the acrylamide gel.

The extracellular region of neurofascin contains 15 potential asparagine-linked glycosylation sites throughout the polypeptide and several putative O-linked sites particularly in the PAT domain. The first, the second and the sixth Ig-related domain do not contain a putative N-glycosylation site. To analyze the type of carbohydrate modifications and their contribution to the molecular mass by biochemical methods, purified neurofascin components were subjected to enzymatic and chemical deglycosylation methods (Fig. 6 *A*), followed by the analysis of peanut lectin binding (Fig. 6 *B*). Peanut lectin was used since this lectin has been previously applied in combination with neuraminidase treatment to identify O-linked carbohydrates on the low-density lipoprotein receptor (Russell et al., 1984; Yamamoto et al., 1984) and NCAM (Walsh et al., 1989). As shown in Fig. 6 *A* (lane *4* and *5*) all neurofascin components are susceptible to N-glycosidase F (PNGase F) cleavage. The molecular mass shifts considerably and differences from 12 to 23 kD are observed. Assuming a molecular mass of 2.5 kD for a N-linked oligosaccharide chain, this result indicates that not all 15 N-glycosylation sites predicted by the cDNA sequence are used. Samples treated by N-glycosidase F for 16 h, however, still bind peanut lectin suggesting the presence of oligosaccharides not susceptible to N-glycosidase F digestion, possibly O-linked carbohydrates, on neurofascin components (Fig. 6 *B*, lanes *4* and *5*). To further characterize the type of N-linked oligosaccharides present, neurofascin components were subjected to endoglycosidase H (endo H) digestion. This enzyme is known to cleave high-mannose or hybrid-type oligosaccharides, while complex type chains are not removed. In contrast to N-glycosidase F treatment, the mobility of all neurofascin components is only slightly increased (Fig. 6 *A*, lane *6*) indicating that most of the N-linked carbohydrates are of the complex type. Neuraminidase digestion results in a minimal reduction in molecular mass of the neurofascin components with one exception: the mobility of the 92-kD component decreases (Fig. 6 *A*, lane 2). This unusual migration behavior in SDS-PAGE might be due to the loss of negative charge after neuraminidase treatment suggesting that this neurofascin component has a high content of sialic acid. A similar abnormal migration behavior has also been observed for glycophorin A, the major sialoglycoprotein of

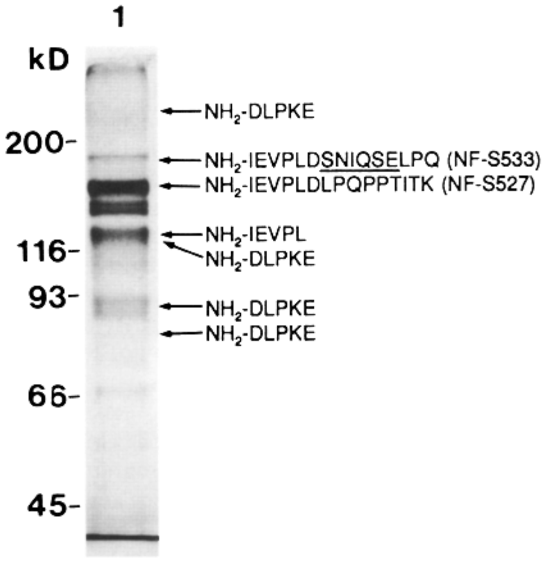


*Figure 5.* Neurofascin components resolved in SDS-PAGE and their $NH_2$-terminal amino acid sequences. Neurofascin was isolated by immunoaffinity chromatography from detergent extracts of plasma membrane preparations from adult chicken brain and analysed by SDS-PAGE (7% acrylamide). Protein bands were visualized by silver staining. $NH_2$-terminal amino acid sequences were obtained after electroelution or blotting of neurofascin components on a ProBlott membrane followed by analysis on a gas-phase sequenator. The peptide segment expressed only in NF-S533 but not in NF-S527 is underlined. Molecular mass standards are indicated at the left of the panel.

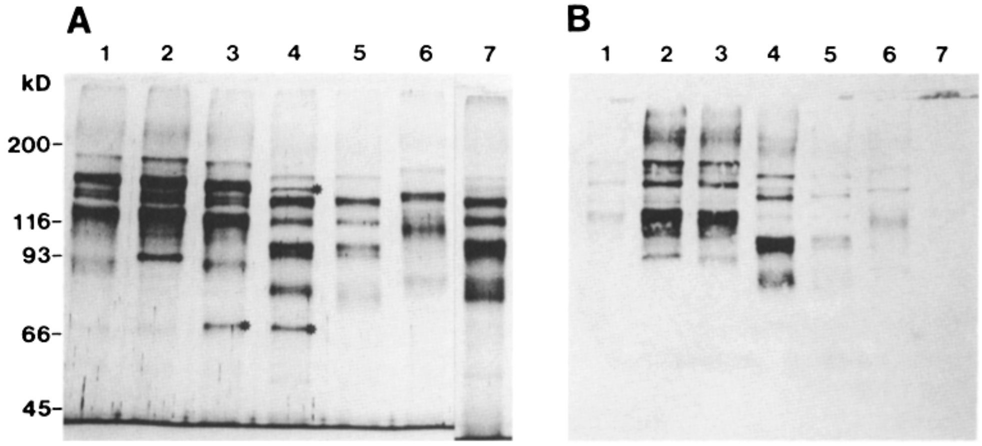


*Figure 6.* Deglycosylation of neurofascin components by N-glycosidase F (*PNGase F*), endoglycosidase H, neuraminidase, O-glycosidase, and trifluoromethanesulfonic acid (*TFMS*) and analysis of peanut lectin binding. Neurofascin was isolated by immunoaffinity chromatography from adult chicken brains and was subjected to enzymatic and chemical deglycosylation. Sialic acid was removed by digestion with neuraminidase (from Arthrobacter) (lane *2*), O-linked oligosaccharides of NeuNAc-Gal-GalNAc type by neuraminidase and O-glycosidase (lane *3*), N-glycosidically linked carbohydrates by PNGase F (lane *5*), and N-linked high-mannose and hybrid-type oligosaccharides by endo H (lane *6*). Lane *4* shows neurofascin components after treatment with neuraminidase, O-glycosidase, and PNGase F. Removal of O- and N-linked oligosaccharides was obtained by treatment with TFMS (lane *7*). The control samples were incubated under identical conditions without enzymes (lane *1*) or with TFMS that had been previously neutralized (not shown). Samples were resolved by SDS-PAGE (7%) and visualized by silver staining (*A*) or analyzed for peanut lectin binding after transfer to a nitrocellulose membrane (*B*). Labeling of biotinylated peanut lectin was visualized by alkaline phosphatase coupled to avidin. Asterisks in *A* indicate bands originating from the O-glycosidase enzyme preparation. Molecular mass standards are indicated at the left of the panel.

the red-cell membrane (Gahmberg and Andersson, 1992). The removal of distal sialic acid by neuraminidase treatment results in an increased binding of peanut lectin to neurofascin components in particular to the 250-300-kD band (Fig. 6 *B*, lanes *2* to *4*). Neuraminidase digestion followed by O-glycosidase treatment, resulting in the removal of O-glycosidically linked sugars of the NeuNAc-Gal-GalNAc type, slightly increases the electrophoretic mobiltity of neurofascin components (Fig. 6, *A* and *B*, lane *3*). This suggests that this type of O-linked sugar chains does not contribute much to the molecular mass of neurofascin components. Treatment of neurofascin components with trifluoromethanesulfonic acid (TFMS), a chemical reagent known to remove both N- and O-linked oligosaccharides (Edge et al., 1981), shows like the N-glycosidase F cleavage, a marked reduction in molecular mass (Fig. 6 *A*, lane *7*) and the complete loss of binding of peanut lectin to neurofascin bands (Fig. 6 *B*, lane *7*). Comparison of the molecular mass components observed upon N-glycosidase F digestion with those obtained by TFMS treatment (compare lane *5* and *7* of Fig. 6 *A*) reveals an additional band at 160 kD in the TFMS sample indicating the presence of O-linked oligosaccharides. The origin of this band is not clear but might represent intact neurofascin containing the PAT domain. The molecular masses of the other major neurofascin components observed by TFMS treatment do not or only slightly differ from those observed upon N-glycosidase F digestion (compare lane *5* and *7* of Fig. 6 *A*). We therefore conclude that O-glycosidic linked oligosaccharides only slightly effect the migration behavior of these neurofascin components in SDS-PAGE.

### *Neurofascin Forms with Nr-CAM and L1 (Ng-CAM) a Subgroup within the Immunoglobulin Superfamily*

The arrangement of multiple Ig- and FNIII-like domains in the neurofascin polypeptide (Fig. 7) resembles that also found in other axon-associated glycoproteins including NCAM (Cunningham et al., 1987; Walsh and Doherty, 1991), L1 (Ng-CAM) (Moos et al., 1988; Burgoon et al., 1991; Prince et al., 1991; Miura et al., 1991; Hlavin and Lemmon, 1991), F11 (Brümmendorf et al., 1989; Gennarini et al., 1989; Ranscht, 1988), TAG-1 (Furley et al., 1990), and Nr-CAM (Grumet et al., 1991) in vertebrates and fasciclin II (Harrelson and Goodman, 1988) and neuroglian (Bieber et al., 1989) in invertebrates. A direct sequence comparison of neurofascin with these molecules reveals the highest degree of sequence similarity with chicken Nr-CAM, chicken Ng-CAM, and mouse L1 including a related overall domain organization (Table I and Fig. 8). As indicated by the quality ratio of the Gap program (GCG program, University of Wisconsin) the highest similarity is obtained between neurofascin and Nr-CAM. The latter might be identical to the chicken protein designated Bravo (De la Rosa, 1990). Furthermore, neurofascin is more strongly related to mouse L1 than to Ng-CAM which might represent the species homologue of mouse L1 in the chicken. F11 and TAG-1, two GPI-anchored proteins which themselves form a subgroup within the Ig superfamily, show both a weaker relationship to neurofascin. The lowest degree of sequence similarity between neurofascin and other members of the neural Ig/FNIII-like proteins is observed with NCAM (Table I).

To further characterize the similarity between the individual domains in neurofascin, Nr-CAM, Ng-CAM and L1 quality ratios were calculated using the Gap program (GCG program, University of Wisconsin) and sequence alignments were performed (Table II and Fig. 8). This comparison shows that each neurofascin Ig-like domain is most closely related to the corresponding domain in Nr-CAM, Ng-CAM, or L1. Among these four polypeptides the second Ig-like domains are the most conserved and most interestingly the center of this domain contains a highly conserved stretch of 17 amino acid residues (amino acid residues 161 to 177 in neurofascin) suggesting that this Ig-like domain might serve for an identical or a similar function in the four proteins. Furthermore and most interestingly, neurofascin and Nr-CAM express at the COOH-terminal end of this domain a 20-

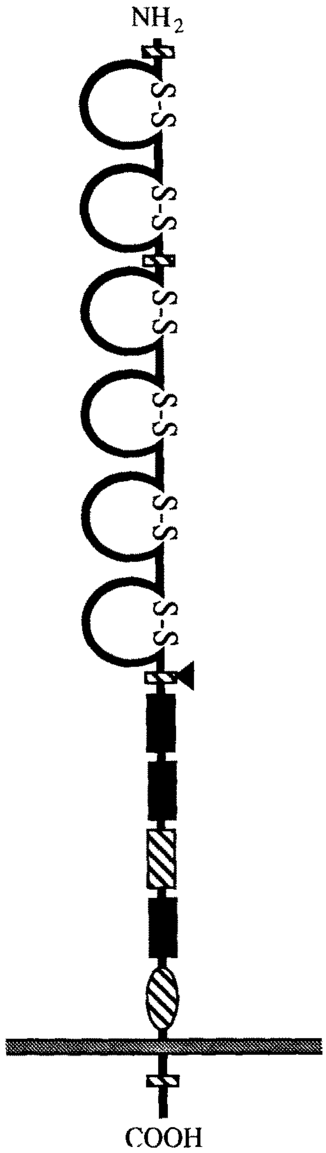


*Figure 7.* Basic domain organization of the neurofascin polypeptide. Ig-like domains of the C2 subcategory (Williams and Barclay, 1988) are shown as loops and are closed by putative disulphide bridges. FNIII-related repeats are represented as rectangles and the segment rich in proline, alanine, and threonine is indicated by an ellipse. The cytoplasmic domain is indicated by a short line at the COOH terminus. The third FNIII-like repeat and the PAT domain are hatched to indicate that they might be alternatively spliced; other potential pre-mRNA splice variants in the Ig-, between the Ig- and the FNIII-like region and in the cytoplasmic domain are indicated by small hatched boxes. The black arrowhead indicates a major proteolytic cleavage site.

amino acid–long stretch which may be alternatively spliced implying an important function of this segment in these two proteins (Figs. 2 and 8; Grumet et al., 1991). The lowest level of similarity is found between the sixth Ig-like domains. The fibronectin type III-like repeats appear slightly less conserved among neurofascin and Nr-CAM, but again each neurofascin FNIII-like domain is most closely related to its counterpart in Nr-CAM, Ng-CAM and L1. This colinear relationship of the individual Ig-and FNIII-like domains suggest an evolutionary origin of these molecules from a common ancestor (Hortsch and Goodman, 1991; Edelman and Cunningham, 1990).

The cytoplasmic tails are the most conserved domains among the four proteins, and there are two regions of increased sequence relationship evident (Fig. 8). One is close to the plasma membrane spanning segment, and the second near the COOH terminus contains a string of 12 amino acid residues (QFNEDGSFIGQY) that is also, except for one amino acid position, detected in the long form of the invertebrate cell adhesion molecule neuroglian (Bieber et al., 1989; Hortsch et al., 1990). The cytoplasmic tetrapeptide RSLE which is present in Ng-CAM but not in Nr-CAM might be alternatively spliced in neurofascin as it has been recently described for mammalian L1 (Miura et al., 1991; Harper et al., 1991; Prince et al., 1991). On the other hand, the cytoplasmic pentapeptide TFGEY which is conserved among Nr-CAM, Ng-CAM, and L1 is contracted to SFY in neurofascin.

*Table I. Relationship of Neurofascin and Other Axon-associated Ig/FNIII-like Proteins Expressed in the Vertebrate Nervous System and Implicated in Axonal Growth*

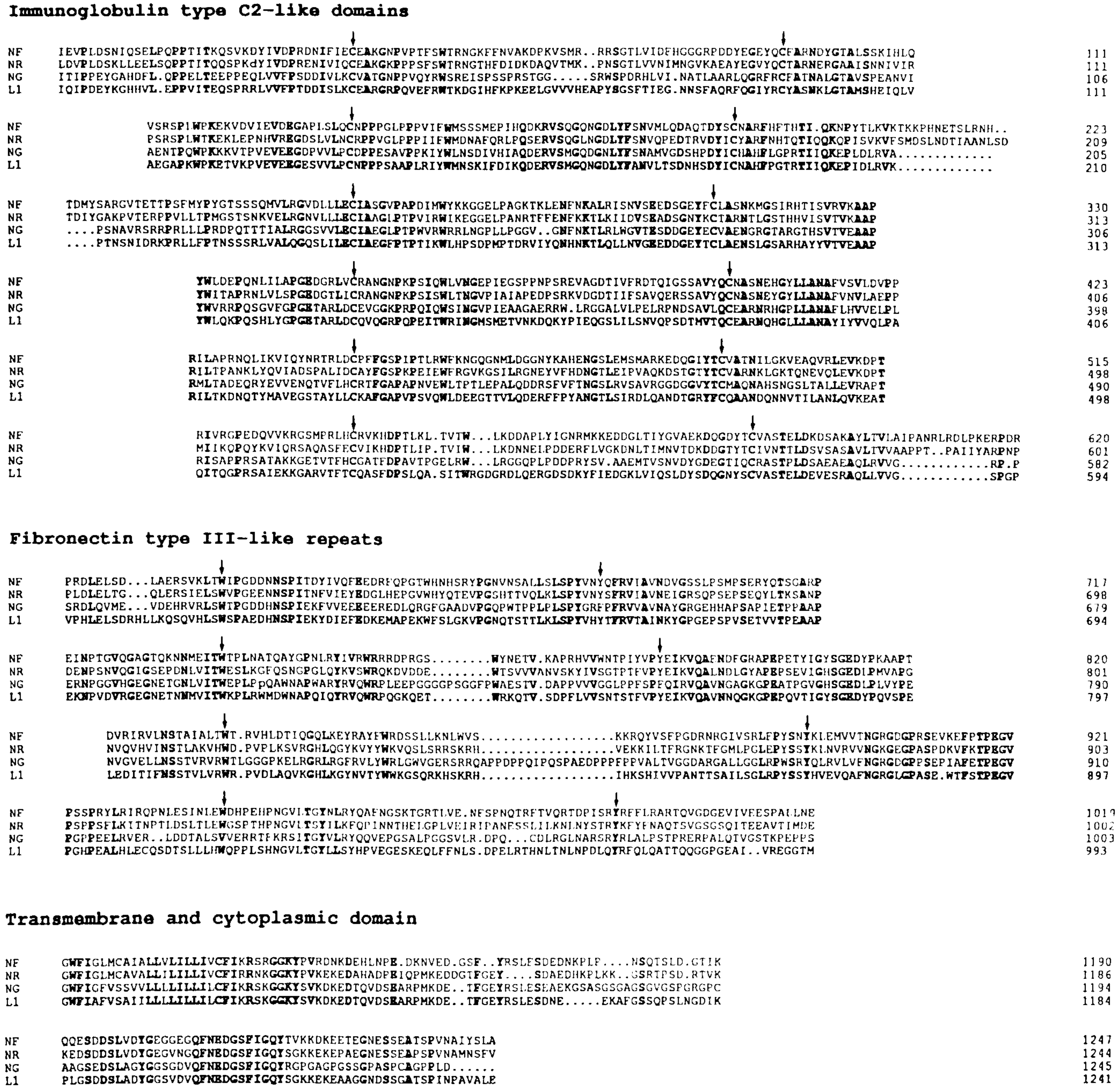
| | NF | NR | NG | L1 | F11 | TAG-1 | NCAM |
|---|---|---|---|---|---|---|---|
| NF | 1.5 | | | | | | |
| NR | 0.868 | 1.5 | | | | | |
| NG | 0.624 | 0.665 | 1.5 | | | | |
| L1 | 0.697 | 0.728 | 0.798 | 1.5 | | | |
| F11 | 0.503 | 0.476 | 0.496 | 0.497 | 1.5 | | |
| TAG-1 | 0.491 | 0.494 | 0.492 | 0.512 | 0.882 | 1.5 | |
| NCAM | 0.376 | 0.371 | 0.374 | 0.373 | 0.368 | 0.356 | 1.5 |

L1 and TAG-1 sequences are from mouse and rat, respectively, whereas the other proteins are from chick. Each value represents the quality ratio of pairwise compared proteins using the Gap program (GCG, University of Wisconsin).

## *Discussion*

Neurofascin is a chick neurite-associated surface glycoprotein implicated in axon extension as demonstrated by classical antibody perturbation experiments in two distinct in vitro bioassays (Rathjen et al., 1987*a*). The primary structure of neurofascin reported here reveals that it is a new member of the immunoglobulin superfamily containing both multiple Ig- and FNIII-like domains. Its close structural relationship to L1 together with its timing and pattern of expression in developing axon tracts also suggest that it may be involved in aspects of axonal growth during embryonic development as it has been shown more extensively for L1 by several independent assays (Rathjen and Schachner, 1984; Hoffman et al., 1986; Fischer et al., 1986; Stallcup et al., 1985; Rathjen et al., 1987*b*; Chang et al., 1987; Lagenaur and Lemmon, 1987; Lemmon et al., 1989; Landmesser et al., 1988; Chang et al., 1990; Kuhn et al., 1991).

The $NH_2$-terminal half of neurofascin contains like other axon-associated glycoproteins six Ig-like domains of the C2 subcategory (Williams and Barclay, 1988), and the second domain is the most conserved when compared with L1, Ng-CAM, and Nr-CAM. In ICAM-1 (CD54) the most $NH_2$-terminal located Ig-like domains have been implicated in binding with LFA-1 and Mac-1, two proteins belonging to the integrin protein family (Staunton et al., 1990; Diamond et al., 1991), and in NCAM a heparin-binding domain has been mapped to its second Ig-like domain (Cole et al., 1989). Axonal recognition molecules might use their individual Ig-like domains to mediate specific interactions with other cell surface or extracellular matrix proteins. It remains to be seen whether corresponding regions in neurofascin are also involved in interactions with other proteins expressed on axons. A common feature of several members of the Ig superfamily on cells of the immune system is that they associate specifically with other members of the Ig superfamily within the same or across plasma membranes to regulate cel-



```
Immunoglobulin type C2-like domains

                                              ↓                                                        ↓
NF      IEVPLDSNIQSELPQPPTITKQSVKDYIVDPRDNIFIECEAKGNPVPTFSWTRNGKFFNVAKDPKVSMR..RRSGTLVIDFHGGGRPDDYEGEYQCFARNDYGTALSSKIHLQ  111
NR      LDVPLDSKLLEELSQPPTITQQSPKdYIVDPRENIVIQCEAKGKPPPSFSWTRNGTHFDIDKDAQVTMK..PNSGTLVVNIMNGVKAEAYEGVYQCTARNERGAAISNNIVIR  111
NG      ITIPPEYGAHDFL.QPPELTEEPPEQLVVFPSDDIVLKCVATGNPPVQYRWSREISPSSPRSTGG.....SRWSPDRHLVI.NATLAARLQGRFRCFATNALGTAVSPEANVI  106
L1      IQIPDEYKGHHVL.EPPVITEQSPRRLVVFPTDDISLKCEARGRPQVEFRWTKDGIHFKPKEELGVVVHEAPYSGSFTIEG.NNSFAQRFQGIYRCYASNKLGTAMSHEIQLV  111

                                              ↓                                                  ↓
NF                  VSRSPIWPKEKVDVIEVDEGAPLSLQCNPPPGLPPPVIFWMSSSMEPIHQDKRVSQGQNGDLYFSNVMLQDAQTDYSCNARFHFTHTI.QKNPYTLKVKTKKPHNETSLRNH..  223
NR                  PSRSPLWTKEKLEPNHVREGDSLVLNCRPPVGLPPPIIFWMDNAFQRLPQSERVSQGLNGDLYFSNVQPEDTRVDYICYARFNHTQTIQQKQPISVKVFSMDSLNDTIAANLSD  209
NG                  AENTPQWPKKKVTPVEVEEGDPVVLPCDPPESAVPPKIYWLNSDIVHIAQDERVSMGQDGNLYFSNAMVGDSHPDYICHAHFLGPRTIIQKEPLDLRVA............  205
L1                  AEGAPKWPKETVKPVEVEEGESVVLPCNPPPSAAPLRIYWMNSKIFDIKQDERVSMGQNGDLYFANVLTSDNHSDYICNAHFPGTRTIIQKEPIDLRVK............  210

                                              ↓                                               ↓
NF       TDMYSARGVTETTPSFMYPYGTSSSQMVLRGVDLLLECIASGVPAPDIMWYKKGGELPAGKTKLENFNKALRISNVSEEDSGEYFCLASNKMGSIRHTISVRVKAAP  330
NR       TDIYGAKPVTERPPVLLTPMGSTSNKVELRGNVLLLECIAAGLPTPVIRWIKEGGELPANRTFFENFKKTLKIIDVSEADSGNYKCTARNTLGSTHHVISVTVKAAP  313
NG       ....PSNAVRSRRPRLLLPRDPQTTTIALRGGSVVLECIAEGLPTPWVRWRRLNGPLLPGGV..GNFNKTLRLWGVTESDDGEYECVAENGRGTARGTHSVTVEAAP  306
L1       ....PTNSNIDRKPRLLFPTNSSSRLVALQGQSLILECIAEGFPTPTIKWLHPSDPMPTDRVIYQNHNKTLQLLNVGEEDDGEYTCLAENSLGSARHAYYVTVEAAP  313

                                               ↓                                                 ↓
NF                        YWLDEPQNLIILAPGEDGRLVCRANGNPKPSIQWLVNGEPIEGSPPNPSREVAGDTIVFRDTQIGSSAVYQCNASNEHGYLLANAFVSVLDVPP  423
NR                        YWITAPRNLVLSPGEDGTLICRANGNPKPSISWLTNGVPIAIAPEDPSRKVDGDTIIFSAVQERSSAVYQCNASNEYGYLLANAFVNVLAEPP  406
NG                        YWVRRPQSGVFGPGETARLDCEVGGKPRPQIQWSINGVPIEAAGAERRW.LRGGALVLPELRPNDSAVLQCEARNRHGPLLANAFLHVVELPL  398
L1                        YWLQKPQSHLYGPGETARLDCQVQGRPQPEITWRINGMSMETVNKDQKYPIEQGSLILSNVQPSDTMVTQCEARNQHGLLLANAYIYVVQLPA  406

                                              ↓                                                ↓
NF                       RILAPRNQLIKVIQYNRTRLDCPFFGSPIPTLRWFKNGQGNMLDGGNYKAHENGSLEMSMARKEDQGIYTCVATNILGKVEAQVRLEVKDPT  515
NR                       RILTPANKLYQVIADSPALIDCAYFGSPKPEIEWFRGVKGSILRGNEYVFHDNGTLEIPVAQKDSTGTYTCVARNKLGKTQNEVQLEVKDPT  498
NG                       RMLTADEQRYEVVENQTVFLHCRTFGAPAPNVEWLTPTLEPALQDDRSFVFTNGSLRVSAVRGGDGGVYTCMAQNAHSNGSLTALLEVRAPT  490
L1                       RILTKDNQTYMAVEGSTAYLLCKAFGAPVPSVQWLDEEGTTVLQDERFFPYANGTLSIRDLQANDTGRYFCQAANDQNNVTILANLQVKEAT  498

                                              ↓                                                    ↓
NF                        RIVRGPEDQVVKRGSMPRLHCRVKHDPTLKL.TVTW...LKDDAPLYIGNRMKKEDDGLTIYGVAEKDQGDYTCVASTELDKDSAKAYLTVLAIPANRLRDLPKERPDR  620
NR                        MIIKQPQYKVIQRSAQASFECVIKHDPTLIP.TVIW...LKDNNELPDDERFLVGKDNLTIMNVTDKDDGTYTCIVNTTLDSVSASAVLTVVAAPPT..PAIIYARPNP  601
NG                        RISAPPRSATAKKGETVTFHCGATFDPAVTPGELRW...LRGGQPLPDDPRYSV.AAEMTVSNVDYGDEGTIQCRASTPLDSAEAEAQLRVVG............RP.P  582
L1                        QITQGPRSAIEKKGARVTFTCQASFDPSLQA.SITWRGDGRDLQERGDSDKYFIEDGKLVIQSLDYSDQGNYSCVASTELDEVESRAQLLVVG............SPGP  594

Fibronectin type III-like repeats

                             ↓                                                 ↓
NF       PRDLELSD...LAERSVKLTWIPGDDNNSPITDYIVQFEEDRFQPGTWHNHSRYPGNVNSALLSLSPYVNYQFRVIAVNDVGSSLPSMPSERYQTSGARP  717
NR       PLDLELTG...QLERSIELSWVPGEENNSPITNFVIEYEDGLHEPGVWHYQTEVPGSHTTVQLKLSPYVNYSFRVIAVNEIGRSQPSEPSEQYLTKSANP  698
NG       SRDLQVME...VDEHRVRLSWTPGDDHNSPIEKFVVEEEEEREDLQRGFGAADVPGQPWTPPLPLSPYGRFPFRVVAVNAYGRGEHHAPSAPIETPPAAP  679
L1       VPHLELSDRHLLKQSQVHLSWSPAEDHNSPIEKYDIEFEDKEMAPEKWFSLGKVPGNQTSTTLKLSPYVHYTFRVTAINKYGPGEPSPVSETVVTPEAAP  694

                             ↓                                                         ↓
NF       EINPTGVQGAGTQKNNMEITWTPLNATQAYGPNLRYIVRWRRRDPRGS........WYNETV.KAPRHVVWNTPIYVPYEIKVQAFNDFGRAPEPETYIGYSGEDYPKAAPT  820
NR       DENPSNVQGIGSEPDNLVITWESLKGFQSNGPGLQYKVSWRQKDVDDE........WTSVVVANVSKYIVSGTPTFVPYEIKVQALNDLGYAPEPSEVIGHSGEDIPMVAPG  801
NG       ERNPGGVHGEGNETGNLVITWEPLPpQAWNAPWARYRVQWRPLEEPGGGGPSGGFPWAESTV.DAPPVVVGGLPPFSPFQIRVQAVNGAGKGPEATPGVGHSGEDLPLVYPE  790
L1       EKNPVDVRGEGNETNNMVITWKPLRWMDWNAPQIQYRVQWRPQGKQET........WRKQTV.SDPFLVVSNTSTFVPYEIKVQAVNNQGKGPEPQVTIGYSGEDYPQVSPE  797

                             ↓                                                                            ↓
NF            DVRIRVLNSTAIALTWT.RVHLDTIQGQLKEYRAYFWRDSSLLKNLWVS..................KKRQYVSFPGDRNRGIVSRLFPYSNYKLEMVVTNGRGDGPRSEVKEFPTPEGV  921
NR            NVQVHVINSTLAKVHWD.PVPLKSVRGHLQGYKVYYWKVQSLSRRSKRH..................VEKKILTFRGNKTFGMLPGLEPYSSYKLNVRVVNGKGEGPASPDKVFKTPEGV  903
NG            NVGVELLNSSTVRVRWTLGGGPKELRGRLRGFRVLYWRLGWVGERSRRQAPPDPPQIPQSPAEDPPPFPPVALTVGGDARGALLGGLRPWSRYQLRVLVFNGRGDGPPSEPIAFETPEGV  910
L1            LEDITIFNSSTVLVRWR.PVDLAQVKGHLKGYNVTYWWKGSQRKHSKRH..................IHKSHIVVPANTTSAILSGLRPYSSYHVEVQAFNGRGLGPASE.WTFSTPEGV  897

                             ↓                                                   ↓
NF       PSSPRYLRIRQPNLESINLEWDHPEHPNGVLTGYNLRYQAFNGSKTGRTLVE.NFSPNQTRFTVQRTDPISRYRFFLRARTQVGDGEVIVEESPALLNE  1019
NR       PSPPSFLKITNPTLDSLTLEWGSPTHPNGVLTSYILKFQPINNTHELGPLVEIRIPANESSLILKNLNYSTRYKFYFNAQTSVGSGSQITEEAVTIMDE  1002
NG       PGPPEELRVER..LDDTALSVVERRTFKRSITGYVLRYQQVEPGSALPGGSVLR.DPQ...CDLRGLNARSRYRLALPSTPRERPALQTVGSTKPEPPS  1003
L1       PGHPEALHLECQSDTSLLLHWQPPLSHNGVLTGYLLSYHPVEGESKEQLFFNLS.DPELRTHNLTNLNPDLQYRFQLQATTQQGGPGEAI..VREGGTM  993

Transmembrane and cytoplasmic domain

NF      GWFIGLMCAIALLVLILLIVCFIKRSRGGKYPVRDNKDEHLNPE.DKNVED.GSF..YRSLFSDEDNKPLF....NSQTSLD.GTIK  1190
NR      GWFIGLMCAVALLILILLIVCFIRRNKGGKYPVKEKEDAHADPEIQPMKEDDGTFGEY....SDAEDHKPLKK..GSRTPSD.RTVK  1186
NG      GWFIGFVSSVVLLLLILLILCFIKRSKGGKYSVKDKEDTQVDSEARPMKDE..TFGEYRSLESEAEKGSASGSGAGSGVGSPGRGPC  1194
L1      GWFIAFVSAIILLLLILLILCFIKRSKGGKYSVKDKEDTQVDSEARPMKDE..TFGEYRSLESDNE.....EKAFGSSQPSLNGDIK  1184

NF      QQESDDSLVDYGEGGEGQFNEDGSFIGQYTVKKDKEETEGNESSEATSPVNAIYSLA  1247
NR      KEDSDDSLVDYGEGVNGQFNEDGSFIGQYSGKKEKEPAEGNESSEAPSPVNAMNSFV  1244
NG      AAGSEDSLAGYGGSGDVQFNEDGSFIGQYRGPGAGPGSSGPASPCAGPPLD......  1245
L1      PLGSDDSLADYGGSVDVQFNEDGSFIGQYSGKKEKEAAGGNDSSGATSPINPAVALE  1241
```

*Figure 8.* Alignment analysis of amino acid sequences of individual domains of chicken neurofascin (*NF*), Nr-CAM (*NR*), Ng-CAM (*NG*), and mouse L1 using the PileUP program (GCG, University of Wisconsin). Characteristic cysteine residues in the Ig-like domains and tryptophan and tyrosine residues in the FNIII-related repeats are indicated by arrows. Residues shared by the four proteins are printed in bold and the amino acid positions are given on the right.

lular interactions (Williams and Barclay, 1988). For the neural members of the Ig superfamily, it has recently been demonstrated that L1 and axonin-1 bind to each other to induce axon outgrowth (Kuhn et al., 1991). Axonin-1 which exists as secreted and GPI-anchored form represents the chick homologue of rat TAG-1 (Furley et al., 1990; Zuellig et al., 1992). It is therefore conceivable that structurally related molecules in the same subgroup of neurofascin may interact with other Ig-related proteins to serve related functions in different parts of the nervous system. However, although L1 and neurofascin are very similar no homophilic or heterophilic binding to F11, NCAM, or L1 could be demonstrated for neurofascin so far (unpublished observations).

The function of the FNIII-like repeats found in several proteins involved in axonal growth are not well understood, however, certain repeats contain the tripeptide RGD that in extracellular matrix proteins such as fibronectin is involved in specific cell binding (Pierschbacher and Ruoslahti, 1984). In neurofascin the third FNIII-related repeat contains, like the corresponding domain in Ng-CAM, this peptide sequence in an environment that resembles the RGD region in fibronectin. However, at present it is unclear whether such structure in neurofascin or in any other axon-associated gly-

*Table II. Relationship between Individual Ig- (A), FNIII-like (B) and the Cytoplasmic Domains (C) of Neurofascin (NF), Nr-CAM (NR), Ng-CAM (NG), and Mouse L1*

| | | NF-I | NF-II | NF-III | NF-IV | NF-V | NF-VI |
|---|---|---|---|---|---|---|---|
| A | NF-I | 1.5 | | | | | |
| | -II | 0.383 | 1.5 | | | | |
| | -III | 0.387 | 0.374 | 1.5 | | | |
| | -IV | 0.429 | 0.349 | 0.414 | 1.5 | | |
| | -V | 0.363 | 0.360 | 0.482 | 0.408 | 1.5 | |
| | -VI | 0.301 | 0.350 | 0.387 | 0.455 | 0.473 | 1.5 |
| | NR-I | **1.040** | 0.382 | 0.354 | 0.499 | 0.330 | 0.321 |
| | -II | 0.422 | **0.999** | 0.466 | 0.329 | 0.342 | 0.321 |
| | -III | 0.398 | 0.349 | **0.977** | 0.401 | 0.466 | 0.397 |
| | -IV | 0.462 | 0.370 | 0.458 | **1.132** | 0.449 | 0.538 |
| | -V | 0.404 | 0.392 | 0.515 | 0.410 | **0.904** | 0.410 |
| | -VI | 0.296 | 0.333 | 0.451 | 0.413 | 0.471 | **0.786** |
| | NG-I | **0.565** | 0.319 | 0.389 | 0.416 | 0.374 | 0.322 |
| | -II | 0.449 | **0.892** | 0.345 | 0.359 | 0.337 | 0.308 |
| | -III | 0.414 | 0.304 | **0.735** | 0.440 | 0.478 | 0.445 |
| | -IV | 0.417 | 0.332 | 0.426 | **0.828** | 0.462 | 0.446 |
| | -V | 0.387 | 0.426 | 0.510 | 0.437 | **0.641** | 0.435 |
| | -VI | 0.341 | 0.409 | 0.454 | 0.446 | 0.427 | **0.552** |
| | L1 -I | **0.625** | 0.308 | 0.385 | 0.423 | 0.413 | 0.342 |
| | -II | 0.456 | **0.922** | 0.387 | 0.319 | 0.302 | 0.329 |
| | -III | 0.420 | 0.429 | **0.818** | 0.432 | 0.503 | 0.426 |
| | -IV | 0.401 | 0.446 | 0.387 | **0.781** | 0.423 | 0.453 |
| | -V | 0.444 | 0.414 | 0.486 | 0.593 | **0.640** | 0.372 |
| | -VI | 0.353 | 0.346 | 0.385 | 0.435 | 0.424 | **0.571** |
| B | NF-I | 1.5 | | | | | |
| | -II | 0.396 | 1.5 | | | | |
| | -III | 0.439 | 0.342 | 1.5 | | | |
| | -IV | 0.373 | 0.308 | 0.349 | 1.5 | | |
| | NR-I | **0.974** | 0.441 | 0.387 | 0.396 | | |
| | -II | 0.431 | **0.858** | 0.357 | 0.355 | | |
| | -III | 0.418 | 0.330 | **0.762** | 0.357 | | |
| | -IV | 0.366 | 0.303 | 0.336 | **0.805** | | |
| | -V | 0.340 | 0.320 | 0.315 | 0.377 | | |
| | NG-I | **0.740** | 0.413 | 0.349 | 0.408 | | |
| | -II | 0.458 | **0.732** | 0.403 | 0.343 | | |
| | -III | 0.454 | 0.325 | **0.622** | 0.329 | | |
| | -IV | 0.343 | 0.316 | 0.358 | **0.417** | | |
| | -V | 0.365 | 0.308 | 0.347 | 0.353 | | |
| | L1 -I | **0.798** | 0.374 | 0.368 | 0.421 | | |
| | -II | 0.407 | **0.869** | 0.385 | 0.319 | | |
| | -III | 0.435 | 0.388 | **0.663** | 0.314 | | |
| | -IV | 0.410 | 0.292 | 0.346 | **0.543** | | |
| | -V | 0.322 | 0.332 | 0.301 | 0.333 | | |

| | | NF | NR | NG | L1 |
|---|---|---|---|---|---|
| C | NF | 1.5 | | | |
| | NR | 1.063 | 1.5 | | |
| | NG | 0.812 | 0.826 | 1.5 | |
| | L1 | 1.054 | 0.978 | 1.065 | 1.5 |

Each value represents the quality ratio of pairwise compared domains using the Gap program (GCG, University of Wisconsin). Values indicating the highest similarity are printed in bold. Exact amino acid positions are given in Figs. 2 and 8.

coprotein including TAG-1 and Ng-CAM is implicated in axon extension or cell binding.

Close to the plasma membrane spanning region some variant forms of neurofascin contain an unusual 75-amino acid residues-long segment rich in proline, alanine, and threonine which might be extensively O-glycosylated. A similar structural motif near the membrane bound domain has been described for several other cell surface proteins including the low density lipoprotein (LDL) receptor (Yamamoto et al., 1984; Russell et al., 1984), the decay accelerating factor (DAF) (Medhof et al., 1987), the platelet glycoprotein Ib (Lopez et al., 1987), and a specific form of NCAM (Dickson et al., 1987; Walsh et al., 1989). While N-linked sugars have been shown to modulate the homophilic binding activities of NCAM (Rutishauser et al., 1988; Walsh and Doherty, 1991), the function of O-linked oligosaccharides remains less well understood. The O-glycosylation could give NCAM and neurofascin a specific conformation, in particular to induce a longer stiff structure which would extend their $NH_2$-terminal Ig-like region well above the axonal surface as has also been proposed for the functional domain of the LDL receptor and DAF (Jentoft, 1990). The extension of the Ig-like region above the axonal glycocalyx might allow neurofascin to interact with other macromolecules in the environment of an extending axon which otherwise are not accessible.

The cytoplasmic segments represent the most conserved regions between neurofascin, Nr-CAM, Ng-CAM, L1, and the *Drosophila* protein neuroglian implying that they may be critical for the process of neurite outgrowth possibly by interacting with cytoskeletal or other intracellular proteins. This notion is in line with the finding that L1 co-localizes with actin in the filopodia of extending growth cones (Letournou and Shattuck, 1989). Evidence that the interaction of cell adhesion molecules with the cytoskeleton is crucial for their function has been provided by the work on another family of adhesion proteins expressed in the nervous system, the cadherins (Takeichi, 1991). Truncation of the cytoplasmic region of E-cadherin leads to a loss of its binding activity (Nagafuchi and Takeichi, 1988) and proteins have been described, designated catenin-$\alpha$, -$\beta$, and -$\gamma$, that associate with the intracellular domain of cadherins (Ozawa et al., 1989). Such proteins have so far not been detected for the L1 group of molecules while the 261-amino acid residues-long insert in the cytoplasmic tail of NCAM-180 was found to interact with spectrin (Pollenberg et al., 1987). There are other indications that the cytoplasmic segment might be required for the function of L1 including the activation of intracellular second messenger systems (Schuch et al., 1989) and phosphorylation by a specific kinase (Sadoul et al., 1989).

The domain arrangement and the overall amino acid identity indicates that neurofascin, Nr-CAM, and L1(Ng-CAM) form a subgroup within the Ig superfamily in vertebrates. Despite their similarities, however, neurofascin differs from both in that several variant forms of it might be expressed and that it contains a 75-amino acid residues-long segment rich in proline, alanine, and threonine. Whether additional members of this subgroup, not detected by the immunological approach, are expressed at a much lower abundance or on specific subsets of axons during development remains to be seen. As discussed elsewhere, the colinear relationship of the individual Ig- and FNIII-related domains in these proteins also suggests an evolutionary origin from a common ancestor (Grenningloh et al., 1990; Edelman and Cunningham, 1990). F11 and TAG-1 which are most similar to each other form a second neural subgroup within the vertebrate Ig superfamily. Both proteins comprise six Ig- and four

FNIII-related domains and are attached to the plasma membrane via GPI and are involved in axonal growth (Rathjen et al., 1987*b*; Chang et al., 1987; Brümmendorf et al., 1989; Gennarini et al., 1989; Ranscht, 1988; Wolff et al., 1989; Furley et al., 1990; Gennarini et al., 1991; Dodd et al., 1988; Stoeckli et al., 1991; Zuellig et al., 1992).

It is evident that the in vitro antibody perturbation experiments used to monitor neurofascin function provide an approximation of function (Rathjen et al., 1987*a*). All these assays are indirect in that they are dependent on the use of specific antibody reagents. However, binding of even monovalent antibodies to the cell surface may nonspecifically interfere with neighboring proteins and should therefore be considered presumptive until further confirmation is obtained by other independent methods. A direct demonstration of axonal outgrowth on purified neurofascin has so far failed in contrast to L1 (our unpublished observations). One reason might be that purified neurofascin is very sensitive to degradation leading to its inactivation. Alternatively, there might exist several forms of neurofascin with distinct or contrasting functions. The complexity of the neurofascin structure requires an alternative system to further study its biological function. The expression of different forms or of specific segments of neurofascin cDNA in cell lines might resolve in which mode neurofascin participates in neurite extension as it has been revealed for NCAM (Doherty et al., 1990; Doherty et al., 1991).

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