Structural Requirements for Association of Neurofascin with Ankyrin*

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Xu Zhang, Jonathan Q. Davis, Scott Carpenter, and Vann Bennett‡

From the Howard Hughes Medical Institute and Departments of Cell Biology and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

This paper presents the first structural analysis of the cytoplasmic domain of neurofascin, which is highly conserved among the LICAM family of cell adhesion molecules, and describes sequence requirements for neurofascin-ankyrin interactions in living cells. The cytoplasmic domain of neurofascin dimerizes in solution, has an asymmetric shape, and exhibits a reversible temperature-dependent β-structure. Residues Ser56–Tyr81 are necessary for ankyrin binding but do not contribute to either dimerization or formation of structure. Transfected neurofascin recruits GFP-tagged 270-kDa ankyrinG to the plasma membrane of human embryo kidney 293 cells. Deletion mutants demonstrate that the sequence Ser56–Tyr81 contains the major ankyrin-recruiting activity of neurofascin. Mutations of the FIGQY tyrosine (Y81H/A/E) greatly impair neurofascin-ankyrin interactions. Mutation of human L1 at the equivalent tyrosine (Y1229H) is responsible for certain cases of mental retardation (Van Camp, G., Fransen, E., Vits, L., Raes, G., and Willems, P. J. (1996) Hum. Mutat. 8, 391). Mutations F77A and E73Q greatly impair ankyrin binding activity, whereas mutation D74N and a triple mutation of D57N/D58N/D62N result in less loss of ankyrin binding activity. These results provide evidence for a highly specific interaction between ankyrin and neurofascin and suggest that ankyrin association with L1 is required for L1 function in humans.

L1, CHL1, neurofascin, NrCAM, and NgCAM in vertebrates and neuroglin in Drosophila are members of the LICAM family of cell adhesion molecules (1, 2). These proteins possess variable ectodomains that engage in homophilic as well as heterophilic interactions and have in common a conserved cytoplasmic domain that binds to the membrane skeletal protein ankyrin (1, 3, 4). LICAM family members are abundant in brain tissue (3, 4) and participate in diverse cellular activities including axon fasciculation, myelination, synaptogenesis, and axonal guidance (1, 5, 6). Mutations in the human L1 gene are responsible for developmental abnormalities including mental retardation and hydrocephalus (7–9).

The cytoplasmic domains of LICAM cell adhesion molecules contain a highly conserved sequence that has been identified as a binding site for members of the ankyrin family of membrane skeletal proteins (3, 4, 10–12). Association of LICAM molecules with ankyrin was first characterized for neurofascin, which was originally identified as a brain protein that associated with ankyrin-coupled affinity columns (3). Subsequent studies demonstrated that other LICAM family molecules including L1, NrCAM, and neuroglian also have ankyrin binding activity in their cytoplasmic domains (4, 10, 12). The membrane binding domain of ankyrin has been demonstrated to have two distinct binding sites for neurofascin and other membrane proteins and is proposed to form lateral complexes between ion channels and cell adhesion molecules as well as to couple these proteins to the spectrin-based membrane skeleton (13–15). Physiologically relevant sites for interactions between ankyrin and LICAM molecules include nodes of Ranvier and axon initial segments, where neurofascin and NrCAM are concentrated and co-localized with specialized isoforms of ankyrin, 270/480-kDa ankyrinG (16). In addition, L1 and ankyrinG are co-localized and believed to associate with each other in unmyelinated axons based on loss of L1 in premyelinated axon tracts of ankyrinG(−/−) mice.1

Ankyrin binding activity of neurofascin requires a highly conserved sequence from Ser56 to Tyr81 in the cytoplasmic domain and in particular the sequence FIGQY (Phe77–Tyr81) that is present in all LICAM family members (11, 12). Internal deletion of these sequences abolishes ankyrin binding. In addition, the tyrosine residue in the FIGQY sequence has been identified as the major tyrosine phosphorylation site of neurofascin (11). Phosphorylation of the FIGQY tyrosine eliminates ankyrin binding of neurofascin in vitro and reduces coupling of neurofascin to the cytoskeleton in vivo (11). Inhibition of neurofascin-ankyrin interaction by tyrosine phosphorylation also results in dissociation of cell-cell adhesion mediated by homophilic interactions between neurofascin molecules expressed in cultured cells (18).

Questions remaining unanswered concern the oligomeric state and folding of the neurofascin cytoplasmic domain and the influence of tyrosine phosphorylation and mutations on these parameters. Furthermore, since previous studies on neurofascin-ankyrin interaction are largely based on in vitro binding assays (3, 4, 11, 14), it is not clear whether the high or low affinity interactions identified in vitro actually occur in living cells.

This study presents analysis of primary and secondary structural requirements for association between neurofascin and 270-kDa ankyrinG. Ankyrin-neurofascin interactions were detected based on ability of transfected neurofascin to recruit GFP-tagged 270-kDa ankyrinG to the plasma membrane of human embryo kidney 293 cells. Results of this assay demonstrate that the FIGQY tyrosine residue as well as certain other aromatic and negatively charged residues in the sequence Ser56–Tyr81 are essential for the neurofascin-ankyrin interaction. Moreover, mutation of the tyrosine residue in the FIGQY sequence to histidine (Y81H) greatly impairs ankyrin binding

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‡ To whom correspondence should be addressed. Tel.: 919-684-3538; Fax: 919-684-3590; E-mail: benne012@mc.duke.edu.

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activity of neurofascin. An equivalent mutation in the human L1 gene (Y1229H) results in mental retardation and hydrocephalus (19). These results provide evidence for a highly specific interaction between ankyrin and neurofascin involving residues conserved among L1CAM family members and suggest that ankyrin association with L1 is required for L1 function in humans.

MATERIALS AND METHODS

Preparation of cDNA Constructs—Assembly of the full-length cDNA construct of 270-kDa ankyrinG (Ank270) was achieved by ligating the first half of the membrane-binding domain, which was isolated from an adult rat brain 5′-stretch plus cDNA library (CLONTECH) by PCR2 and constructed with a number of overlapping fragments. The identity of expressed neurofascin cytoplasmic domain polypeptides was confirmed by N-terminal sequencing (see "Materials and Methods").

### Table I

| Property                  | Native (S56–Y81) | ΔS56–Y81 |
|---------------------------|------------------|----------|
| Sedimentation coefficient | 1.5              | 1.6      |
| Partial specific volume, v | 0.73 cm³/g       | 0.73 cm³/g |
| Stokes radius, Rₜ         | 3.1 nm           | 3.0 nm   |
| Mᵣ, calculated            | 20,000           | 20,000   |
| Mᵣ, actual                | 12,186           | 10,705   |
| Frictional ratio, f/f₀     | 1.7              | 1.6      |

* From sedimentation velocity on a Beckman ultracentrifuge.

* Estimated from the amino acid composition.

* The Stokes radius was determined from gel filtration on a calibrated Superose 12 column (see "Materials and Methods").

* Calculated from the equation below and rounded to two significant figures.

\[ Mᵣ = \frac{6\pi NRₛₚ₂₀,w}{1 - \nuₚ₂₀,w} \] (Eq. 1)

* Calculated from the amino acid sequence.

* Calculated from the following equation.

\[ \frac{f}{f₀} = Rₜ \left( \frac{4\pi N \nu}{3Mᵣ(\nu + 8\nu)} \right)^{1/3} \] (Eq. 2)

![270 kDa AnkyrinG](image)

![Neurofascins](image)

**Fig. 2.** Schematic diagram of cDNA constructs used in transfection experiments. Ank270-GFP represents the full-length 270-kDa ankyrinG with a GFP tag at its C terminus. Construct HA-NF is full-length neurofascin with the HA epitope at the N terminus. Deletion of the extracellular domain results in construct Epo-NF(E21-A109). Deletion of most of the cytoplasmic domain encompassing from Glu21 to Ala109 results in construct HA-NF(E21-A109). The numbers assigned to the amino acid residues are based on the published method (11), in which the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSSGG; see also Fig. 2) has been assigned as the number 1 residue. Epo-NF(E21-A109) is a chimeric protein with the cytoplasmic domain of erythropoietin receptor replaced by a part of the cytoplasmic domain of neurofascin containing the sequence from Glu21 to Ala109.

![Association of Neurofascin with Ankyrin](image)
Association of Neurofascin with Ankyrin

Point mutations of the cytoplasmic domain of neurofascin were prepared using the QuickChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing (20). The full-length cDNA construct of neurofascin (Epo-NF(E21-A109) was amplified at the N terminus (11) was cut out by HindIII–NotI digestion of pBluescript KS vector (Stratagene) and subcloned into the corresponding sites of pEGFP-N1 vector (CLONTECH). The confirmed construct (HA-NF; Fig. 1) does not contain the EGFP sequence and is driven by a cytomegalovirus promoter. All other neurofascin constructs were prepared from construct HA-NF unless indicated. The cytoplasmic domain-deleted neurofascin HA-NFΔE21-A109 was made by replacing the Scal–NotI fragment of construct HA-NF with a PCR-amplified fragment containing the sequence of the Scal–ApoI fragment with a stop codon following the ApoI site (Fig. 2). The extracellular domain-truncated neurofascin (HA-NFΔΔE); Fig. 2) was prepared through two steps. First, the 5'-untranslated region of neurofascin, the start codon, the signal peptide, and HA tag were PCR-amplified and subcloned into the BglII–HindIII sites of the pEGFP-N1 vector. Then the transmembrane and cytoplasmic domains were introduced by PCR into the HindIII–NotI sites of the first step construct. The chimeric protein with the cytoplasmic domain of erythropoietin receptor replaced by a part of the cytoplasmic domain of neurofascin (Epo-NF(E21-A109); Fig. 2) was prepared by replacing the HindIII–ApoI fragment of HA-NF with a PCR-amplified sequence containing the extracellular and transmembrane domains of the erythropoietin receptor, which was kindly provided by Dr. Harvey Lodish (MIT).

Neurofascin constructs with deletions from the C terminus of the cytoplasmic domain of HA-NFΔΔE were purified from the soluble fraction of lysed bacteria using a nickel–NTA affinity column (Qiagen) while keeping in frame with the downstream EGFP protein. For immunofluorescence, transfected 293 cells were fixed in 2% paraformaldehyde for 10 min and then incubated with blocking buffer (10% normal goat serum and 2% bovine serum albumin in phosphate-buffered saline) for another 5 min before applying the primary antibody against HA epitope (Babco). After 3 h of incubation with the primary antibody, cells were washed three times with phosphate-buffered saline and subjected to secondary stain using tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibody for 1 h. All immunostaining procedures were performed at room temperature. In the case of co-transfection of Ank270-GFP and Epo-NF(E21-A109), 0.1% Triton X-164 was included in the blocking buffer, and the primary stain was an antibody specific for the F11GQY sequence in the cytoplasmic domain of neurofascin. All immunofluorescence experiments were recorded using a Zeiss LSM 410 confocal microscope. Experiments were replicated at least three times.

**Determination of Physical Properties of the Cytoplasmic Domain of Neurofascin—**DNA constructs of the neurofascin cytoplasmic domain were amplified by PCR using 186-kDa rat neurofascin cDNA as a template (19) with ScI–NotI and a SphI restriction sites for subcloning. The PCR products were restricted and ligated into a Pet vector with a C-terminal histidine tag (Novagen Pet 28b(+)). Cytoplasmic domain lacking the histidine tag was also expressed and exhibited the same properties as His tag constructs. Plasmids were transformed into BL21 DE3 (λ) E. coli and expressed with isopropyl-1-thio-β-D-galactopyranoside induction. Expressed proteins with histidine tags were purified from the soluble fraction of lysed bacteria using a nickel-

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**Fig. 3. Endogenous spectrin of 293 cells is insufficient to recruit transfected ankyrin to the plasma membrane.** A, immunostaining of endogenous spectrin in 293 cells, which is predominantly localized at the plasma membrane. B, immunostaining of endogenous ankyrin, which is also concentrated at the plasma membrane. C, transfected 270-kDa ankyrin (Ank270-GFP) visualized by GFP signal is distributed throughout the cytoplasm of 293 cells.
nitrilotriacetic acid-agarose affinity column (Qiagen). The eluate from the nickel column was then dialyzed and applied to a Mono S high pressure liquid chromatography ion exchanger column, which was subsequently eluted with a sodium chloride salt gradient. Proteins lacking a histidine tag were isolated using Mono Q ion exchange chromatography and gel filtration on Superose 12 in addition to Mono S ion exchange chromatography. The identity of purified polypeptides was established by N-terminal sequencing following transfer to polyvinylidene difluoride paper (3, 13). The purity of polypeptides was at least 90% based on SDS-polyacrylamide gel electrophoresis.

The Stokes radii ($R_s$) of cytoplasmic domains were estimated by gel filtration on a Superose 12 column equilibrated with 10 mM sodium phosphate, 100 mM NaCl, 0.5 mM dithiothreitol, 1 mM NaN$_3$, pH 7.4, and calibrated with the following protein standards: ferritin ($R_s = 6.1$ nm), catalase ($R_s = 5.2$ nm), bovine serum albumin ($R_s = 3.5$ nm), ovalbumin ($R_s = 3.05$ nm), and cytochrome c ($R_s = 2$ nm). The sedimentation coefficients were determined by sedimentation equilibrium on a Beckman XL-A Optima analytical centrifuge, and the sedimentation patterns were analyzed by the Ideal 1 software program from Beckman. The measurements of Table I were repeated three times with comparable results. Values are presented for a single representative experiment.

RESULTS

Secondary Structure and Oligomeric State of the Cytoplasmic Domain of Neurofascin—The secondary structure of the cytoplasmic domain of native neurofascin expressed in bacteria was evaluated by CD spectroscopy (Fig. 1). At 5 °C, the dominant negative peak at 200 nm in the CD spectrum (Fig. 1A) suggested the native cytoplasmic domain is predominantly a random coil (21). Hydrodynamic measurements of the Stokes radius ($R_s = 3.1$ nm) and the frictional ratio ($f/f_o = 1.7$) also indicate an extended conformation of the cytoplasmic domain of native neurofascin (Table I). At higher temperatures (37 and 65 °C), however, there is evidence of structure, as demonstrated by the reduction of absorbance above 210 nm at higher temperatures (Fig. 1A). Difference spectra between 65 and 5 °C revealed a curve consistent with β-structure with a positive peak at 195 nm as well as a negative peak at 220 nm (Fig. 1C). The appearance of β-structure is fully reversible upon lowering the temperature (data not shown). This temperature dependence implies hydrophobic interactions involved in stabilizing β-structure in the cytoplasmic domain of neurofascin (21).

The sequence Ser$_{56}$–Tyr$_{81}$ is necessary for ankyrin binding to intact neurofascin expressed in neuroblastoma cells (11). The contribution of residues Ser$_{56}$–Tyr$_{81}$ to the secondary structure of the cytoplasmic domain of neurofascin expressed in bacteria was evaluated by measuring the CD spectrum of the cytoplasmic domain with internal deletion of Ser$_{56}$–Tyr$_{81}$ (Fig. 1B). The appearance of a positive peak at 195 nm and a negative peak at 220 nm in the difference spectrum, which is almost identical to that of native neurofascin, indicates that the sequence Ser$_{56}$–Tyr$_{81}$ does not contribute to formation of the temperature-dependent secondary structure.

The cytoplasmic domain of neurofascin exists as a dimer or in a monomer-dimer equilibrium, based on molecular weight determined by sedimentation equilibrium measurements (Table I). The cytoplasmic domain with internal deletion of Ser$_{56}$–Tyr$_{81}$ also behaves as a dimer (Table I). Sedimentation measurements were performed at 25 °C, which is a condition with minimal temperature-induced secondary structure detected by CD spectroscopy. Therefore, assembly of neurofascin cytoplasmic domain with internal deletion of Ser$_{56}$–Tyr$_{81}$ is predominantly localized in the cytoplasm of transfected cells. Transfected ankyrin (Ank270-GFP) is recruited to the plasma membrane, although the majority of the expressed Epo-NF(E21-A109) is localized in the cytoplasm of transfected cells. Transfected ankyrin (Ank270-GFP) is predominantly localized in the cytoplasm instead of at the plasma membrane. Double labeling of co-transfection of extracellular domain-truncated neurofascin (HA-NF(E21-A109)) and Ank270-GFP. Ank270-GFP is associated with the plasma membrane. D, co-transfection of chimeric protein Epo-NF(E21-A109) and Ank270-GFP. Epo-NF(E21-A109) recruits Ank270-GFP to the plasma membrane, although the majority of the expressed Epo-NF(E21-A109) is localized in the cytoplasm of transfected cells.

![Fig. 4](image.png)

**Fig. 4.** The cytoplasmic domain of neurofascin is sufficient and necessary for recruitment of co-transfected ankyrin to the plasma membrane. A, double labeling of co-transfection of neurofascin (HA-NF) and 270-kDa ankyrin$_B$ (Ank270-GFP) in 293 cells. Transfected ankyrin (Ank270-GFP) is recruited to the plasma membrane. B, double labeling of co-transfection of the cytoplasmic domain-deleted neurofascin (HA-NF(E21-A109)) and Ank270-GFP. Ank270-GFP is predominantly localized in the cytoplasm instead of at the plasma membrane. C, double labeling of co-transfection of extracellular domain-truncated neurofascin (HA-NF(ΔEC)) and Ank270-GFP. Ank270-GFP is associated with the plasma membrane. D, co-transfection of chimeric protein Epo-NF(E21-A109) and Ank270-GFP. Epo-NF(E21-A109) recruits Ank270-GFP to the plasma membrane, although the majority of the expressed Epo-NF(E21-A109) is localized in the cytoplasm of transfected cells. Epo-NF(E21-A109) recruits Ank270-GFP to the plasma membrane, although the majority of the expressed Epo-NF(E21-A109) is localized in the cytoplasm of transfected cells.
The sequence of the cytoplasmic domain of neurofascin and its mutants

| Consensus: | 21 | 31 | 51 | 61 | 71 | 81 | 101 |
|------------|----|----|----|----|----|----|----|
| HA-NF:     |    |    |    |    |    |    |    |
| HA-NF(E21-A109): |    |    |    |    |    |    |    |
| HA-NF(37-A109): |    |    |    |    |    |    |    |
| HA-NF(5-A109): |    |    |    |    |    |    |    |
| HA-NF(S27-A109): |    |    |    |    |    |    |    |
| HA-NF(S77-Y81): |    |    |    |    |    |    |    |
| HA-NF(S77-A109): |    |    |    |    |    |    |    |
| HA-NF(S56-Y81): |    |    |    |    |    |    |    |
| HA-NF(E21-E55): |    |    |    |    |    |    |    |
| HA-NF(E21-A70): |    |    |    |    |    |    |    |
| HA-NF(E21-Q70): |    |    |    |    |    |    |    |
| HA-NF(Y16-F): |    |    |    |    |    |    |    |
| HA-NF(Y16-A): |    |    |    |    |    |    |    |
| HA-NF(Y16-I): |    |    |    |    |    |    |    |
| HA-NF(Y18-H): |    |    |    |    |    |    |    |
| HA-NF(Y18-I): |    |    |    |    |    |    |    |
| HA-NF(Y18-L): |    |    |    |    |    |    |    |
| HA-NF(Y18-Y): |    |    |    |    |    |    |    |
| HA-NF(77-A): |    |    |    |    |    |    |    |
| HA-NF(77-I): |    |    |    |    |    |    |    |
| HA-NF(77-L): |    |    |    |    |    |    |    |
| HA-NF(77-Y): |    |    |    |    |    |    |    |
| HA-NF(77-N): |    |    |    |    |    |    |    |
| HA-NF(57,58,62): |    |    |    |    |    |    |    |

The addition of the sequence from Glu21 to Ala109 (Ank270-GFP) was recruited to the plasma membrane by co-transfected ankyrins (see text). The intensity ratio between the GFP signals at the plasma membrane and inside the cytoplasm (MIC) is used as an approximate scale to measure the affinity of the association of 270-kDa ankyrin with co-transfected neurofascins.  

**Fig. 5. Summary of effects of different mutations in the cytoplasmic domain of neurofascin on membrane recruitment of co-transfected 270-kDa ankyrin.** Numbers for the amino acid residues in the cytoplasmic domain of neurofascin are assigned based on the method of Garver et al. (11), in which the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSSRGG) has been assigned as the number 1 residue (not shown in the displayed sequence). The COOH terminus residue is Ala109. The consensus sequence is Ser56–Tyr81, which is essential for ankyrin binding to full-length neurofascin (11), contributes to dimerization or to formation of β-structure. The sequence Ser56–Tyr81 thus has little residual ankyrin binding activity (Fig. 6, C). Deletion of a major portion of the cytoplasmic domain of neurofascin extending from Glu21 to the C terminus residue Ala109 (HA-NFΔE21-A109; Fig. 5) abolished ankyrin recruitment to the plasma membrane (Fig. 4B). The numbers assigned to the amino acid residues in the cytoplasmic domain of neurofascin are based on the convention where the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSSRGG; Fig. 5) is residue 1 (11). The addition of the sequence from Glu21 to Ala109 to the cytoplasmic domain-deleted erythropoietin receptor resulted in a chimeric protein (Epo-NF(E21-A109)) with full activity in recruiting co-transfected ankyrin to the plasma membrane (Fig. 4D). These data indicate that the sequence from Glu21 to Ala109 of the cytoplasmic domain of neurofascin determines activity in binding to ankyrin.

**The Sequence Ser26–Tyr81 Contains the Major Ankyrin-binding Site of Neurofascin**—In the *in vivo* assay described above was used to evaluate the effect on ankyrin binding of various internal deletions in the cytoplasmic domain of neurofascin. Internal deletion from Ser26 to Tyr31 (HA-NFΔS56-Y81) (Fig. 5) abolished recruitment of co-transfected ankyrin to the plasma membrane in the *in vivo* assay (Fig. 6A), as observed previously in direct binding assays (11). Two other mutants with shorter internal deletions, one from Glu70 to Tyr81 (HA-NFΔ(Δ70-Y81)) and the other from Phe72 to Tyr81 (HA-NFΔ(Δ77-Y81)) (Fig. 5), also eliminated the ability of neurofascin to recruit co-transfected ankyrin to the plasma membrane (Fig. 6, B and C, respectively). Neurofascin with internal deletion of Phe72–Tyr81 retained about 20% ankyrin binding activity compared with the native protein in *in vitro* binding assays (11). However, the cell assay developed in this study failed to detect this residual ankyrin binding activity (Fig. 6C). These results in the cytoplasmic domain of neurofascin preceding Ser26 and following Tyr81 do not contribute significantly to ankyrin binding activity. As demonstrated above using constructs Epo-NF(E21-A109) and HA-NFΔE21-A109 (Fig. 5), the sequence before Glu21 that includes the extracellular and transmembrane domains and part of the cytoplasmic domain (K1-P20) was neither necessary (Fig. 4D) nor sufficient (Fig. 4B) for recruitment of ankyrin to the plasma membrane. A neurofascin mutant with an internal deletion of the sequence
from Glu\textsuperscript{21} to Glu\textsuperscript{55} (HA-NA(\Delta E21-E55)) retained full activity to recruit transfected ankyrin to the plasma membrane (Fig. 7A). Deletion of the sequence following Tyr\textsuperscript{81} (HA-NA(\Delta Q70-Y81)) also exerted little effect on recruitment of ankyrin to the plasma membrane (Fig. 8A).}

While residues outside of the critical Ser\textsuperscript{56}-Tyr\textsuperscript{81} stretch are not required for recruitment of ankyrin to the plasma membrane, sequences within Ser\textsuperscript{56}-Tyr\textsuperscript{81} are essential for activity. Internal deletions extending from Glu\textsuperscript{21} to Gly\textsuperscript{64} (HA-NA(\Delta E21-G64)) or from Glu\textsuperscript{21} to Gly\textsuperscript{70} (HA-NA(\Delta E21-Q70)) (Fig. 5) greatly impair ankyrin-recruiting activity (Fig. 7, B and C, respectively). Deletion of residues from Phe\textsuperscript{77} to Ala\textsuperscript{109} (HA-NA(\Delta F77-Y81)) also eliminate the ability of the mutant neurofascin to recruit co-transfected Ank270-GFP to the plasma membrane. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

**Fig. 6.** The sequence from Ser\textsuperscript{56} to Tyr\textsuperscript{81} in the cytoplasmic domain of transfected neurofascin is necessary for recruitment of co-transfected ankyrin to the plasma membrane. Internal deletion of the sequence from Ser\textsuperscript{56} to Tyr\textsuperscript{81} (HA-NA(\Delta S56-Y81)) (A) and internal deletion from Gln\textsuperscript{70} to Tyr\textsuperscript{81} (HA-NA(\Delta Q70-Y81)) (B) abolish the ability of the mutant neurofascin to recruit co-transfected Ank270-GFP to the plasma membrane. C, internal deletion of the FIGQY sequence (HA-NA(\Delta F77-Y81)) also eliminate the ability of the mutant neurofascin to recruit Ank270-GFP to the plasma membrane. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

**Fig. 7.** The sequence following Ser\textsuperscript{56} is sufficient for recruitment of co-transfected ankyrin to the plasma membrane. A, neurofascin mutant with internal deletion from Glu\textsuperscript{21} to Glu\textsuperscript{55} (HA-NA(\Delta E21-E55)) retains full ability to recruit co-transfected Ank270-GFP to the plasma membrane. B and C, internal deletions from Glu\textsuperscript{21} to Gly\textsuperscript{64} (HA-NA(\Delta E21-G64)) and from Glu\textsuperscript{21} to Gly\textsuperscript{70} (HA-NA(\Delta E21-Q70)), respectively, abolish ankyrin-recruiting activity. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.
abolishes ankyrin binding activity (11, 18). Mutation of Tyr81 to Phe 77 (HA-NF(Y81F)) retains full ankyrin-recruiting activity. These experiments demonstrated a critical role for the FIGQY tyrosine in neurofascin-ankyrin interactions. The clinical importance of the tyrosine residue in the FIGQY sequence is emphasized by the finding that a mutation in the cytoplasmic domain of human L1 molecule with the corresponding tyrosine residue changed to histidine (Y1229H) is responsible for the development of mental retardation and hydrocephalus (19). An equivalent mutation in neurofascin (Y81H) was prepared and evaluated for its activity to recruit co-transfected ankyrin to the plasma membrane. Mutation of Tyr81 to histidine greatly impairs ankyrin recruitment activity in 293 cells (Fig. 9D). This result suggests that disruption of the interaction between L1 and ankyrin may be the molecular basis for symptoms of patients with the L1 Y1229H mutation.

Interaction between Neurofascin and Ankyrin Involves Conserved Aromatic and Negatively Charged Residues within the Gln70–Tyr81 Stretch—Other aromatic residues in addition to Tyr81 in the highly conserved Q70FNEDGFIGQY81 sequence also participate in association between neurofascin and ankyrin. F77A (HA-NF(F77A)) and F71A (HA-NF(F71A)) (Fig. 5) mutations both impair ankyrin recruitment to the plasma membrane (Fig. 10, A and B, respectively). Moreover, mutation F71L (HA-NF(F71L)), compared with mutation F71A, does not significantly improve ankyrin recruitment to the plasma membrane (Fig. 10C), suggesting that the function of the aromatic group in neurofascin-ankyrin interactions cannot be replaced by a hydrophobic side chain.

Electrostatic interactions between neurofascin and ankyrin have been inferred from in vitro binding assays based on sensitivity to salt (13, 14). Neutralizing a negative charge by mutation of Glu73 to glutamine (HA-NF(E73Q)) abolished the ankyrin-recruiting activity of the mutant neurofascin (Fig. 11A). However, mutation of Asp74 to asparagine (HA-NF(D74N)) had much less effect on neurofascin-ankyrin association (Fig. 11B). Changing all three aspartic acid residues at Asp77, Asp79, and Asp82 to asparagine (HA-NF(D57,58,62N)) had little effect on recruiting ankyrin to the plasma membrane (Fig. 11C). These data (summarized in Fig. 5) indicate that electrostatic interactions between neurofascin and ankyrin are confined to specific charged residues and provide evidence for a high degree of specificity in the interaction between neurofascin and ankyrin.

DISCUSSION

This paper presents the first structural analysis of the conserved cytoplasmic domain of the L1CAM family of cell adhesion molecules and provides a detailed structural and functional analysis of neurofascin-ankyrin interactions in living cells. The cytoplasmic domain of neurofascin dimerizes in solution, has an asymmetric shape, and exhibits a reversible temperature-dependent β-structure. The sequence from Ser86 to Tyr81, which is necessary for ankyrin binding, does not contribute to either dimerization or formation of structure in the cytoplasmic domain of neurofascin. A qualitative assay for evaluation of neurofascin-ankyrin interactions in living cells has been developed. Using this assay, we confirmed results of previous in vitro binding assays in living cells (11) and provided additional evidence that the sequence from Ser86 to Tyr81 contains the major ankyrin-binding site. The FIGQY tyrosine (Tyr81), which is the site for tyrosine phosphorylation that abolishes ankyrin binding (11), has been demonstrated to be critical for neurofascin-ankyrin interactions. Mutation of the FIGQY tyrosine to histidine greatly impairs neurofascin-ankyrin interactions. An equivalent mutation in the human L1
molecule (Y1229H) is responsible for certain cases of hydrocephalus and mental retardation (19). Since L1 and neurofascin share a conserved cytoplasmic binding site for ankyrin (4, 11), disruption of L1-ankyrin interactions may be the molecular basis for pathology due to this L1 mutation (Y1229H). Other conserved aromatic and negatively charged residues in the sequence Ser56–Tyr81 are also shown to contribute to neurofascin-ankyrin interactions. Mutation E73Q, which has a minimal alteration of the side chain, greatly impairs ankyrin binding activity, whereas a triple mutation of D57N/D58N/D62N resulted in no loss of ankyrin binding activity. These data provide strong evidence for a high degree of specificity in the interaction between neurofascin and ankyrin.

The new assay for evaluation of neurofascin-ankyrin interactions in co-transfected 293 cells is rapid and effective, although qualitative, and has several potential applications. This assay could be used to screen various point mutations in ankyrin as well as in the L1CAM family of cell adhesion molecules for their effects on ankyrin-L1CAM interactions in living cells. The membrane recruitment assay could also be used to identify potential proteins such as protein-tyrosine kinases and protein-tyrosine phosphatases that are involved in regulation of association of neurofascin with ankyrin (11). The clinical importance of ankyrin-L1CAM interactions has been exemplified by the point mutation Y1229H in the human L1 gene. Moreover, the membrane recruitment assay could be applied to screen pharmaceutical drugs that can modulate ankyrin-L1CAM interactions and perhaps cause developmental defects in the nervous system. Nonetheless, the assay has drawbacks due to lack of quantitative information and does not provide a dissociation constant \( K_d \) describing the affinity of association of mutant neurofascins with ankyrin. In addition, without a careful control of the amount of cDNAs used in co-transfection experiments, one species of cDNA could be overexpressed and complicate subsequent analysis. By using the same type of vector, the same promoter and 3'-untranslated region, and carefully controlled cDNA ratios, our results can be successfully reproduced.

Previous studies of ankyrin-neuroglian interactions in Drosophila S2 tissue culture cells have demonstrated that ankyrin selectively associates with neuroglian at sites of cell-cell contact but not other regions of the plasma membrane (10, 12). These observations suggest that recruitment of ankyrin by neuroglian requires activation of neuroglian by extracellular interactions. However, our study demonstrates that recruitment of transfected ankyrin to the plasma membrane of cultured human embryonic kidney cells (293 cells) is solely governed by the presence of the cytoplasmic domain of neurofascin localized at the plasma membrane and is independent of whether the co-transfected cell is isolated or has contact with other cells. The basis for these differences may reflect specialized behavior among L1 CAM family members, differences between S2 cells and human 293 cells, and differences between 270-kDa ankyrinG and the ankyrin expressed in S2 cells.

An unexpected result of this study is identification of the cytoplasmic domain of neurofascin as a dimer in solution. The effects of dimerization of neurofascin on ankyrin binding and on lateral organization of neurofascin in the plane of the membrane have yet to be determined. Since the ankyrin-binding sequence from Ser56-Tyr81 does not contribute to dimerization and sequences outside the Ser56-Tyr81 sequence do not contribute to ankyrin binding, it is likely that formation of neurofascin dimers does not directly affect ankyrin binding, at least at the qualitative level detectable in the membrane recruitment assay. However, dimerization of neurofascin provides a potential mechanism for formation of neurofascin-ankyrin polymers. Considered together with the multiple binding sites for neurofascin in ankyrinR (13, 14), the existence of neurofascin dimers...
implies that neurofascin and ankyrin are able to form lateral complexes containing multiple copies of neurofascin, ankyrin, and possibly other ankyrin-binding membrane proteins. These complexes could be further immobilized by coupling to the spectrin-based membrane skeleton through the spectrin-binding domain of ankyrin. The ability to form such large immobilized complexes between ankyrin and neurofascin could be important for the assembly of specialized membrane domains such as axon initial segments and nodes of Ranvier, where these proteins are localized (16)3.

Lack of discernible secondary structure in the sequence Ser56–Tyr81 indicates that effects of various deletions and mutations in this sequence on ankyrin binding are more likely exerted by direct perturbation of the contact site with ankyrin rather than by disrupting the conformation of the cytoplasmic domain of neurofascin. Atomic structure of ankyrin repeats of the transcription factor GAPB-β revealed that the protein binding site in ankyrin repeats is configured as a tandem array of extended loops, where the tips of these loops provide the binding interface (17). Extrapolation of this information regarding the ankyrin repeats of GAPB-β to ankyrin suggests the sequence Ser56–Tyr81 in the cytoplasmic domain of neurofascin also is in contact with predicted loops. This prediction ultimate

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mately can be evaluated by determining the structure of ankyrin-neurofascin complexes. It may also be possible to mutate ankyrin at predicted loop sites and “repair” loss of binding of certain neurofascin mutations as well as create ankyrins specifically lacking neurofascin binding activity.

In summary, this study presents a structural and functional analysis of the interaction between ankyrin and the cytoplasmic domain of neurofascin in living cells. Since the ankyrin-binding site is conserved among members of L1CAM family of cell adhesion molecules, our results with neurofascin are likely to apply to other L1CAM molecules.

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