Tyrosine Phosphorylation at a Site Highly Conserved in the L1 Family of Cell Adhesion Molecules Abolishes Ankyrin Binding and Increases Lateral Mobility of Neurofascin

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Abstract. This paper presents evidence that a member of the L1 family of ankyrin-binding cell adhesion molecules is a substrate for protein tyrosine kinase(s) and phosphatase(s), identifies the highly conserved FIGQY tyrosine in the cytoplasmic domain as the principal site of phosphorylation, and demonstrates that phosphorylation of the FIGQY tyrosine abolishes ankyrin-binding activity. Neurofascin expressed in neuroblastoma cells is subject to tyrosine phosphorylation after activation of tyrosine kinases by NGF or bFGF or inactivation of tyrosine phosphatases with vanadate or dephostatin. Furthermore, both neurofascin and the related molecule Nr-CAM are tyrosine phosphorylated in a developmentally regulated pattern in rat brain. The FIGQY sequence is present in the cytoplasmic domains of all members of the L1 family of neural cell adhesion molecules. Phosphorylation of the FIGQY tyrosine abolishes ankyrin binding, as determined by coimmunoprecipitation of endogenous ankyrin and in vitro ankyrin-binding assays. Measurements of fluorescence recovery after photobleaching demonstrate that phosphorylation of the FIGQY tyrosine also increases lateral mobility of neurofascin expressed in neuroblastoma cells to the same extent as removal of the cytoplasmic domain. Ankyrin binding, therefore, appears to regulate the dynamic behavior of neurofascin and is the target for regulation by tyrosine phosphorylation in response to external signals. These findings suggest that tyrosine phosphorylation at the FIGQY site represents a highly conserved mechanism, used by the entire class of L1-related cell adhesion molecules, for regulation of ankyrin-dependent connections to the spectrin skeleton.

Vertebrate L1, neurofascin, neuroglial cell adhesion molecule (Ng-CAM),[1] Ng-CAM–related cell adhesion molecule (Nr-CAM), and *Drosophila* neuroglian are members of a family of nervous system cell adhesion molecules that possess variable extracellular domains comprised of Ig and fibronectin type III domains and a relatively conserved cytoplasmic domain (Grumet, 1991; Hortsch and Goodman, 1991; Rathgen and Jessel, 1991; Sonderegger and Rathgen, 1992; Hortsch, 1996). Members of this family, including a number of alternatively spliced forms, are abundant in the nervous system during early development as well as in adults. Neurofascin and Nr-CAM, for example, constitute ~0.5% of the total membrane protein in adult brain (Davis et al., 1993; Davis and Bennett, 1994). Cellular functions attributed to the L1 family include axon fasciculation (Stallcup and Beasley, 1985; Landmesser et al., 1988; Brummendorf and Rathjen, 1993; Bastmeyer et al., 1995; Itoh et al., 1995; Magyar-Lehmann et al., 1995), axonal guidance (van den Pol and Kim, 1993; Liljelund et al., 1994; Brittis and Silver, 1995; Brittis et al., 1995; Lochter et al., 1995; Wong et al., 1996), neurite extension (Chang et al., 1987; Felsenfeld et al., 1994; Hankin and Lagenaur, 1994; Ignelzi et al., 1994; Williams et al., 1994*a*,*b*,*c*,*d*; Doherty et al., 1995; Zhao and Siu, 1995), a role in long term potentiation (Luthl et al., 1994), synaptogenesis (Itoh et al., 1995), and myelination (Wood et al., 1990). The potential clinical importance of this group of proteins has been emphasized by the findings that mutations in the L1 gene on the X chromosome are responsible for developmental anomalies including hydrocephalus and mental retardation (Rosenthal et al., 1992; Jouet et al., 1994; Wong et al., 1995).

The conserved cytoplasmic domains of L1 family members include a binding site for the membrane skeletal protein ankyrin. This interaction was first described for neurofascin (Davis et. al., 1993) and subsequently has been

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1. *Abbreviations used in this paper*: Ng-CAM, neuroglial cell adhesion molecule; Nr-CAM, Ng-CAM–related cell adhesion molecule.

observed for L1, Nr-CAM (Davis and Bennett, 1994), and *Drosophila* neuroglian (Dubreuil et al., 1996). The membrane-binding domain of ankyrin contains two distinct sites for neurofascin and has the potential to promote lateral association of neurofascin and presumably other L1 family members (Michaely and Bennett, 1995). Nodes of Ranvier are physiologically relevant axonal sites where ankyrin and L1 family members collaborate, based on findings of colocalization of a specialized isoform of ankyrin with alternatively spliced forms of neurofascin and Nr-CAM in adults (Davis et al., 1996) as well as in early axonal developmental intermediates (Lambert, S., J. Davis, P. Michael, and V. Bennett. 1995. *Mol. Biol. Cell.* 6:98a).

L1, after homophilic and/or heterophilic binding, participates in signal transduction pathways that ultimately are associated with neurite extension and outgrowth (Ignelzi et al., 1994; Williams et al., 1994*a*,*b*,*c*,*d*; Doherty et al., 1995). L1 copurifies with a serine–threonine protein kinase (Sadoul et al., 1989) and is phosphorylated on a serine residue that is not conserved among other family members (Wong et al., 1996). L1 pathway(s) may also involve G proteins, calcium channels, and tyrosine phosphorylation (Williams et al., 1994*a*,*b*,*c*,*d*; Doherty et al., 1995). After homophilic interactions, L1 directly activates a tyrosine signaling cascade after a lateral association of its ectodomain with the fibroblast growth factor receptor (Doherty et al., 1995). Antibodies against L1 have also been shown to activate protein tyrosine phosphatase activity in growth cones (Klinz et al., 1995). However, details of the downstream substrates of L1-promoted phosphorylation and dephosphorylation and possible roles of the cytoplasmic domain are not known.

Tyrosine phosphorylation is well established to modulate cell–cell and cell–extracellular matrix interactions involving integrins and their associated proteins (Akiyama et al., 1994; Arroyo et al., 1994; Schlaepfer et al., 1994; Law et al., 1996) as well as the cadherins (Balsamo et al., 1996; Krypta et al., 1996; Brady-Kalnay et al., 1995; Shibamoto et al., 1995; Hoschuetzky et al., 1994; Matsuyoshi et al., 1992). For example, the adhesive functions of the calcium-dependent cadherin cell adhesion molecule are mediated by a dynamic balance between tyrosine phosphorylation of β-catenin by TrkA and dephosphorylation via the LAR-type protein tyrosine phosphatase (Krypta et al., 1996). In this example the regulation of binding among the structural proteins is the result of a coordination between classes of protein kinases and protein phosphatases.

This study presents evidence that neurofascin, expressed in a rat neuroblastoma cell line, is a substrate for both tyrosine kinases and protein tyrosine phosphatases at a tyrosine residue conserved among all members of the L1 family. Site-specific tyrosine phosphorylation promoted by both tyrosine kinase activators (NGF and bFGF) and protein tyrosine phosphatase inhibitors (dephostatin and vanadate) is a strong negative regulator of the neurofascin–ankyrin binding interaction and modulates the membrane dynamic behavior of neurofascin. Furthermore, neurofascin and, to a lesser extent Nr-CAM, are also shown here to be tyrosine phosphorylated in developing rat brain, implying a physiological relevance to this phenomenon. These results indicate that neurofascin may be a target for the coordinate control over phosphorylation that is elicited by protein kinases and phosphatases during in vivo tyrosine phosphorylation cascades. The consequent decrease in ankyrin-binding capacity due to phosphorylation of neurofascin could represent a general mechanism among the L1 family members for regulation of membrane–cytoskeletal interactions in both developing and adult nervous systems.

Materials and Methods

SDS-PAGE and Immunoblotting

SDS–polyacrylamide gel electrophoresis and Western blotting, after transfer of resolved proteins to nitrocellulose, were performed as previously described (Davis and Bennett, 1983, 1984). Briefly, immunoblots were incubated overnight at 4°C with each primary antibody, washed, and then incubated with ^{125}I-radiolabeled protein A for 2 h at room temperature. Blots were visualized via autoradiography. The primary antibodies used included the anti-phosphotyrosine polyclonal (1:1,000; Upstate Biotechnology Inc., Lake Placid, NY), a mucin-specific neurofascin polyclonal (1:1,000; Davis et al., 1996), the Nr-CAM–specific polyclonal (1:1,000; Davis et al., 1993), and the brain ankyrin-specific polyclonal antibodies (1:1,000; Davis and Bennett, 1984).

Construct Generation and Transfection of B104 Cells

A 600-bp ApaI–EcoRI fragment of wild-type neurofascin cDNA, which encodes most of the cytoplasmic domain sequence, was subcloned into pBluescript vector (Stratagene, La Jolla, CA). All deletions and point mutations were carried out using the Exsite PCR-based mutagenesis kit (Stratagene). The HA epitope was inserted by PCR six amino acids downstream of the signal peptide. Mutated constructs were confirmed by DNA sequencing before the wild-type ApaI–EcoRI sequence was substituted by the mutated fragment. Both HA epitope-tagged wild-type and cytoplasmic domain-truncated neurofascin were subcloned into pCMV vector at HindIII and BamHI sites. The pCMV vector was derived from pBC12 (Cullen, 1986) with the following modifications: (*a*) the human IL-2 cDNA sequence (760–1437 bp) was deleted and replaced by a multiple cloning site containing NheI, SmaI, SalI, and XhoI restriction sites; (*b*) the BamHI fragment of the Neo^R gene with an SV40 promotor was inserted into the BglII site. All the mutated neurofascin cDNA constructs were subcloned into the pC13 CAT vector at HindIII and NotI sites. The pC13 CAT vector was derived from pOP13 CAT (Stratagene) with the RSV promotor of pOP13 CAT being replaced by the CMV promotor from pBC12 at SacII and BglII sites.

Transfection of B104 cells was carried out using lipofectamine (GIBCO BRL, Gaithersburg, MD). For each 35-mm dish, 5 μg of DNA and 10 μl (2 mg/ml) of lipofectamine were used following the instructions provided by GIBCO BRL. 2 d after transfection, 1.5 μg/ml of geneticin (GIBCO BRL) was added to the media, and colonies of neoresistance cells were visible 1–2 wk afterward. Approximately 24 single colonies for each construct were chosen and expanded for Western blot analysis to examine levels of neurofascin expression. Clones with highest expression levels were used for subsequent studies.

Immunoprecipitation of Epitope-tagged Neurofascin

After lysis of cells with a modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM vanadate, 1 mM benzamidine, 1 mM PMSF, aprotinin [10 μg/ml], and leupeptin [10 μg/ml]), neurofascin was immunoprecipitated overnight at 4°C from 250–500 μg crude cell lysate using an HA-specific monoclonal antibody (BAbCO, Berkeley, CA). Immune complexes were captured with either protein A–Sepharose (Pierce, Rockford, IL) for SDS-PAGE analysis or with protein A–latex beads for use in the in vitro ankyrin-binding assays.

In Vitro Ankyrin Binding Assays

The ability of neurofascin to bind ankyrin was quantitated using an in vitro ankyrin-binding assay. Beads coated with protein A were used to immunoadsorb HA-tagged neurofascin and subsequently used as substrates for binding of radiolabeled ankyrin. Antibody-coated beads were prepared by coupling 1 mg protein A (Pierce, Rockford, IL) to 100 μg latex beads (epoxy-activated, 0.4-μm-diam MMA/GMA copolymer beads from

Bangs Laboratories, Carmel, IN) for 72 h at 4°C with end over end mixing in a buffer containing 10 mM Hepes, pH 8.5, 1 mM EGTA, 1 M NaCl, and 1 mM NaN_3. Unreacted sites were quenched by adding Tris, pH 8.0, to 100 mM concentration and incubating overnight at 4°C. Nonspecific protein sites were then blocked by the addition of 10 mg/ml bovine serum albumin for 2 h. Neurofascin was immunoprecipitated as described above, and the immune complexes were captured with 20 μl protein A–latex beads. The amount of each expressed neurofascin isoform used for an assay was normalized after Phosphorimage scanning (Molecular Dynamics, Inc., Sunnyvale, CA) of crude neurofascin immunoblots of cell extracts for each respective transfected cell line.

The 82-kD membrane binding domain of $ankyrin_B$ was expressed and purified as described (Davis et al., 1993). Purified 82-kD ankyrin was then radiolabeled with ^{125}I–Bolton-Hunter reagent (ICN Biochemicals, Irvine, NY) as previously described for ankyrin and other proteins (Davis et al., 1993; Davis and Bennett 1983, 1984). Individual neurofascin immunoprecipitates were incubated with 20 nM radiolabeled ankyrin plus a given amount of unlabeled 82-kD ankyrin in a buffer containing 10 mM sodium phosphate, pH 7.0, 100 mM NaCl, 1 mM EDTA, 2 mg/ml bovine serum albumin, 0.1% Triton X-100, and 1 mM NaN_3 for 3 h at 4°C. After incubation the beads were centrifuged over a 10% sucrose cushion for 15 min at 10,000 *g* to separate bound from free radioactivity, and the beads were assayed for ^{125}I in a γ counter. Each plot depicts one representative complete assay, whereas individual points were determined from the average of duplicate assays for two individual immunoprecipitates.

Cell Culture

Transfected and untransfected B104 cells were cultured in high glucose DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell culture reagents were purchased from GIBCO BRL. Transfected cells were additionally cultured in 250 μg/ml neomycin (Sigma Chemical Co., St. Louis, MO). For pharmacological treatments, cells were grown to 50–70% confluency. Cells were cultured on serum-free media for 12 h before treatment with either NGF or bFGF. Immediately preceding lysis, cells were washed with ice-cold phosphate-buffered saline.

Fluorescence Recovery after Photobleaching

HA epitope-tagged full-length neurofascin, full cytoplasmic-truncated neurofascin, and the FIGQY to F tyrosine mutant were either untreated or treated with 100 ng/ml NGF for 30 min and then immunolabeled with the HA-specific monoclonal antibody and an FITC-tagged secondary antibody. Cells were subsequently monitored using an Odyssey confocal imaging system (Noran Inc., Middleton, WI) mounted on an inverted Nikon Diaphot microscope in combination with an imaging board (Bitflow, Woburn, MA) and eye imager calculator software (IO Industries, ON, Canada), a system that captures images every 16.7 ms. These instruments were purchased and assembled by Dr. Tobias Meyer (Duke University Medical Center, Durham, NC). FITC was excited at 488 nm and was monitored above 515 nm. FITC was photobleached using a focused UV laser at 365 nm. UV laser estimated intensity is 1 mW with an opening time of 10 ms. Fluorescence measurements were determined by the National Institutes of Health Image method of analysis. Triplicate measurements were taken from each image and averaged followed by subtraction of average background fluorescence.

Detergent Extraction Experiments

Denoted neurofascin-expressing B104 cells were either untreated or treated with 100 ng/ml NGF for 90 min. Control cells were first fixed with 2% paraformaldehyde for 20 min at 4°C and then treated with a PBS blocking buffer (150 mM NaCl, 30 mM sodium phosphate, pH 7.4, 1% BSA, and 10% normal goat serum) for 30 min. Control cells were subsequently treated with 0.2% Triton X-100 in the PBS blocking solution for 10 min at room temperature and washed with a 0.2% Tween-20 solution (in the PBS blocking buffer). Alternatively, cells were first treated with 0.2% Triton X-100 in DMEM cell culture media (GIBCO BRL) for 10 min at room temperature and then fixed with 2% paraformaldehyde for 20 min at 4°C. Subsequently, cells were treated with PBS blocking buffer for 30 min and washed with a 0.2% Tween-20 solution.

After fixation, all cells were incubated overnight at 4°C with the HA-specific monoclonal antibody (1:1,000). Cells were washed in 0.2% Tween-20 buffer and then incubated with anti–rabbit FITC-conjugated secondary antibody (1:2,000) for 2 h at 4°C. Finally, cells were washed with the 0.2% Tween-20 solution. Confocal images were obtained with an LSM Zeiss confocal microscope. Fluorescence measurements (intensity/μm^2) were obtained from entire cells, and data was analyzed with a Zeiss analysis software package. Fluorescence intensities for cells treated with 0.2% Triton X-100 before paraformaldehyde fixation are presented relative to fluorescence intensities for cells that were paraformaldehyde fixed before Triton X-100 extraction.

Analysis of Levels of Phosphotyrosine-immunoreactive Neurofascin and Nr-CAM in Developing Rat Brain

Rat brain lysates were prepared for embryonic days 15, 17, and 20, postnatal days 1, 2, 5–8, 10, 14, and 15, as well as adult rat. Brains were sonicated in the modified RIPA buffer previously mentioned. Crude lysates (100 μg) from each day were subjected to SDS-PAGE, and subsequent immunoblots were probed with either the mucin-specific neurofascin polyclonal or an anti–Nr-CAM polyclonal anitbody. Immunoblots were scanned via a Phosphorimager to determine the amount of neurofascin and Nr-CAM expressed each day over the developmental period. Subsequently, lysates were immunoprecipitated either for normalized amounts of neurofascin or Nr-CAM, and resulting blots were probed with the anti-phosphotyrosine antibody. Alternatively, lysates were immunoprecipitated with the anti-phosphotyrosine antibody (after normalization for neurofascin or Nr-CAM levels), and subsequent blots were probed with either neurofascin or Nr-CAM–specific polyclonal antibodies.

Results

Neurofascin Is Subject to In Vivo Tyrosine Phosphorylation That Inhibits Ankyrin-binding Activity

Neurofascin and other members of the L1 family of nervous system cell adhesion molecules have four highly conserved tyrosine residues in their cytoplasmic domains. The possibility that one or more of these tyrosines are substrates for phosphorylation by protein kinases was evaluated using HA epitope-tagged neurofascin stably transfected into the B104 rat neuroblastoma cell line. Transfected cells were first treated with activators of receptor tyrosine kinases (NGF and bFGF) or inhibitors of protein tyrosine phosphatases (vanadate and dephostatin), after which neurofascin was immunoprecipitated using the HA-specific monoclonal antibody. Subsequent immunoblots were probed with an anti-phosphotyrosine antibody (Fig. 1 *A*). The protein tyrosine phosphatase inhibitor dephostatin (10 μM for 30 min) resulted in strong anti-phosphotyrosine immunoreactivity (Fig. 1 *A*, lane *2*), as did sodium metavanadate (data not shown). Furthermore, incubation of the transfected cells with either nerve growth factor (100 ng/ml for 30 min; Fig. 1 *A*, lane *3*) or basic fibroblast growth factor (50 ng/ml for 30 min; Fig. 1 *A*, lane *4*) also generated a strong immunoreactive pool of tyrosine-phosphorylated neurofascin. Fig. 1 *B* quantitates the extent of tyrosine phosphorylation of neurofascin after treatment of transfected cells with either protein tyrosine phosphatase inhibitors (vanadate and dephostatin) or tyrosine kinase activators (NGF and bFGF) over an extended concentration range. These results demonstrate that neurofascin is a target for both protein tyrosine kinases and phosphatases in vivo when the relevant enzymes are pharmacologically perturbed in a dose-dependent manner.

The question of whether tyrosine phosphorylation modulates ankyrin-binding activity of neurofascin was evaluated by determining the effects of phosphorylation on the

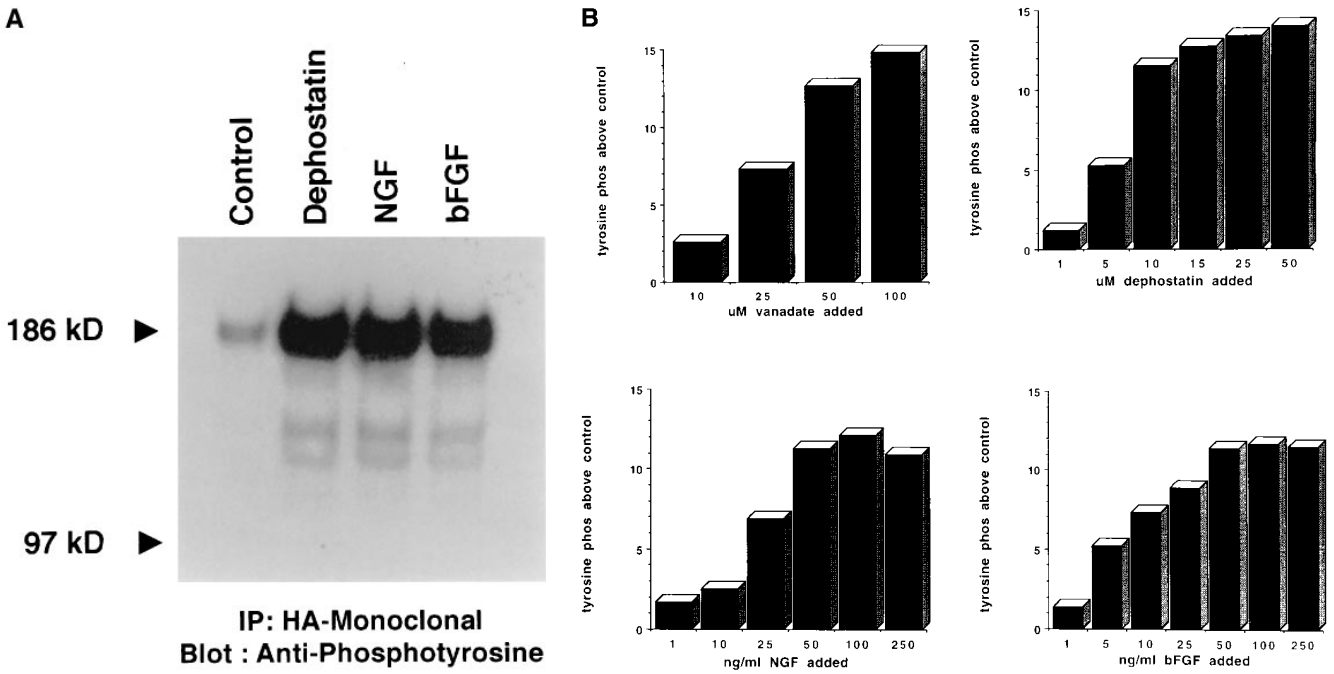

Figure 1. Neurofascin is phosphorylated in vivo by activation of tyrosine kinase signaling cascades or inactivation of tyrosine phosphatases. (*A*) HA epitope-tagged neurofascin was expressed in the rat neuroblastoma B104 cell line. Transfected cells were treated with one of the following agents for 30 min: dephostatin (10 μM), NGF (100 ng/ml), or bFGF (50 ng/ml). Neurofascin was subsequently immunoprecipitated from 500 μg crude cell extract using an HA-specific monoclonal antibody. Immune complexes were then electrophoresed on SDS-PAGE and transferred to nitrocellulose. Immunoblots were probed overnight at 4°C with a phosphotyrosine-specific polyclonal antibody followed by incubation with ^{125}I-radiolabeled protein A. Blots were visualized via autoradiography. (*B*) HA epitope-tagged neurofascin was expressed in the rat neuroblastoma B104 cell line. Transfected cells were treated with one of the following agents (at the noted concentrations) for 30 min: vanadate, dephostatin, NGF, or bNGF. Neurofascin was immunoprecipitated from 500 μg crude cell lysate using the HA-specific monoclonal antibody. Immune complexes were electrophoresed on SDS-PAGE and transferred to nitrocellulose. Blots were probed with the phosphotyrosine-specific polyclonal antibody followed by incubation with ^{125}I-radiolabeled protein A. Subsequently, blots were subjected to Phosphorimage scanning to quantitate the extent of tyrosine phosphorylation of full length neurofascin under the given treatment conditions.

ability of neurofascin to coimmunoprecipitate endogenous ankyrin expressed in B104 cells (Fig. 2 *A*). Neurofascin immunoprecipitates were simultaneously enriched for polypeptides of 210 and 190 kD as well as a doublet at 102 and 104 kD that crossreact with a brain ankyrin-specific polyclonal antibody (ankyrin$_B$; Fig. 2 *A*, lane *2*). The 210- and 190-kD crossreacting polypeptides are of the expected size for ankyrin with membrane-binding, spectrin-binding, and COOH-terminal domains. The smaller polypeptides presumably represent either alternatively spliced variants missing one or more of these domains or proteolytically cleaved forms of the 210- and 190-kD polypeptides. Activation of tyrosine kinases (Fig. 2 *A*, *NGF* or *bFGF*; lanes *4* and *5*) or inactivation of tyrosine phosphatases (Fig. 2 *A*, *dephostatin*; lane *3*) completely eliminate the ability of neurofascin to coimmunoprecipitate 210- and 190-kD ankyrin as well as the 102 and 104-kD crossreacting polypeptides.

Coimmunoprecipitation experiments provide qualitative evidence that tyrosine phosphorylation of neurofascin abolishes association with endogenous ankyrin in the context of intact cells. These results do not, however, address whether other cellular factors such as phosphotyrosine-binding proteins are required for inhibition. Effects of tyrosine phosphorylation of neurofascin on binding to ankyrin were evaluated quantitatively in isolation from other cytosolic proteins in an in vitro ankyrin-binding assay. Neurofascin was immunoprecipitated using the HA-specific monoclonal antibody and adsorbed to protein A–coated beads, extensively washed, and then incubated with radiolabeled ankyrin in a binding assay described in Materials and Methods.

Neurofascin isolated from cells treated with NGF and dephostatin exhibits an ~40% reduction in ankyrin-binding activity compared to neurofascin isolated from untreated cells (Fig. 2 *B*). Scatchard plots (Fig. 2 *C*) demonstrate that the inhibition is due to a reduction in capacity (intercept with the X axis) with no change in affinity (slope). Tyrosine phosphorylation thus decreased the total ankyrin-binding capacity, while the ankyrin-binding affinity was unaltered. Such a result can be interpreted to indicate that tyrosine phosphorylation of a given neurofascin molecule completely abolishes its ability to bind ankyrin, but only a subpopulation of neurofascin molecules are tyrosine phosphorylated. Possible explanations for only partial phosphorylation and, thus, only a partial reduction in the ankyrin-binding capacity of the total neurofascin population include dephosphorylation during isolation, expression of neurofascin in amounts that exceed the capacity of tyrosine kinases, and/or the presence of a subpopulation of neurofascin in a cellular compartment that does not con-

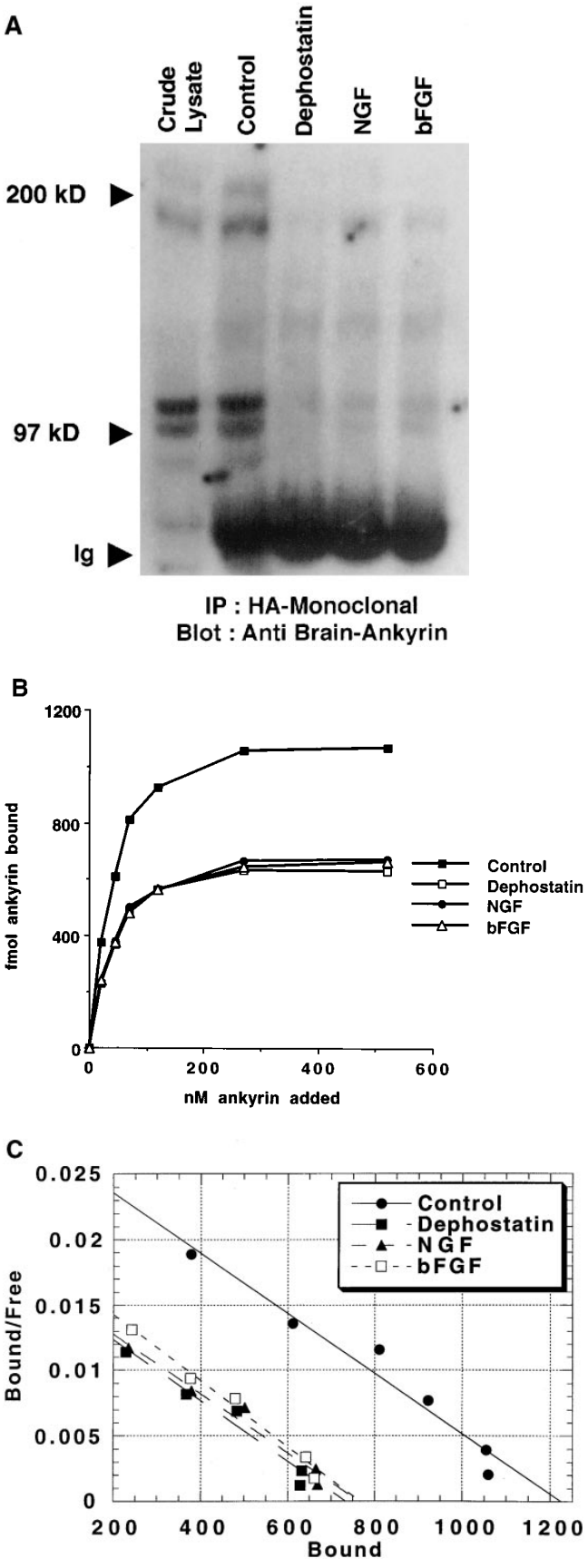

Figure 2. In vivo activation of tyrosine phosphorylation of neurofascin decreases ankyrin binding capacity. (*A*) Neurofascin-transfected cells were treated with one of the following agents for 30 min: dephostatin (10 μM), NGF (100 ng/ml), or bFGF (50 ng/ml). HA-labeled neurofascin (250 μg crude cell lysate) was subsequently immunoprecipitated as described above. Immune complexes were captured on protein A–Sepharose beads and electrophoresed on SDS-PAGE. Resolved proteins were transferred to nitrocellulose and incubated overnight at 4°C with a brain ankyrin-specific polyclonal antibody. After incubation with ^{125}I-labeled protein A (2 h at 4°C), immunoblots were visualized with autoradiography. (*B*) Neurofascin-transfected cells were treated as described. HA-labeled neurofascin was immunoprecipitated, and immune complexes were captured on protein A–labeled latex beads, washed, and then incubated for 180 min at 4°C with 20 nM ^{125}I-radiolabeled, bacterially expressed 82K membrane-binding domain of the ankyrin$_B$ isoform plus a given amount of unlabeled 82K ankyrin$_B$. After incubation the immune complexes were centrifuged over a 10% sucrose cushion to separate bound and free radioactivity, and the beads were counted on a γ counter to assess levels of ankyrin binding. Each point was obtained by averaging duplicate assays from individual immunoprecipitations. (*C*) The ankyrin binding assay data from above was converted into a form suitable for Scatchard analysis. The tyrosine phosphorylation–induced changes in the Scatchard plots (downward shift with no change in slope) demonstrate that the total ankyrin binding capacity is decreased with no change in ankyrin binding affinity. This indicates that phosphorylation of a given neurofascin molecule abolishes its ability to bind ankyrin but does not alter the affinity of remaining neurofascin molecules that have not been phosphorylated and, thus, maintain ankyrin-binding competence.

tain appropriate tyrosine kinase activity. All three of these possibilities could be operative and therefore contribute to partial phosphorylation of isolated neurofascin.

Highly Conserved FIGQY Residues in the Cytoplasmic Domain of Neurofascin Are Required for Ankyrin Binding

The finding that tyrosine phosphorylation abolished ankyrin-binding activity suggested the possibility that definition of the ankyrin-binding determinants of neurofascin would also lead to identification of the specific site of tyrosine phosphorylation. The ankyrin-binding site of neurofascin has been mapped previously to a 35–amino acid stretch in the COOH-terminal portion of the cytoplasmic domain encompassing two highly conserved tyrosine residues (Davis and Bennett, 1994). Deletion constructs targeted to this general location, as well as the full length and fully truncated constructs (Fig. 3 *A*), were evaluated for ankyrin-binding activity as determined by coimmunoprecipitation of endogenous ankyrin (Fig. 3 *B*) and by direct measurement of ankyrin-binding activity of isolated neurofascin (Fig. 3 *C*).

Neurofascin with a complete truncation of the cytoplasmic domain exhibits no detectable coimmunoprecipitation with endogenous ankyrin isoforms (Fig. 3 *B*, lane *4*) and has no binding activity in ankyrin-binding assays (Fig. 3 *C*). Deletion of the COOH-terminal 28 residues up to, but not including, the FIGQ sequence had no effect on ankyrin binding, while deletion of an additional 11 residues resulted in a major loss of ankyrin-binding activity (Garver, T., and Q. Ren, unpublished observations). These results were the basis for a second generation of internally de-

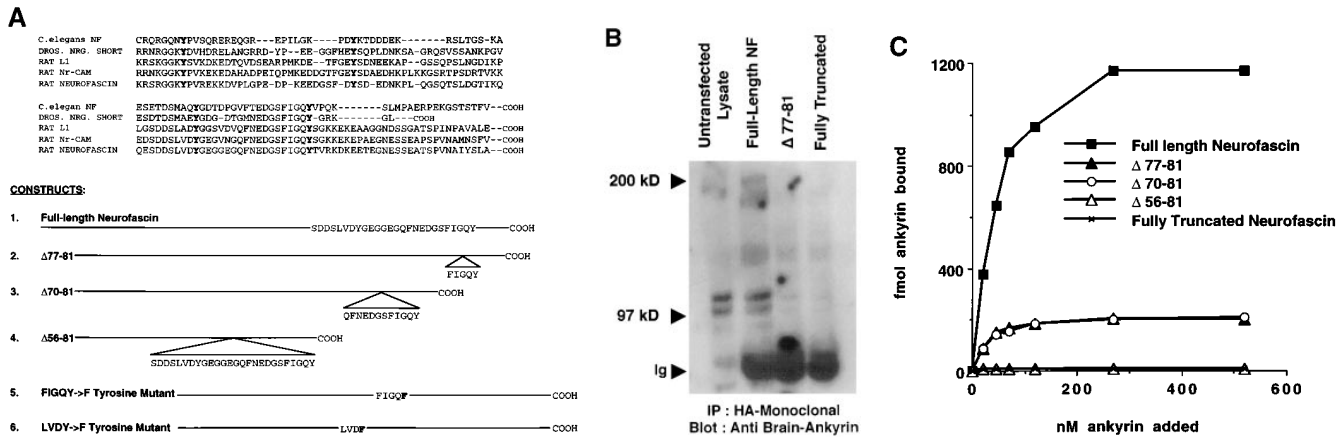

Figure 3. The highly conserved FIGQY amino acid stretch in the cytoplasmic domain of neurofascin is responsible for ankyrin binding. (*A*) Cytoplasmic sequences of L1 family cell adhesion molecules and definition of the neurofascin cytoplasmic domain constructs. (*B*) Epitope-tagged neurofascin was immunoprecipitated from 250 μg crude cell lysate, as previously described. Immune complexes were captured on protein A–Sepharose beads and electrophoresed on SDS-PAGE. Resolved proteins were transferred to nitrocellulose and incubated overnight at 4°C with a brain ankyrin-specific polyclonal antibody. After incubation with ^{125}I-labeled protein A (2 h at 4°C), immunoblots were visualized by autoradiography. (*C*) HA-epitope tagged neurofascin was expressed in B104 cells and subsequently immunoprecipitated from 250 μg crude cell lysate as described previously. Captured immune complexes (on protein A–labeled latex beads) were then used for the in vitro ankyrin binding assays that have been described above.

leted constructs. Deletion of FIGQY region alone leads to loss of ability to coimmunoprecipitate ankyrin (Fig. 3 *B*, lane *3*) and an 80% decrease in the level of in vitro ankyrin binding after immunoadsorption of HA-tagged neurofascin to protein A–coated beads (Fig. 3 *C*). Neurofascin with a deletion of an additional seven residues (QFNED-FIGQY) exhibited a similar 80% reduction in the ankyrin-binding activity as compared to deletion of FIGQY alone (Fig. 3 *C*). However, an internal deletion of 25 residues encompassing SDDSLVDY . . . FIGQY (56–81) resulted in a complete loss of ankyrin-binding activity (Fig. 3 *C*).

Phosphorylation of the FIGQY Tyrosine Regulates Ankyrin Binding of Neurofascin

The results with deletion mutants implicated the five residues, FIGQY, as essential for full ankyrin binding, with the NH_2-terminal residues SLVDY potentially playing a secondary role, and suggested that phosphorylation of one or both of these tyrosines could be the basis for regulation of ankyrin-binding activity. The role of these tyrosines in regulation was evaluated by site-directed mutagenesis of each to phenylalanine (Fig. 3 *A*) followed by evaluation of tyrosine phosphorylation and ankyrin-binding activity. These constructs were stably transfected in B104 cells that were subsequently treated with NGF, bFGF, or dephostatin. The LVDY to F tyrosine mutant has a tyrosine phosphorylation pattern (Fig. 4 *A*) similar to that seen for the full length, native neurofascin (Fig. 1 *A*). However, the FIGQY to F tyrosine mutant is significantly less phosphorylated under the same pharmacological paradigm. Fig. 4 *B* quantitatively depicts the effects of the treatments on tyrosine phosphorylation. These results identify the FIGQY tyrosine residue as the primary site of tyrosine phosphorylation under the given treatment conditions.

Upon establishing differential phosphorylation patterns for the two tyrosine residues, we next inspected whether these two tyrosines are differentially responsible for the modulation of ankyrin binding. After treatment of the respective transfected cells, neurofascin was immunoprecipitated and used for the in vitro ankyrin-binding assays. The ankyrin-binding competence of the LVDY to F tyrosine mutated form of neurofascin is negatively regulated by tyrosine phosphorylation to a similar extent as native neurofascin, as determined by the ability to coimmunoprecipitate endogenous ankyrin (Fig. 4 *C*) and in ankyrin-binding assays (Fig. 4 *D*). However, the FIGQY to F neurofascin mutant isolated from cells treated with NGF, bFGF, or dephostatin retains full ankyrin-binding competence based on the ability to coimmunoprecipitate endogenous ankyrin (Fig. 4 *C*) and in ankyrin-binding assays (Fig. 4 *D*). Therefore, the FIGQY tyrosine residue appears to be both the primary site of tyrosine phosphorylation as well as the phosphorylated site responsible for inhibition of ankyrin-binding activity.

Lateral Mobility of Neurofascin in Living Cells Is Increased by Phosphorylation of the FIGQY Tyrosine

Consequences of ankyrin binding and tyrosine phosphorylation on the dynamic behavior of neurofascin in living cells were evaluated using the technique of FRAP. HA-tagged neurofascin constructs transfected in B104 cells were labeled for fluorescence measurements using a monoclonal antibody against the HA epitope and an FITC-labeled secondary antibody. The extent and rate of recovery of fluorescence at the site of bleaching provide insight about the mobile fraction and the diffusion properties of immunolabeled neurofascin molecules.

Native neurofascin exhibits essentially no recovery of fluorescence after photobleaching, implying its involvement in highly constraining intermolecular interactions (Fig. 5 *A*). In contrast, neurofascin with a fully truncated cytoplasmic domain exhibits a 10–15% recovery after photobleaching

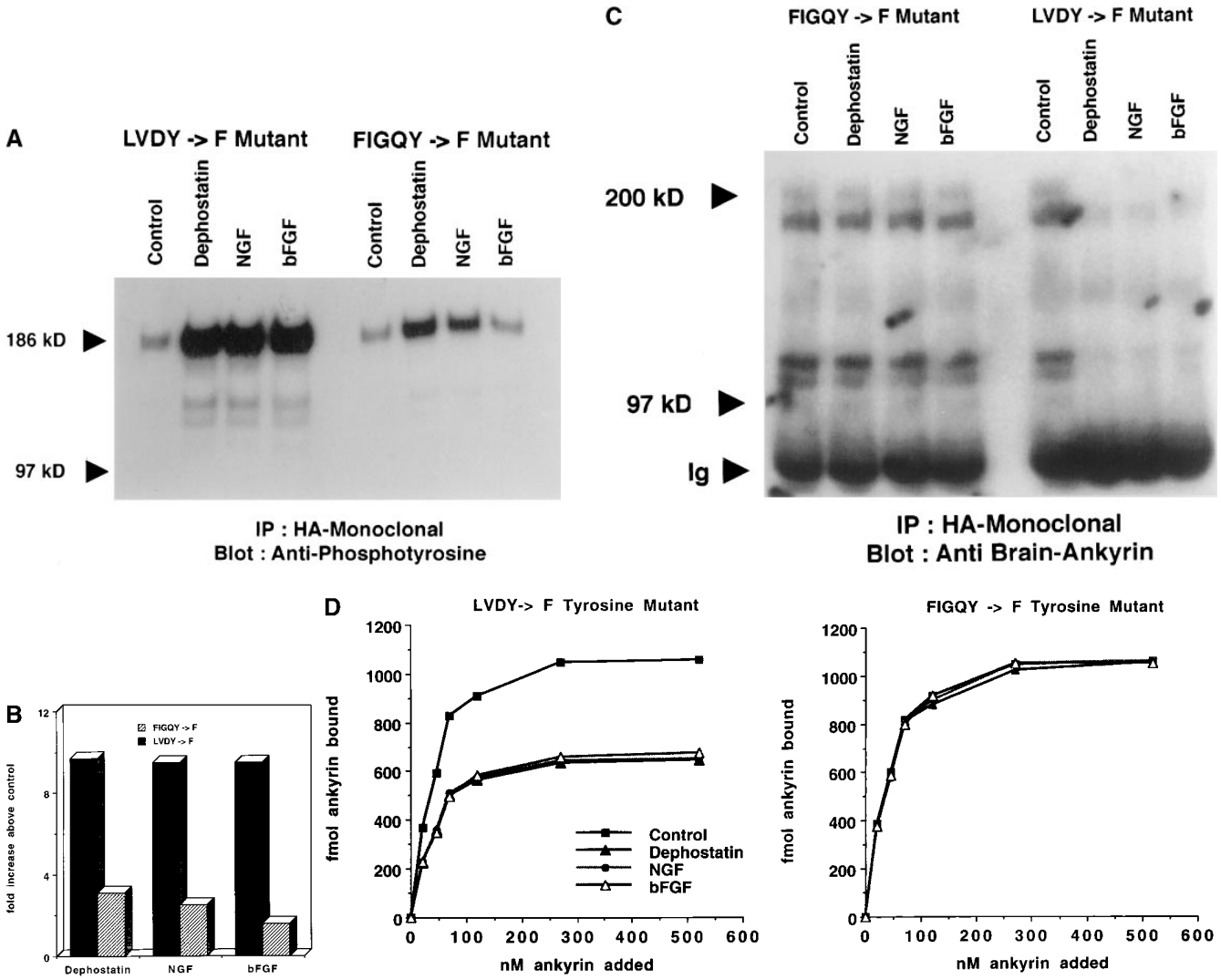

Figure 4. FIGQY is the primary site of tyrosine phosphorylation on neurofascin and decreases ankyrin-binding capacity in a site-specific manner. (*A*) Neurofascin was immunoprecipitated after a 30-min treatment of the LVDY to F and the FIGQY to F neurofascin transfected cells with one of the following agents: dephostatin (10 μM), NGF (100 ng/ml), or bFGF (50 ng/ml). Subsequent immunoblots were probed with the anti-phosphotyrosine antibody overnight at 4°C followed by ^{125}I-labeled protein A. (*B*) The immunoblot visualized above was subjected to Phosphorimage scanning to quantitate the extent of tyrosine phosphorylation of full length neurofascin under the given treatment conditions. The phosphotyrosine signals for both tyrosine mutants are expressed relative to the signals obtained from treatment of B104 cells expressing native, full length neurofascin (Fig. 1 *A*). All cells were treated during the same experiment. (*C*) Neurofascin was immunoprecipitated from each treatment group for each denoted cell line. Immune complexes were electrophoresed on SDS-PAGE, and resolved proteins were transferred to nitrocellulose. Blots were incubated with the brain ankyrin-specific polyclonal antibody followed by ^{125}I-labeled protein A. (*D*) Neurofascin was immunoprecipitated following noted treatments from each cell line. Immune complexes, captured via protein A-labeled latex beads, were then used in the ankyrin binding assay that has been described above. The LVDY to F tyrosine mutant, with an intact FIGQY tyrosine residue, is subject to ankyrin binding regulation via tyrosine phosphorylation, whereas the FIGQY to F tyrosine mutant is resistant to phosphorylation-induced decreases in the in vitro ankyrin binding assays.

(Fig. 5 *A*), indicating that the cytoplasmic domain of neurofascin makes a small but significant contribution to the restriction in lateral mobility. The effect of the cytoplasmic domain presumably results from connections to the spectrin skeleton through ankyrin binding. Interactions involving the ectodomain and/or membrane-spanning domain of neurofascin, however, probably participate in the majority of restriction in the lateral mobility of neurofascin.

Treatment of cells expressing full length neurofascin with NGF under conditions that promote phosphorylation of the FIGQY tyrosine releases all of the restrictions attributable to the cytoplasmic domain (Fig. 5 *A*). The fraction that recovers increased from 0 to ~10–15% of original fluorescence levels. The FIGQY tyrosine is responsible for effects of NGF-promoted tyrosine phosphorylation since the FIGQY to F mutant is resistant to NGF treatment (Fig. 5 *B*) and exhibits the same parameters of fractional recovery of fluorescence as neurofascin in untreated cells.

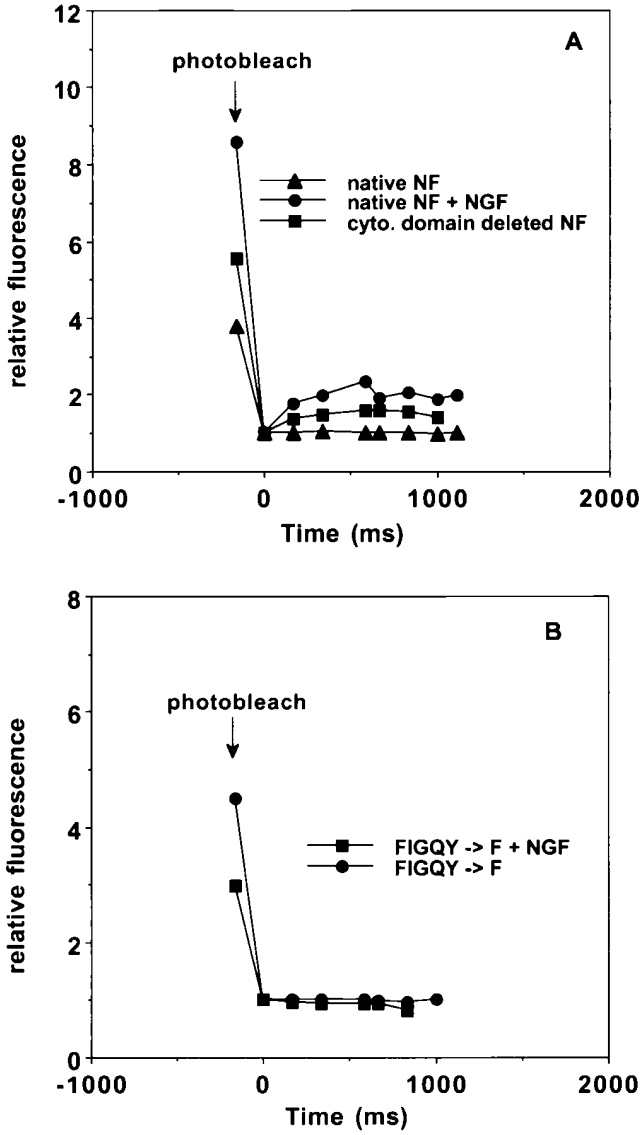

Figure 5. Lateral mobility of neurofascin is increased by site-specific phosphorylation of FIGQY tyrosine. B104 cells expressing either native neurofascin or the cytoplasmic domain deleted neurofascin (*A*) or the FIGQY to F tyrosine mutant form of neurofascin (*B*) were either untreated or treated with 100 ng/ml NGF for 30 min and then immunolabeled with the HA-specific monoclonal antibody and an FITC-tagged secondary antibody. Cells were subsequently monitored using an Odyssey confocal imaging system (Noran Inc.) mounted on an inverted Nikon Diaphot microscope in combination with an imaging board (Bitflow) and eye imager calculator software (IO Industries), a system that captures images every 16.7 ms. FITC was excited at 488 nm and was monitored above 515 nm. FITC was photobleached using a focused UV laser at 365 nm. Fluorescence measurements were determined by the National Institutes of Health Image method of analysis.

Phosphorylation of the FIGQY Tyrosine Increases Detergent Extractability of Neurofascin

Effects of tyrosine phosphorylation on the static in vivo interaction between forms of expressed neurofascin with detergent-insoluble components of the spectrin-based cytoskeleton were evaluated by analyzing the changes in rhodamine-labeled, HA epitope-tagged neurofascin intensity after Triton X-100 extraction. Detergent-extraction experiments allow one to examine the steady state interaction between neurofascin and ankyrin and the factors that may modulate this interaction. B104 cells expressing either native neurofascin, cytoplasmic domain-deleted neurofascin, or the FIGQY to F tyrosine mutant form of neurofascin were either untreated or treated with 100 ng/ml NGF for 90 min (Fig. 6, *A–D*). Cells (extracted with 0.2% Triton X-100 before fixation) expressing full length neurofascin exhibited a 65% retention of fluorescence, as compared to cells fixed before detergent treatment. The fluorescence was reduced to 28% after treatment with NGF. Neurofascin lacking the cytoplasmic domain was retained at only 10% in the presence of detergent, indicating that NGF abolished most, but not all, of the interactions of the cytoplasmic domain with detergent-insoluble proteins. Interestingly, the reduction in fluorescence due to NGF-induced tyrosine phosphorylation of native neurofascin is quantitatively similar to the reduction in the in vitro binding capacity of neurofascin after tyrosine phosphorylation (Fig. 2 *B*). In contrast to native neurofascin, cells expressing the FIGQY to F tyrosine mutant neurofascin were unaffected by NGF treatment and retained ~65% of the label in the presence and absence of NGF, once again indicating that this site is the primary one responsible for phosphotyrosine-dependent regulation of neurofascin–ankyrin interactions.

Neurofascin and Nr-CAM Are Tyrosine Phosphorylated in a Developmentally Regulated Pattern in Rat Brain

We wished to determine whether neurofascin and the related molecule Nr-CAM are tyrosine phosphorylated in vivo to establish a physiological correlation for what we have observed in vitro. Consequently, we looked at each molecule during neuronal development in rat to see if either or both of these molecules exhibit a period of phosphotyrosine immunoreactivity. Fig. 7, *A* (*neurofascin*) and *B* (*Nr-CAM*) demonstrate that both of these molecules are tyrosine phosphorylated in vivo in a time-dependent fashion, with the maximal level of phosphotyrosine immunoreactivity present during the embyronic period. Phosphorimage scanning (data not shown) indicates there is nearly a threefold reduction in Nr-CAM tyrosine phosphorylation from embryonic day 15 to adult brain. In comparison, neurofascin phosphotyrosine immunoreactivity is intense at embryonic day 15. However, this signal is dramatically reduced (an ~20-fold decline from embryonic day 15 to adult) over the denoted developmental period. With respect to neurofascin, it is interesting to note that the 186-kD isoform is the preferential splice form to be tyrosine phosphorylated, even when minimally expressed, as in embryonic day 15. Immunoprecipitation first with the anti-phosphotyrosine antibody followed by immunoblotting with either neurofascin or Nr-CAM antibodies rendered qualitatively similar results (data not shown).

Discussion

This paper presents evidence that a member of the L1

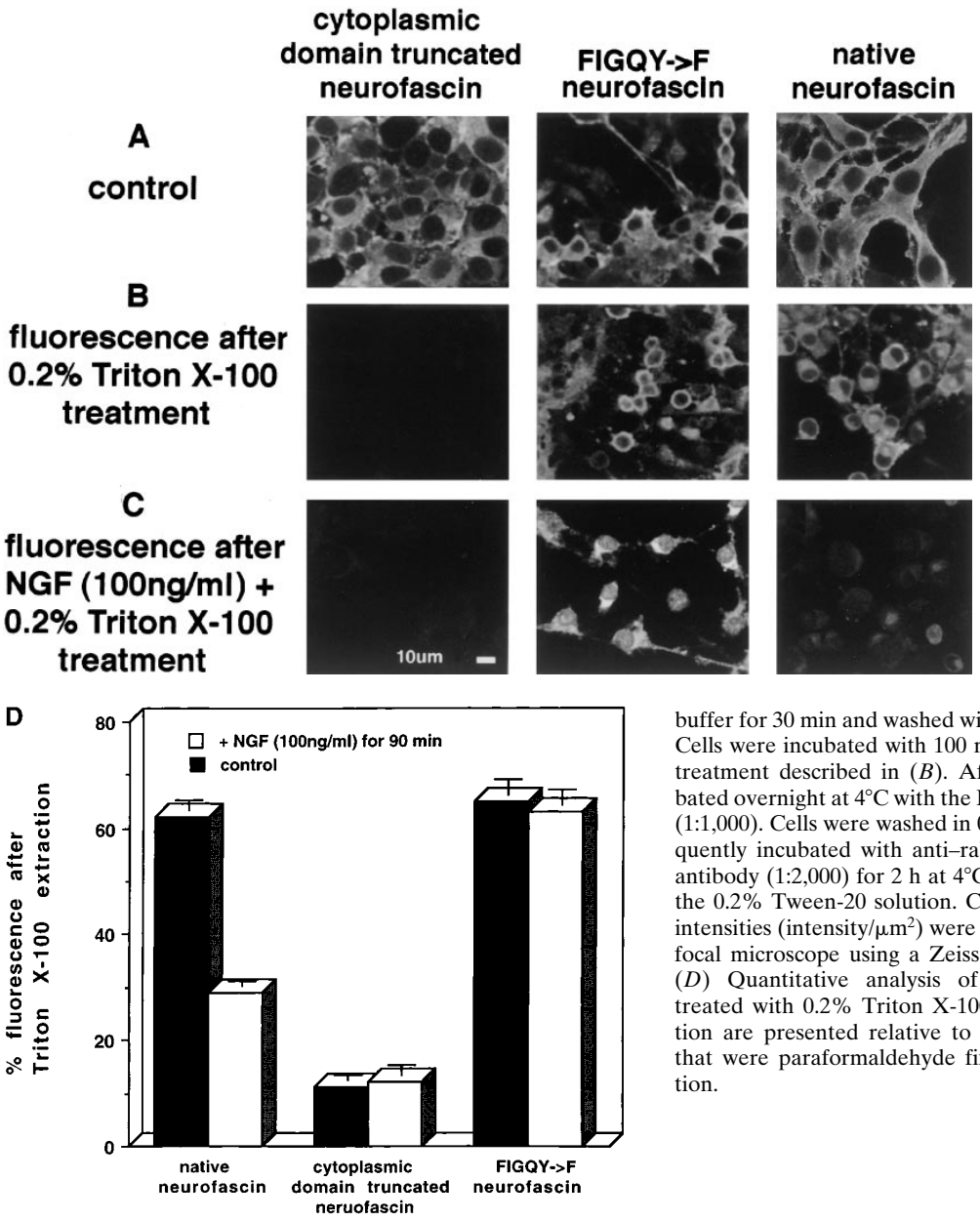

Figure 6. Phosphorylation of the FIGQY tyrosine increases detergent extractability of neurofascin. (*A*) Control cells were first fixed with 2% paraformaldehyde for 20 min at 4°C and then treated with a PBS blocking buffer (150 mM NaCl, 30 mM sodium phosphate, pH 7.4, 1% BSA, and 10% normal goat serum) for 30 min. Cells were subsequently treated with 0.2% Triton X-100 in the PBS blocking solution for 10 min at room temperature and washed with a 0.2% Tween-20 solution. (*B*) Cells were first treated with 0.2% Triton X-100 in DMEM cell culture media (GIBCO BRL) for 10 min at room temperature and then subsequently fixed with 2% paraformaldehyde for 20 min at 4°C. Cells were then incubated with PBS blocking buffer for 30 min and washed with a 0.2% Tween-20 solution. (*C*) Cells were incubated with 100 ng/ml NGF for 90 min before the treatment described in (*B*). After fixation, all cells were incubated overnight at 4°C with the HA-specific monoclonal antibody (1:1,000). Cells were washed in 0.2% Tween-20 buffer and subsequently incubated with anti–rabbit FITC-conjugated secondary antibody (1:2,000) for 2 h at 4°C. Finally, cells were washed with the 0.2% Tween-20 solution. Confocal images and fluorescence intensities (intensity/μm^2) were obtained with an LSM Zeiss confocal microscope using a Zeiss data analysis software package. (*D*) Quantitative analysis of fluorescence intensities. Cells treated with 0.2% Triton X-100 before paraformaldehyde fixation are presented relative to fluorescence intensities for cells that were paraformaldehyde fixed before Triton X-100 extraction.

family of cell adhesion molecules is a substrate for protein tyrosine kinase(s) and phosphatase(s), identifies the highly conserved FIGQY tyrosine in the cytoplasmic domain as the site of phosphorylation, and demonstrates that phosphorylation of the FIGQY tyrosine abolishes ankyrin-binding activity. Phosphorylation of the FIGQY tyrosine increases lateral mobility of neurofascin expressed in neuroblastoma cells to the same extent as removal of the cytoplasmic domain and increases detergent extractability of neurofascin. Ankyrin binding thus modulates the dynamic behavior of neurofascin and is the target for regulation by tyrosine phosphorylation–dephosphorylation in response to external signals. We have further demonstrated that two L1 family members, neurofascin and Nr-CAM, are both tyrosine phosphorylated in vivo during embryonic development, indicating that the functions attributed to tyrosine phosphorylation, as determined from our in vitro studies, are likely to be relevant in vivo as well.

The FIGQY sequence is present in cytoplasmic domains of all members of the L1 family of nervous system cell adhesion molecules, including a recently described *Caenorhabditis elegans* partial cDNA sequence. Moreover, ankyrin-binding activity has been directly demonstrated for mammalian neurofascin, L1 and Nr-CAM (Davis and Bennett, 1994), as well as *Drosophila* neuroglian (Dubreuil et al., 1996). The findings of this study, therefore, may reflect a highly conserved mechanism, used by the entire class of L1-related cell adhesion molecules, of ankyrin-dependent connections to the spectrin skeleton regulated via site-specific tyrosine phosphorylation.

The interaction of neurofascin with ankyrin is the first example of an ankyrin–membrane protein association that is reversible and subject to dynamic control. The potential for localized activation or inhibition of cell adhesion molecule interactions with ankyrin could be important for processes such as neuronal development, synaptic plasticity,

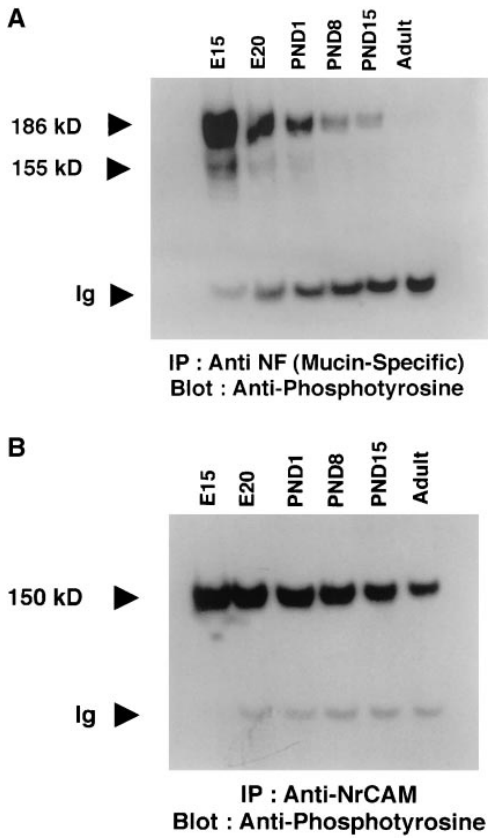

Figure 7. Neurofascin and Nr-CAM are tyrosine phosphorylated in developing rat brain. Neurofascin (*A*) or Nr-CAM (*B*) were immunoprecipitated, from each noted time point, with either the mucin-specific neurofascin polyclonal antibody (*A*) or the Nr-CAM–specific polyclonal antibody (*B*) after normalization for expressed neurofascin or Nr-CAM levels, respectively (see Materials and Methods). Resolved proteins were transferred to nitrocellulose, and blots were incubated overnight at 4°C with the anti-phosphotyrosine antibody.

and localized assembly of cell adhesion molecule–based structures in axons. The observation that Nr-CAM and, more dramatically, neurofascin are preferentially tyrosine phosphorylated during fetal development indicates phosphorylation may play a key role in modulating cytoskeletal and membrane dynamics during assembly of the nervous system. Neurofascin and Nr-CAM are targeted to nodes of Ranvier in myelinated axons where the voltage-dependent sodium channel and an isoform of ankyrin are highly concentrated (Davis et al., 1996). Neurofascin and Nr-CAM are detected early during the development of nodes of Ranvier and may direct the subsequent targeting of ankyrin and the voltage-dependent sodium channel to this site (Lambert, S., J. Davis, P. Michael, and V. Bennett. 1995. *Mol. Biol. Cell.* 6:98a). Dephosphorylation by a protein tyrosine phosphatase localized at nodes of Ranvier is an attractive mechanism for localized activation of ankyrin-binding activity of neurofascin and Nr-CAM. The biological significance of a reversible ankyrin-binding activity for L1-like cell adhesion molecules could be evaluated using FIGQY to F mutants, which are resistant to inhibition of ankyrin binding by tyrosine phosphorylation.

Consequences of ankyrin association with neurofascin include the potential for stabilizing laterally associated neurofascin oligomers in addition to providing a connection to spectrin. The membrane-binding domain of ankyrin contains two independent binding sites for neurofascin, and, in principle, could promote assembly of neurofascin dimers (Michaely and Bennett, 1995). Transcellular interactions of neurofascin, by analogy with cadherins, could be promoted by lateral association of neurofascin molecules. These considerations suggest the testable hypotheses that ankyrin binding could enhance cell–cell interactions mediated by neurofascin and other L1 family members and that elevation of tyrosine phosphorylation would reverse activation of cell adhesion by ankyrin.

A question remains how the phosphorylation event prohibits neurofascin–ankyrin association. The fact that the key tyrosine residue is directly adjacent to the primary amino acid sequence established in this study as required for ankyrin binding may mean that tyrosine phosphorylation of neurofascin blocks ankyrin binding directly through steric hinderance or by the introduction of electrostatic repulsion. Another possibility is that ankyrin associates preferentially with neurofascin dimers and that phosphorylation inhibits neurofascin dimerization. These issues could be addressed by characterizing the stoichiometries of neurofascin–ankyrin complexes in solution.

A possible consequence of tyrosine phosphorylation of neurofascin, in addition to inhibition of ankyrin binding, is the activation of a phosphotyrosine-specific binding protein. However, consensus sequences for SH2 and PTB proteins differ from the neurofascin sequence flanking the FIGQY region. Putative phosphotyrosine-binding proteins specific for phosphorylated members of the L1 family, therefore, may be distinct from currently established candidates.

Previous studies (Williams et al., 1994*a*,*b*,*c*,*d*; Doherty et al., 1995; Dubreuil et al., 1996) have recognized that L1 family members can directly act as signal transducers. We are providing evidence of an L1 family member as a substrate for enzyme(s) in tyrosine signaling cascade(s). Therefore, it will be important in future work to elucidate the specific tyrosine protein kinases and phosphatases that control the phosphorylation state of neurofascin. Both NGF and bFGF promoted phosphorylation of neurofascin, suggesting either neurofascin is recognized by at least two distinct receptor protein kinases or that other protein kinase(s) activated downstream are involved. Another point of interest includes determining which protein tyrosine phosphatase(s) are involved in dephosphorylating neurofascin. Recent work has focused on a class of receptor protein tyrosine phosphatases (for review see Brady-Kalnay et al., 1995). Some integral protein tyrosine phosphatases (LAR-PTP, for example) are homologous to cell adhesion molecules of the Ig superfamily. Interestingly, Krypta et al. (1996) have shown that a direct association between LAR-PTP and cadherins helps to control cadherin adhesive functions. Although the cadherins are distinct from the L1 family, this direct association may represent a common mechanism by which cell adhesion molecules, through lateral association with a structurally related receptor protein tyrosine phosphatase, may be dephosphorylated.

Modulation of the interaction between ankyrin and the

various members of L1 cell adhesion molecules may play a critical role in mediating reversible assembly of a variety of transcellular complexes in the developing and adult nervous systems. The next step will be to extend the findings of this study to more physiologically relevant systems. Both tyrosine phosphorylation-resistant neurofascin mutants and forms of neurofascin lacking ankyrin-binding activity should provide precisely defined molecular tools for future work.

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