Intracellular Signaling Is Changed after Clustering of the Neural Cell Adhesion Molecules Axonin-1 and NgCAM during Neurite Fasciculation

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Abstract. Neural cell adhesion molecules of the immunoglobulin/fibronectin type III family on axons have been implicated in promotion of neurite outgrowth, fasciculation, and the mediation of specific cell adhesion. The present study demonstrates that two of these molecules on dorsal root ganglion neurons are associated with distinct protein kinases, axonin-1 with the src-related nonreceptor tyrosine kinase fyn and NgCAM with a casein kinase II–related activity and a serine/threonine kinase related to S6 kinase. When neurites grew without contacts involving axonin-1 and NgCAM, strong fyn kinase activity was associated with axonin-1, whereas the NgCAM-associated kinase activities were low. Clustering of axonin-1 with NgCAM induced by the formation of cell–cell contacts correlated with a reduction of the axonin-1–associated fyn activity and an increased phosphorylation of NgCAM by the associated casein kinase II–related activity. Thus, axonin-1 and NgCAM trigger distinctive intracellular signals during in vitro differentiation depending on their state of association.

THE correct connection of neurons with their targets involves axon guidance information provided by soluble molecules or guidance cues presented locally on cellular surfaces or in the extracellular matrix (Dodd and Jessell, 1988; Jessell, 1988; Hynes and Lander, 1992; Luo et al., 1993; Goodman and Shatz, 1993). Such molecular guidance cues are thought to be recognized by receptor proteins of the axonal surface. A major subgroup of these is represented by the immunoglobulin/fibronectin type III (IgFnIII)[1] class of molecules, named after their common structural features (Williams and Barclay, 1988). Molecules of this class have been implicated in the promotion of neurite outgrowth (Rathjen, 1991), neurite fasciculation (Rathjen and Jessell, 1991), and the mediation of specific cell adhesion (Friedlander et al., 1989). Extracellular interactions of IgFnIII molecules are transduced into intracellular signals (Schuch et al., 1989; Doherty et al., 1991; Lemmon et al., 1992; Williams et al., 1992, 1994*a*; Zisch et al., 1995). A large body of evidence has now directly linked IgFnIII cell adhesion molecule (CAM)-stimulated neurite outgrowth to calcium influx through N- and L-type calcium channels (Doherty et al., 1991; Saffell et al., 1992; Williams et al., 1992). The FGF-receptor tyrosine kinase and nonreceptor tyrosine kinases of the src family as well as casein kinase II–related and other serine/threonine kinases have been implicated as proximal components of the signal transduction pathways (Sadoul et al., 1989; Bixby and Jhabvala, 1993; Beggs et al., 1994; Williams et al., 1994*b*; Ignelzi et al., 1994; Zisch et al., 1995: Wong et al., 1996*a*,*b*).

A relatively well-characterized group of IgFnIII CAMs includes axonin-1, F11(contactin), NgCAM, and NrCAM (Bravo). An intriguing property of this subset of IgFnIII molecules is their complex binding pattern. Each member of this subgroup is involved in heterophilic binding to a specific set of ligands. Axonin-1 interacts with NgCAM (Kuhn el al., 1991) and NrCAM (Suter et al., 1995), whereas NgCAM also binds to F11(contactin) (Brümmendorf et al., 1993), laminin (Grumet et al., 1993*a*), and the proteoglycans 3F8, neurocan, and phosphocan (Grumet et al., 1993*b*; Friedlander et al., 1994; Milev et al., 1994). In addition, axonin-1 and NgCAM exhibit homophilic interactions (Lemmon et al., 1989; Rader et al., 1993).

Based on their localization on the surface of growth cones and axons and the combinatorial possibilities of their interactions, these molecules have been suggested to form distinctly composed complexes at sites of cell–cell contact which regulate pathfinding or cell–cell recognition (Sonderegger and Rathjen, 1992). The identification of signal transduction components associated with these molecules is, therefore, of crucial importance for the understanding of these processes. Here we demonstrate that the neural cell adhesion molecules axonin-1 and NgCAM of chicken embryonic dorsal root ganglia (DRG) neurons are

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1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; DRG, dorsal root ganglia; DST, disuccinimidyl tartarate; Fab, fragment with an antigen binding site; FnIII, fibronectin type III; GPI, glycosyl phosphatidylinositol; OAG, 1-oleoyl 2-acetyl sn glycerin; PVDF, polyvinyl difluoride.

associated with distinctive protein kinases and that axonin-1/NgCAM clusters are formed during the formation of cell–cell contacts that underlie neurite fasciculation. We provide evidence that the formation of axonin-1/NgCAM clusters during neurite fasciculation is accompanied by a reduction of the axonin-1–associated fyn tyrosine kinase activity and a concomitant increase of the casein kinase II–related activity associated with NgCAM.

Materials and Methods

Proteins and Antibodies

Axonin-1 was purified from chicken ocular vitreous fluid as described previously (Ruegg et al., 1989*a*). NgCAM was purified by immunoaffinity chromatography as described by Rathjen et al. (1987). Laminin (mouse, isolated from Engelbreth-Holm-Swarm sarcoma) was purchased from GIBCO-BRL (Gaithersburg, MD). Polyclonal serum R38 against axonin-1 (Ruegg et al., 1989*a*), 3024 against F11 (kindly provided by Fritz G. Rathjen, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany) and against NrCAM (kindly provided by Elisabeth Pollerberg, Max-Planck-Institute of Developmental Biology, Tübingen, Germany) were raised in rabbit. Polyclonal serum G4 against NgCAM was raised in goat (Kuhn et al., 1991). Purified IgG from rabbit serum R028 against chicken NCAM was kindly provided by Urs Rutishauser (Case Western Reserve University, Cleveland, OH). Affinity-purified rabbit antibodies (IgG) FYN 3 (specific for p60fyn from chicken, mouse, rat, and human), N-16 (specific for p60src from chicken, mouse, rat, and human) and anti-yes (specific for p62yes from chicken, mouse, rat, and human) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies lack any detectable cross-reactivity with other members of the src-family. Affinity-purified rabbit IgGs A-17 anti-p125 FAK (specific for FAK of mouse, rat, human, and chicken origin), anti-Rsk (specific for p90 Rsk), and anti-p70 S6 kinase (specific for p70 S6 kinase) were from Santa Cruz Biotechnology. Affinity-purified rabbit anti-phosphotyrosine IgG (Pasquale and Singer, 1988) was kindly provided by Elena Pasquale (La Jolla Cancer Research Foundation, La Jolla, CA). Monoclonal antibodies, M Ig6, V1F1, X9F6, and X9A9 against axonin-1, 12-1-4E-311 against NgCAM, 5e against chicken NCAM (a kind gift from Urs Rutishauser), and 2B3 against NrCAM (kindly provided by William Dreyer, California Institute of Technology, Pasadena, CA) were affinity purified as described earlier (Stoeckli et al., 1991). Coupling of the purified IgG (7.5 mg IgG per ml of hydrated Sepharose 4B gel) to CNBr-activated Sepharose 4B was carried out according to the manufacturer's recommendations. Protein A coupled to Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). FITC-conjugated streptavidin, peroxidase-conjugated anti-mouse IgG fragment with an antigen binding site (Fab), anti-sheep/goat IgG Fab fragments, and anti-rabbit IgG secondary antibodies for enhanced chemiluminescence (ECL) detection were from Boehringer Mannheim Corp. (Mannheim, Germany).

Reagents

Disuccinimidyl tartarate (DST) was purchased from Pierce Chemical Co. (Rockford, IL). Dimethyl sulfoxide was from Merck (Darmstadt, Germany). Trypsin (bovine) was from GIBCO-BRL. Phosphoserine, phosphothreonine, phosphotyrosine, ATP, cyclic AMP, cyclic GMP, calmodulin, phosphatidyl serine, 1-oleoyl 2-acetyl sn glycerin (OAG), genistein, poly-D-lysine, progesterone, triiodothyronine, putrescine, sodium selenite, corticosterone, casein (dephosphorylated), heparin, PMSF, pepstatin A, leupeptin, aprotinin, 5-fluoro deoxyuridine, CHAPS, high molecular weight standard mixture SDS 7B for SDS-PAGE, and cross-linked phosphorylase b from rabbit muscle were from Sigma Chemical Co. (St. Louis, MO). Triton X-100 and β-octyl glucoside were obtained from Fluka Chemicals Co. (Buchs, Switzerland), CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology, and γ-[^{32}P]ATP (~3,000 Ci/mmol) purchased from Amersham Corp. (Buck, UK). Histone H1 and reagents for enhanced chemiluminescence detection (ECL) were from Boehringer Mannheim Corp. P70 S6/p90 Rsk kinase substrate peptide RRRLSSLRA and synthide-2 PLARTLSVAGLPGKK (peptide substrate for Ca^{2+}/calmodulin kinase II and Raf-1) were from Santa Cruz Biotechnology. L-[^{35}S]methionine (translation grade) was from New England Nuclear/Du Pont (Wilmington, DE). Polygram thin-layer plates (0.1 mm cellulose) were from Macherey and Nagel (Dueren, Switzerland). X-ray films X-OMAT were from Eastman Kodak Company (Rochester, NY).

Cell Culture

DRG were dissected from embryonic day-10 chick embryos (Sonderegger et al., 1985). Cells were dissociated by trypsinization and subsequent tituration. The serum-free defined medium used for all cultures was described by Stoeckli et al. (1996). To obtain cultures primarily consisting of neurons, freshly dissociated cells were plated on plastic in a high density (20,000 cells/cm^2) and incubated for 1 h. The nonadherent neurons were gently removed and subsequently transferred to the substrate coated dishes in a density of 5,000-10,000 cells/cm^2. Proliferation of nonneuronal cells in cultures kept for more than 15 h was minimized by the addition of 0.12 mM 5-fluoro deoxyuridine.

For ^{35}S labeling, L-[^{35}S]methionine (translation grade) was added 5 h after plating in a concentration of 25 μCi/ml. After a labeling period of 10 h, cells were cultivated for 1 h with unlabeled medium before subsequent treatment.

Specimens for microscopy were fixed in 2% (wt/vol) glutaraldehyde in 50 mM cacodylate, pH 7.2. For light microscopic inspection, an inverted microscope equipped with phase contrast optics was used. For scanning electron microscopy, specimens were prepared by critical point drying and gold coating.

Immunoprecipitation and Immune Complex Kinase Assay

For immune complex assays, 10^6 cells were used in each sample. Cells were washed twice in ice cold Tris buffer (50 mM Tris, pH 7.6) with 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, and solubilized in Triton lysis buffer 0.5% (wt/vol) Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM PMSF in 50 mM Tris, pH 7.6, for 20 min at 4°C. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was transferred to a vial containing 5 μl Sepharose 4B gel with covalently coupled monoclonal antibodies or 5 μg affinity purified rabbit IgG anti-fyn, anti-src, anti-yes, or anti-FAK preabsorbed to 5 μl protein A coupled to Sepharose 4B and incubated for 90 min at 4°C. Immunoprecipitates were washed five times in ice-cold lysis buffer without EDTA, resuspended in a kinase buffer containing 50 mM Hepes, pH 7.3, 100 mM NaCl, and 5 mM $MnCl_2$ (designated as standard kinase buffer). The kinase reaction was carried out on ice for 15 min in 20 μl kinase buffer containing 5 μCi of γ[^{32}P]ATP in a total concentration of 1 μM ATP (2.5×10^5 Ci ^{32}P/mol ATP) except where described differently. The reaction was stopped by washing three times with lysis buffer containing 25 mM EDTA. For reprecipitation experiments, the immune complex was eluted off the first set of immunoprecipitating beads by boiling in 1% (wt/vol) SDS, 1 mM sodium orthovanadate in 10 mM Tris, pH 7.6, for 15 min. The eluted fraction was diluted 10-fold in lysis buffer and subjected to a second round of immunoprecipitation. All immunoprecipitates were eluted with reducing Laemmli sample buffer and the proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels. For the visualization and quantification of ^{32}P-labeled proteins, a phosphoimager and the Image QuaNT software (version 3.3) from Molecular Dynamics (Sunnyvale, CA) were used.

For inhibition assays the tyrosine kinase inhibitor genistein (Akiyama et al., 1987) was added to the kinase assay buffer before the addition of ATP in final concentrations of 10 and 50 μM. Stock solutions of genistein were prepared in DMSO such that the final DMSO concentration was 1% (vol/vol) in each sample. Kinase reaction was carried out as described above.

For an estimation of fyn quantities in the axonin-1 immunocomplex, the amount of ^{32}P incorporated into fyn by its autophosphorylation activity (Kypta et al., 1988) was quantified. In src-family kinases, one site of autophosphorylation per molecule is described (Smart et al., 1981). The kinase assay with the axonin-1 immunocomplex was performed with 1 μM carrier-free γ[^{32}P]ATP (1,500 Ci/mmol). ^{32}P labeling was carried out on ice to saturation (30 min). After SDS-PAGE, the fyn band was excised and ^{32}P quantified in a scintillation counter. The amount of axonin-1 was determined by quantitative Western blot (see below). In the axonin-1 immunocomplexes isolated from cultures after 15 h, 2.1×10^{-3} mol ^{32}P incorporated into fyn per mol axonin-1 were found (SD: ±0.4; $n = 3$). The fact that some fyn autophosphorylation sites may be already in a phosphorylated state and not available for ^{32}P-labeling might lead to an underestimation of fyn in the complex.

Characterization of the NgCAM-associated Serine/Threonine Kinases

NgCAM was immunoprecipitated as described above. To separate fyn from the NgCAM precipitate, Triton X-100 was replaced with β-octyl glucoside in the washing buffer. The phosphorylation reaction was carried out in assay buffer containing either 5 mM $MnCl_2$ alone or in combination with 10 mM $MgCl_2$ with or without 10 mM $CaCl_2$. Cyclic AMP and cyclic GMP were used at 5 and 10 μM in a phosphorylation buffer containing 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, and 100 mM NaCl (Roskosi, 1983). 200 μg/ml histone H1 were included as a substrate for protein kinase A. Calmodulin was used in concentrations of 10 and 50 μg/ml in an assay buffer composed of 50 mM Hepes, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$. The protein kinase C assay was performed according to the protocol of Go et al. (1987) in 50 mM Hepes, pH 7.5, 6.25 mM $MgCl_2$, 0.125 mM $CaCl_2$, 10 μg/ml phosphatidylserine, 1 μg/ml OAG, and 200 μg/ml histone H1, which was included as a substrate for protein kinase C. To make up the solution of phosphatidylserine and the OAG, the lipids were premixed in a small volume of chloroform. The solvent was evaporated under nitrogen. The residue was resuspended in assay buffer and sonicated under nitrogen for 5 min on ice. In all assays the concentration of ATP was 1 μM containing 5 μCi of $\gamma[^{32}P]$ATP in a total volume of 20 μl (2.5×10^5 Ci ^{32}P/mol ATP). Kinase reactions were carried out for 15 min at 4 and 30°C. For both reaction temperatures, identical results were obtained. After the kinase reactions, all proteins were precipitated (Wessel and Fluegge, 1984) and subjected to SDS-PAGE under reducing conditions using 7.5 and 10% polyacrylamide gels. 100 μg/ml heparin was included in the standard kinase buffer for competition assays. The peptide substrates were added in concentrations of 10 μM, 100 μM, and 1 mM, respectively.

Base Hydrolysis of Phosphoproteins and Phosphoamino Acid Analysis

After the in vitro kinase assay (described above), proteins were separated by SDS-PAGE and blotted onto polyvinyl difluoride (PVDF)-membrane (Immobilon; Millipore Corp., Bedford, MA). The proteins transferred to the PVDF-membrane were stained with Ponceau S (Serva Biochemicals, Paramus, NJ). Alkaline hydrolysis of phosphoproteins was performed as described (Kamps, 1991). The PVDF-membrane was incubated with 1.0 M KOH for 4 h at 55°C, neutralized in 1 M Tris, pH 6.5, and finally washed in TBS. The membranes were dried and exposed for autoradiography.

Phosphoamino acid analysis was performed according to Cooper et al. (1983) by thin-layer electrophoresis in one dimension with acidic electrophoresis buffer (0.5% [vol/vol] pyridine, 5% [vol/vol] acetic acid in water, pH 3.5), using 0.1 mm cellulose thin-layer plates.

Chemical Cross-linking and Immunoprecipitation of Cross-linked Material

To obtain equal amounts of axonin-1 (~100 ng) for each cross-linking experiment, 2×10^6 cells per sample were used after 15 h in culture, and 7.5×10^5 cells after 45 h in culture. Cells were washed three times in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and incubated on ice. 100 mM disuccinimidyl tartarate (DST), dissolved in DMSO was added to the cells to a final concentration of 1 mM. Cross-linking was carried out on ice under gentle shaking. The reactions were stopped by adding glycine, pH 8.0, in a final concentration to 50 mM. After quenching for 5 min, the cells were lysed for 1 h in 1 ml of a lysis buffer containing 1% (wt/vol) CHAPS, 0.1% (wt/vol) SDS, 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM PMSF. Lysates were clarified by centrifugation at 12,000 *g* for 30 min at 4°C. The supernatant was transferred to 5 μl anti-axonin-1 mAb coupled to Sepharose 4B (7.5 μg IgG per μl of Sepharose gel) and allowed to tumble at 4°C for 90 min. Immunoprecipitates were washed thoroughly in lysis buffer, followed by 150 mM NaCl in 50 mM Tris, pH 7.6. The protein was eluted in reducing Laemmli buffer and separated by SDS-PAGE using 5% polyacrylamide gels. For molecular mass determination of the cross-linked species, cross-linked phosphorylase b from rabbit muscle was used according to the manufacturer's recommendations (Technical Bulletin No. MWS-877X; Sigma Chemical Co.).

Immunoblotting

After gel electrophoresis proteins were transferred to nitrocellulose. The nitrocellulose was incubated for 2 h in 1% (wt/vol) blocking reagent containing the primary antibodies. Polyclonal anti-axonin-1 serum was used in a dilution of 1:10,000, and polyclonal anti-F11, anti-NgCAM, and anti-NrCAM were diluted 1:5,000. Polyclonal anti-NCAM, anti-FAK, and mAb anti-NgCAM were used in a concentation of 1 μg/ml purified IgG and the polyclonal antibodies anti-fyn, anti-src, and anti-yes in a concentration of 2 μg/ml. Monoclonal antibodies V1F1, X9F6, and X9A9 against axonin-1 were also used at a concentration of 2 μg/ml. For phosphotyrosine detection, purified rabbit antiphosphotyrosine IgG was used in a concentration of 5 μg/ml in 1% (wt/vol) blocking reagent in TBS and incubated at 4°C for 12 h. The immunoblots were washed in TBS and incubated for 1 h with 0.2 μg/ml anti–rabbit IgG, 0.4 μg/ml anti–mouse IgG Fab fragments, or 0.5 μg/ml anti–sheep/goat IgG Fab fragments coupled to peroxidase in 0.5% (wt/vol) blocking reagent in TBS, according to the primary antibody used. For detection, the chemiluminescence system from Boehringer Mannheim was used. Exposure times for the autoradiographs ranged from 10 to 30 s for most blots; longer exposure times are indicated where used. Sequential application of antibodies to the same blot was performed as follows: after probing with one primary antibody, the nitrocellulose was rinsed and incubated in TBS containing 50 mM 2-mercaptoethanol and 1% (wt/vol) SDS for 30 min at 25°C, washed four times for 15 min in a copious volume of TBS, blocked for at least 1 h in 1% blocking reagent in TBS, and reprobed with another primary antibody according to the protocol above. This procedure allowed the serial application of up to five different antibodies.

For the quantification of axonin-1 and NgCAM in the immunoprecipitates, 50, 100, 200, 400, 600, 800, and 1,000 pg of purified axonin-1 or NgCAM were used as standards. For densitometric quantification the Image QuaNT software (version 4.1) from Molecular Dynamics was used.

Oligonucleotides

Two different axonin-1 antisense phosphorothionate oligonucleotides were used. One was localized at the 5′ end, ranging from nucleotides −10 to +12, and contained the translation initiation codon (5′-CAGTGCCTCCCATCCTGTGGCTC-3′). The other was localized at the 3′ end where it overlapped the stop codon (5′-GCTACCTGGAGCTCTGATGG-3′; Zuellig et al., 1992). These oligonucleotides do not match any other nucleotide sequence of known chicken genes. The oligonucleotides were synthetized by Microsynth Inc. (Balgach, Switzerland). After purification by ion exchange chromatography and gel filtration, they were dissolved in quartz distilled water to a concentration of 2 mg/ml. Oligonucleotides were introduced into DRG neurons as described by Aigner and Caroni (1995). Chick DRGs were trypsinized for 15 min at 37°C and titurated (15–20 cycles through a yellow tip [Gilson Medical Elec. Inc., Middleton, WI]) in 50 μl serum-free defined medium containing the oligonucleotides in the concentrations indicated. The cells were kept at 37°C for 30 s, pelleted by centrifugation for 2 min at 320 *g*, washed in medium, centrifuged again, resuspended, and plated as described (see Cell Culture). Cultures were kept for 20 h in serum-free defined medium. For Western blot analysis, cells were lysed in PBS containing 1% (wt/vol) Triton X-100, 1% (wt/vol) CHAPS, 0.2% (wt/vol) SDS. Total protein was isolated from this lysate using the method of Wessel and Fluegge (1984). Within a range of 25–100 μM, a dose-dependent reduction of axonin-1 was observed for both antisense oligonucleotides (data not shown). Applied in a concentration of 100 μM, the 5′ antisense oligonucleotide alone reduced axonin-1 by 87.4% with respect to control values, the 5′ sense oligonucleotide by 11.9%. Application of the 3′ antisense oligonucleotide led to an axonin-1 reduction of 76%, the 3′ sense oligonucleotide to a reduction of 10.3%. The combination of 5′ and 3′ oligonucleotides resulted in an increased reduction of axonin-1 by 94% for the antisense and 14.6% for the sense oligonucleotides. The combination of 5′ and 3′ antisense axonin-1–oligonucleotides reduced NgCAM by 8.1% compared to 12.8% reduction by the sense oligonucleotides. The uptake of oligonucleotides was verified using 5′ biotinylated oligonucleotides (purchased from Microsynth Inc.), which were subsequently detected by FITC-labeled streptavidine. Cell viability was checked by trypan blue exclusion.

Results

The Nonreceptor Tyrosine Kinase fyn Coimmunoprecipitates with Axonin-1 from DRG Neurons

Associations of protein kinases with the neural cell recog-

nition molecules axonin-1 and NgCAM were demonstrated by immunoprecipitation in cultured chicken DRG neurons. Kinase reactions performed with anti–axonin-1 immunoprecipitates prepared from cells cultivated for 15 h resulted in phosphorylation of proteins with apparent molecular masses of 60 and 140 kD (Fig. 1 *A*). The kinase activity associated with axonin-1 incorporated ^{32}P mainly in the form of tyrosine phosphate, as evidenced by the stability of the phosphoproteins towards base hydrolysis, the detection of tyrosine phosphate by Western blot analysis using a specific antiphosphotyrosine antibody (Fig. 1 *B*), and phosphoamino acid analysis (Fig. 1 *C*). The association of the kinase activity with axonin-1 resisted Triton X-100 but was completely abolished by β-octyl glucoside (Fig. 1 *D*). The 60-kD phosphoprotein was identified as the src-related nonreceptor tyrosine kinase fyn by reprecipitation with a polyclonal anti-fyn antibody (Fig. 1 *E*). Fyn was also identified in the axonin-1 immunocomplex by Western blot analysis (Fig. 1 *E*). In contrast, neither src nor yes was found associated with axonin-1, although they were both detected in DRG neurons (Fig. 1 *F*). The kinase activity associated with axonin-1 was inhibited by the tyrosine kinase inhibitor genistein (Akiyama et al., 1987) in a dose-dependent manner. The extent of phosphorylation of p140 was reduced proportionally to the inhibition of fyn in the immunocomplex (Fig. 1 *G*). The stoichiometry of association between fyn and axonin-1 in the axonin-1 immunocomplex was estimated as 1:500. However, it is likely that fyn is underrepresented in the complexes that are analyzed on gels because it may be lost in part from its complex with axonin-1 during the extensive washing procedures of the immunoprecipitation.

The neural cell adhesion molecules NgCAM and NrCAM, previously described as binding partners for axonin-1 (Kuhn et al., 1991; Suter et al., 1995), were investigated for a potential role as adaptors mediating the association of axonin-1 with fyn. Neither NgCAM nor NrCAM were detected in the axonin-1 immunocomplex by Western blot analysis or reprecipitation with antibodies against NgCAM and NrCAM (not shown). NCAM, which had been shown previously to be involved in fyn signaling in neurons (Beggs et al., 1994), and the focal adhesion kinase p125 FAK, a tyrosine kinase involved in the formation of focal adhesion complexes on laminin substratum (Schaller and Parsons, 1994; Miyamoto et al., 1995), were tested as candidates for phosphoprotein p140. Phosphoprotein p140, as obtained by an in vitro kinase reaction with the axonin-1 immunocomplex, was not reprecipitated by polyclonal an-

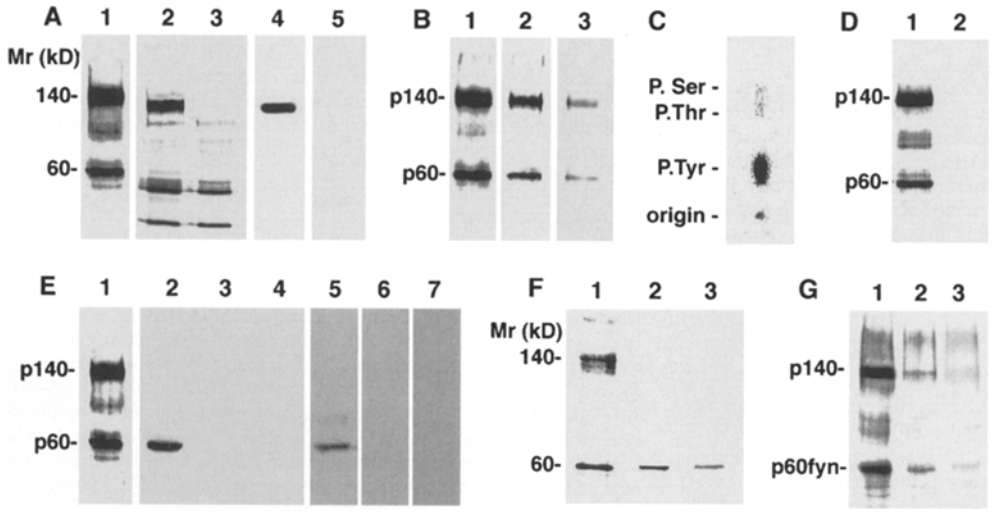

Figure 1. Investigation of axonin-1–associated kinase activity by coimmunoprecipitation. (*A*) Coprecipitation of kinase activity with axonin-1 in DRG neurons. Cell cultures were metabolically labeled with [^{35}S]methionine for 10 h before lysis. Lane *1*, phosphoproteins as visualized by an in vitro kinase assay using γ[^{32}P]ATP with the immunocomplexes of axonin-1. Autoradiography was for 15 h using a 0.1-mm copper foil to shield ^{35}S radiation. Lane *2*, ^{35}S-labeled proteins in the immunocomplex of axonin-1. The immunocomplex was subjected to a kinase assay, as in lane *1*, but nonradioactive ATP was used. Lane *3*, control precipitation using Sepharose 4B visualizes ^{35}S-labeled proteins that bind unspecifically to the Sepharose matrix. Lane *4*, axonin-1 in the axonin-1 precipitate as visualized by Western blot analysis. Lane *5*, control kinase assay on the immunocomplex of NrCAM, using γ[^{32}P]ATP. (*B*) Detection of tyrosine phosphate in the axonin-1–associated phosphoproteins p60 and p140. Lane *1*, the phosphoproteins p60 and p140, as obtained by a kinase assay. Lane *2*, p60 and p140 after alkaline hydrolysis. Lane *3*, tyrosine phosphate detection in p60 and p140 by Western blot analysis using an antiphosphotyrosine antibody. Exposure time of the chemiluminescence detection was 5 min. (*C*) Phosphoamino acid analysis of the phosphoproteins p60 and p140. Proteins of the immunocomplex were hydrolyzed and separated by thin layer electrophoresis using phosphotyrosine, phosphoserine, and phosphothreonine as standards. (*D*) The association of the kinase activity with axonin-1 is maintained in axonin-1 precipitates from Triton X-100 lysates (lane *1*) but not in lysates containing β-octyl glucoside (lane *2*). (*E*) Only fyn but not src and yes are detected in the axonin-1 immunocomplex. The axonin-1 immunocomplex (lane *1*) was disrupted by treatment with SDS. From the eluate, fyn was reprecipitated with a specific anti-fyn antibody (lane *2*). Neither src (anti-src; lane *3*) nor yes (anti-yes; lane *4*) was reprecipitated. Autoradiography was for 10 h for lane *1* and 25 h for lanes *2–4*. In Western blot analysis, the proteins of the axonin-1 immunocomplex were probed with polyclonal antibodies anti-fyn (lane *5*), anti-src (lane *6*), and anti-yes (lane *7*). Exposure time was 5 min for lanes *5–7*. (*F*) Detection of fyn, src, and yes in DRG neurons. Fyn, src and, yes were precipitated with the antibodies anti-fyn (lane *1*), anti-src (lane *2*), and anti-yes (lane *3*) from DRG cultures lysed in Triton X-100 and subjected to an in vitro kinase assay. Autoradiography was for 1 h. (*G*) The kinase activity associated with axonin-1 is inhibited by the tyrosine kinase inhibitor genistein. Before the addition of γ[^{32}P]ATP, the tyrosine kinase inhibitor genistein was added to the kinase assay in the following concentrations: 10 μM (lane *2*) and 50 μM (lane *3*). A kinase assay without genistein in shown in lane *1*.

tibodies against NCAM or p125 FAK, and neither NCAM nor p125 FAK were detected in the immunoprecipitates of axonin-1 by Western blot analysis (not shown).

Two Distinct Serine/Threonine Kinases and the Nonreceptor Tyrosine Kinase fyn Coprecipitate with NgCAM from DRG Neurons

In vitro kinase reactions with the immunocomplex of NgCAM from Triton X-100 extracts of DRG neurons revealed a different pattern of phosphorylated proteins (Fig. 2 *A*). A strongly phosphorylated protein with an apparent molecular mass of 80 kD and weaker bands at apparent molecular masses of 60 and 140 kD were detected. Reimmunoprecipitation using the appropriate antibodies identified the 80-kD phosphoprotein as the transmembrane fragment of NgCAM and the phosphoprotein p60 as fyn (Fig. 2 *B*). In reimmunoprecipitation and Western blot analysis, fyn, but neither src nor yes, was detected in the immunocomplex of NgCAM (Fig. 2 *B*). ^{32}P was incorporated into NgCAM as serine and/or threonine phosphate but as tyrosine phosphate into fyn and p140, as demonstrated by base hydrolysis and detection of tyrosine phosphate using an antiphosphotyrosine antibody (Fig. 2 *C*). The phosphorylation of NgCAM was found to persist after treatment of the NgCAM immunocomplex with β-octyl glucoside, whereas fyn dissociated largely (Fig. 2 *D*). The kinase activity retained in the β-octyl glucoside-treated NgCAM immunocomplex incorporated ^{32}P exclusively as serine and/or threonine phosphate (Fig. 2 *D*).

In summary, distinct kinase activities were present in the NgCAM immunocomplex. The 80-kD fragment of NgCAM was phosphorylated by a serine/threonine kinase activity, whereas fyn and p140 were phosphorylated by a tyrosine kinase activity.

The phosphorylation of L1, the putative mouse homologue of NgCAM, by casein kinase II and S6 kinase has previously been reported (Sadoul et al., 1989; Wong et al., 1996*a*,*b*). For further characterization of the serine/threonine kinase activities coprecipitated with and phosphorylating the 80-kD fragment of NgCAM, the regulation of the kinase activity by different cofactors was evaluated. NgCAM phosphorylation was only detected in presence of Mn^{2+} or Mg^{2+}. Whether or not 10 mM Ca^{2+} was added did not detectably modulate the phosphorylation of NgCAM (Fig. 3 *A*). Phosphorylation of NgCAM and histone H1, which was added to the assay as a substrate for protein kinase A (Roskoski et al., 1983), was not affected by the presence of cAMP and cGMP (Fig. 3 *B*). The presence of calmodulin and Ca^{2+} did not alter NgCAM phosphorylation (Fig. 3 *C*). Addition of phosphatidylserine and OAG, a synthetic diolein analogue, which is known to enhance protein kinase C activity, did not influence the phosphorylation of NgCAM or histone H1 (Fig. 3 *D*). The NgCAM-associated kinase phosphorylated casein with a high efficiency but phosphorylated histone H1 only weakly (Fig. 3 *E*). For further characterization, we investigated the effect of heparin, which is known as a potent and specific inhibitor of casein kinase II (Hathaway and Traugh, 1982, 1983). The quantification of incorporated radioactivity revealed that 1 μg/ml heparin reduced the phosphorylation of casein by 91% and the phosphorylation of NgCAM by

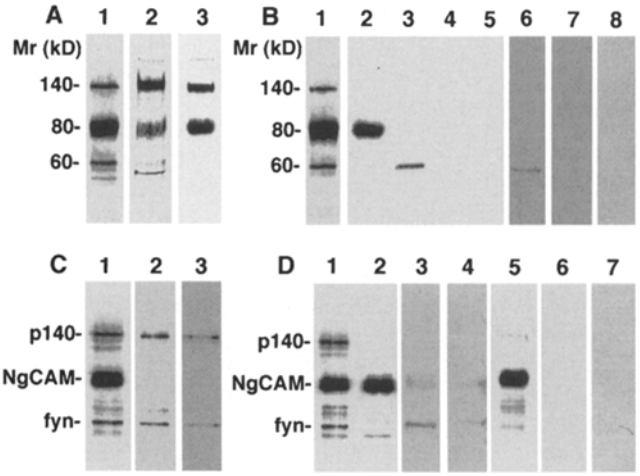

Figure 2. Investigation of NgCAM-associated kinase activity by coimmunoprecipitation. (*A*) Coprecipitation of kinase activity with NgCAM. The cultures were metabolically labeled with [^{35}S]methionine as in Fig. 1 *A*. Lane *1*, the phosphoproteins obtained from an in vitro kinase assay using γ[^{32}P]ATP with an NgCAM immunoprecipitate. Autoradiography was for 20 h shielding ^{35}S radiation in lane *1* with a 0.1-mm copper foil. Lane *2*, ^{35}S-labeled proteins in the NgCAM precipitate. Lane *3*, detection of NgCAM by Western blot. (*B*) Identification of the phosphoproteins p60 and p80 as fyn and the transmembrane fragment of NgCAM, respectively. The NgCAM immunocomplex (lane *1*) was disrupted by treatment with SDS. Reprecipitation was performed using polyclonal anti-NgCAM antiserum (lane *2*) and polyclonal anti-fyn (lane *3*), anti-src (lane *4*), and anti-yes (lane *5*) antibodies. Autoradiography was for 10 h (lane *1*) and 20 h (lanes *2–5*). Lanes *6–8*, reprobing of the NgCAM precipitate on Western blot. Lane *6*, anti-fyn; lane *7*, anti-src; and lane *8*, anti-yes. Exposure time was 10 min for lanes *6–8*. (*C*) Detection of tyrosine phosphate in the phosphoproteins p60 and p140 of the NgCAM precipitate. Lane *1*, phosphoproteins of the NgCAM precipitate. Lane *2*, phosphoproteins after alkaline hydrolysis. Lane *3*, tyrosine phosphate detection on Western blot using a phosphotyrosine antibody. (*D*) The phosphorylation of NgCAM resists, but fyn is dissociated by a solubilization with β-octyl glucoside. Phosphoproteins obtained from a kinase assay on NgCAM precipitates from a Triton X-100 (lane *1*) and a β-octyl glucoside lysate (lane *2*). Detection of fyn in NgCAM precipitates by Western blot analysis using 10 times the amount of material compared to the detection of NgCAM: lane *3*, Triton X-100 lysate; and lane *4*, β-octyl glucoside lysate. The phosphoproteins of an NgCAM immunoprecipitate from β-octyl glucoside lysate (lane *5*) were subjected to base hydrolysis (lane *6*) and phosphotyrosine detection on Western blot (lane *7*). Autoradiography of the untreated control was for 20 h and of the sample subjected to base hydrolysis for 80 h. Exposure of the Western blot was 20 min. Note that the kinase activity retained in the β-octyl glucoside resistant fraction of the NgCAM precipitate incorporates ^{32}P exclusively as serine/threonine phosphate.

60%. A concentration of 100 μg/ml heparin abolished casein phosphorylation completely but reduced NgCAM phosphorylation only by 78% (Fig. 3 *F*). Thus, two distinct serine/threonine kinases were found in the NgCAM precipitate. One phosphorylated casein and exhibited a sensitivity towards low concentrations of heparin and thus was identified as casein kinase II–related kinase. The other phosphorylated NgCAM but not casein and was insensitive to heparin.

For further characterization of the heparin insensitive kinase activity, we tested substrate peptides of different serine/threonine kinases for their capability to complete the in vitro phosphorylation of NgCAM in the presence of 100 μg/ml heparin. We found that NgCAM phosphorylation was specifically inhibited in a dose-dependent manner by the addition of the peptide RRRLSSLRA, which is a substrate for S6 kinases (Pelech et al., 1986; Fig 3 *G*). A peptide concentration of 1 mM resulted in a nearly complete inhibition of NgCAM phosphorylation (Fig. 3 *G*). In contrast, peptide substrates for other independent serine/threonine kinases, such as synthide-2, a substrate for Ca^{2+}/calmodulin kinase II and raf-1, did not affect NgCAM phosphorylation under these conditions (not shown). Together, these results tentatively identified the second kinase activity associated with NgCAM as a S6 kinase-related activity. This conclusion is in line with the demonstration of L1 phosphorylation by S6 kinase (Wong et al., 1996*a*,*b*). Both forms of S6 kinase, p70 S6 kinase and p90 Rsk, were demonstrated to be expressed in DRG neurons (Fig. 3 *H*). Based on these results, we concluded that NgCAM is associated with two distinct serine/threonine kinases, a casein kinase II–related activity, which phosphorylates both casein and NgCAM and is strongly inhibited by low concentrations of heparin, and an S6 kinase-related activity, which phosphorylates NgCAM but not casein and is insensitive to heparin.

The pattern of phosphoproteins generated in the kinase assay of the NgCAM immunocomplex from Triton X-100 extracts included the src family kinase fyn and a phosphoprotein with an apparent molecular mass on SDS-PAGE similar to the phosphoprotein p140 found in association with axonin-1 (Fig. 2 *A*). When the NgCAM immunocomplex was probed on a Western blot, the presence of axonin-1 was revealed (Fig. 4 *A*). In immunoprecipitations of fyn from Triton X-100 lysates, only axonin-1, but not NgCAM, was detected on Western blot (Fig. 4 *B*), though comparable amounts of axonin-1 and NgCAM were detected in the cells (Fig. 4 *B*). This lack of NgCAM in the fyn precipitate could be explained by a much smaller ratio of fyn/NgCAM compared to fyn/axonin-1 or an indirect, axonin-1–mediated coprecipitation of fyn with NgCAM. To investigate this issue, the expression of axonin-1 was reduced with a combination of two axonin-1 antisense oligonucleotides, one directed against the region of the translation initiation site and the other against a sequence overlapping the stop codon. Quantitative Western blot revealed a reduction of axonin-1 by 95% (Fig. 5 *A* and *B*). No reduction of NgCAM expression was observed. In vitro kinase reactions were performed on the immunoprecipitates of axonin-1 and NgCAM obtained from cultures treated with axonin-1 antisense or sense oligonucleotides, as well as from control cultures. In axonin-1 precipitates, the axonin-1 reduction was accompanied by a reduction of ^{32}P incorporation in fyn and p140 as well as the amount of coprecipitated fyn protein (Fig. 5 *C*). In NgCAM immunoprecipitates from lysates of axonin-1–reduced cells, no fyn protein was found, and neither fyn nor p140 were phosphorylated (Fig. 5 *C*). In contrast, the phosphorylation of NgCAM remained unaffected by the axonin-1 antisense

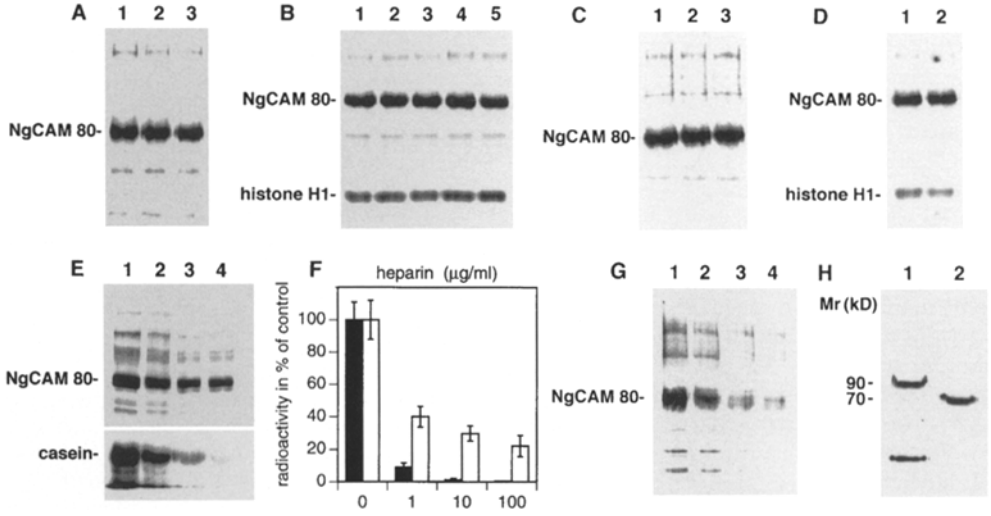

Figure 3. Characterization of the NgCAM phosphorylating kinase activity. (*A*) The inclusion of Mg^{2+} and Ca^{2+} in the kinase assay buffer did not affect NgCAM phosphorylation. Lane *1*, kinase reaction on the NgCAM immunocomplex with 5 mM Mn^{2+}. Lane *2*, 5 mM Mn^{2+} and 10 mM Mg^{2+}. Lane *3*, 5 mM Mn^{2+}, 10 mM Mg^{2+}, and 10 mM Ca^{2+}. (*B*) Cyclic AMP and cyclic GMP did not enhance phosphorylation of NgCAM or histone H1. Lane *2*, NgCAM immunocomplex mixed with histone H1 (200 μg/ml) and 5 μg/ml cAMP. Lane *3*, 10 μg/ml cAMP. Lane *4*, 5 μg/ml cGMP. Lane *5*, 10 μg/ml cGMP. Phosphorylation without cyclic nucleotides is shown in lane *1*. (*C*) The presence of calmodulin and Ca^{2+} does not alter NgCAM phosphorylation. Lane *1*, without calmodulin. Lane *2*, 10 μg/ml calmodulin. Lane *3*, 50 μg/ml calmodulin. Ca^{2+} concentration was 10 mM. (*D*) Activators of protein kinase C did not enhance NgCAM phosphorylation. Lane *1*, control. Lane *2*, 10 μg/ml phosphatidylserine and 1 μg/ml OAG were added to the NgCAM immunocomplex containing histone H1 (200 μg/ml). (*E*) The phosphorylation of NgCAM and casein by the kinase activity found in association with NgCAM in DRG neurons was inhibited by heparin. To the NgCAM immunoprecipitate containing casein (100 μg/ml), the following concentrations of heparin were added: 5 μg/ml (lane *2*), 20 μg/ml (lane *3*), and 100 μg/ml (lane *4*). Phosphorylation without heparin is shown in lane *1*. For autoradiography, the upper part of the gel was exposed for 20 h and the lower part (residing on the same gel) for 1 h. (*F*) Quantification of the results of E. The ^{32}P-labeling of casein (*black bars*) and the 80-kD fragment of NgCAM (*white bars*) was quantified (mean ± SD, $n = 3$). (*G*) The phosphorylation of NgCAM by the second, heparin-insensitive kinase was inhibited by a substrate peptide for S6 kinase. NgCAM precipitates were mixed with the substrate peptide RRRLSSLRA in concentrations of 0 (lane *1*), 10 μM (lane *2*), 100 μM (lane *3*), and 1 mM (lane *4*). Kinase assay was carried out as described above. Autoradiography was for 10 h. (*H*) Both forms of S6 kinase are detected in total cell protein of DRG neurons by Western blot analysis. Lane *1*, p90 Rsk. Lane *2*, P70 S6 kinase.

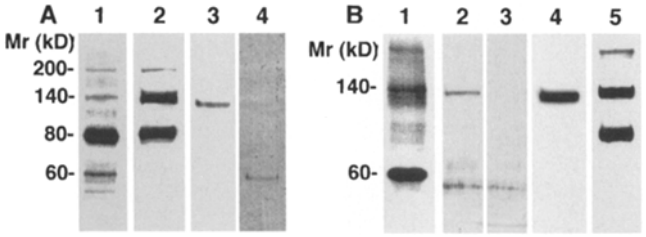

Figure 4. Coimmunoprecipitation. (*A*) Axonin-1 coprecipitated with NgCAM from DRG neurons. Lane *1*, phosphoproteins from an in vitro kinase assay with immunoprecipitates of NgCAM from Triton X-100 lysates of DRG neurons. The NgCAM precipitate was probed with anti-NgCAM (lane *2*), anti–axonin-1 (lane *3*), and anti-fyn (lane *4*). Autoradiograpy was for 20 h (lane *1*) and exposition times 5 s (lane *2*), 30 s (lane *3*), and 10 min (lane *4*). (*B*) Axonin-1, but not NgCAM coprecipitated with fyn. Fyn was precipitated from a Triton X-100 lysate of DRG neurons. Lane *1*, phosphoproteins of a kinase assay performed with fyn immunocomplex. The fyn precipitate was probed for axonin-1 (lane *2*) and NgCAM (lane *3*) on a Western blot. Detection of axonin-1 (lane *4*) and NgCAM (lane *5*) in equal amounts of total cell protein using the same antibodies.

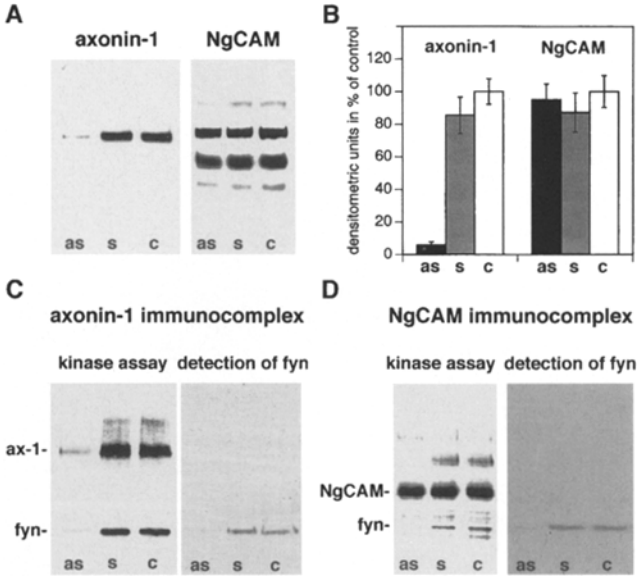

Figure 5. Investigation of the role of axonin-1 for the presence of fyn with immunoprecipitated NgCAM. (*A*) Antisense axonin-1 oligonucleotides reduce axonin-1 in DRG neurons specifically. Cells were treated with antisense– (lanes labeled as) or sense–axonin-1 oligonucleotides (lanes labeled *s*). A culture not treated with oligonucleotides (lanes labeled *c*) was used as an internal control. Axonin-1 and NgCAM were detected in total cell protein by Western blot analysis. One representative example out of three experiments is shown in the gel-panel. (*B*) Densitometric quantification of *A* (*black bars*, antisense; *gray bars*, sense). Normalization was against the untreated controls (*white bars*, defined as 100%). (*C*) Suppression of axonin-1 by axonin-1 antisense oligonucleotides results in disappearance of fyn in the axonin-1 immunoprecipitate. *Left panel*, phosphoproteins of a kinase assay with axonin-1 immunoprecipitates from Triton X-100 lysates of cells treated with antisense and sense axonin-1 oligonucleotides, and an untreated control. *Right panel*, detection of fyn by Western blotting (exposure time of 5 min). Autoradiography was for 10 h. (*D*) Suppression of axonin-1 by antisense oligonucleotides results in disappearance of fyn in the NgCAM immunocomplex. *Left panel*, phosphoproteins of a kinase assay with NgCAM precipitates of cells treated with axonin-1 antisense and sense oligonucleotides, and an untreated control. *Right panel*, detection of fyn by Western blotting as in *B*, using 10 times the amount of material than in the left panel. Exposure time was 10 min.

oligonucleotides (Fig. 5 *D*). These results indicate that the presence of axonin-1 is necessary for the association of fyn with NgCAM but not for the phosphorylation of NgCAM by the associated serine/threonine kinases.

In contrast to the coprecipitation of axonin-1 with NgCAM/anti-NgCAM complexes, no NgCAM was found in the anti–axonin-1 immunocomplex. It is conceivable that a perturbance in the structure of axonin-1 in the complexed form by the anti–axonin-1 antibody results in a destabilization of the axonin-1/NgCAM complex.

The Activities of the Protein Kinases Associated with Axonin-1 and NgCAM Change During In Vitro Differentiation

The axonin-1– and NgCAM-associated protein kinases were studied during neuronal differentiation. As a model system, we used DRG neurons in vitro, which develop extensive neurite fasciculation as the most prominent process of differentiation with time in culture. Several reports have demonstrated that IgFnIII molecules, including axonin-1 and NgCAM, play a pivotal role in the process of neurite fasciculation (Chang et al., 1987; Rathjen et al., 1987, 1991; Ruegg et al., 1989; Honig and Kueter, 1995; Stoeckli and Landmesser, 1995).

Dissociated DRG neurons were cultured on laminin for different times and the cell morphology was examined by light and scanning electron microscopy. After 10–15 h in culture, extensive neurite outgrowth on the substratum was observed, but contacts between neurites or between neurites and cells were rare (Fig. 6, *a* and *b*). Correspondingly, mainly single neurites were found by scanning electron microscopy (Fig. 6 *c*). After 30 h in culture, extensive cell–cell contacts, mainly between neurites of different cells, were observed (Fig. 6, *d–f*). When cultivation was continued, neurites formed fascicles. After 45 and 60 h in culture, thick fascicles with which the membranes of adjacent neurites were in close contact were frequent (Fig. 6, *g–i* and *k–m*). To quantify the extent of fasciculation, we determined the percentage of single neurites in cross sections of the cultures. We found that single neurites decreased from 94% after 15 h in culture to 38% (after 30 h), 14% (after 45 h), and 11% (after 60 h).

Axonin-1 was immunoprecipitated from Triton X-100 extracts of the neuronal cultures, and in vitro kinase assays were performed with the precipitates. The products were subjected to SDS-PAGE. Phosphoproteins were visualized and the incorporated ^{32}P quantified by a phosphoimager (Fig. 7 *A*). Axonin-1 and fyn protein were determined by densitometric quantification of the Western blots (Fig. 7 *B*). The incorporation of ^{32}P into the fyn phosphoprotein was normalized versus the amount of axonin-1 and plotted against time in culture (Fig. 7 *C*). During the initial phase of neurite outgrowth, when the neurons made few contacts, a high fyn activity was associated with axonin-1. Increased neurite fasciculation was accompanied by a reduc-

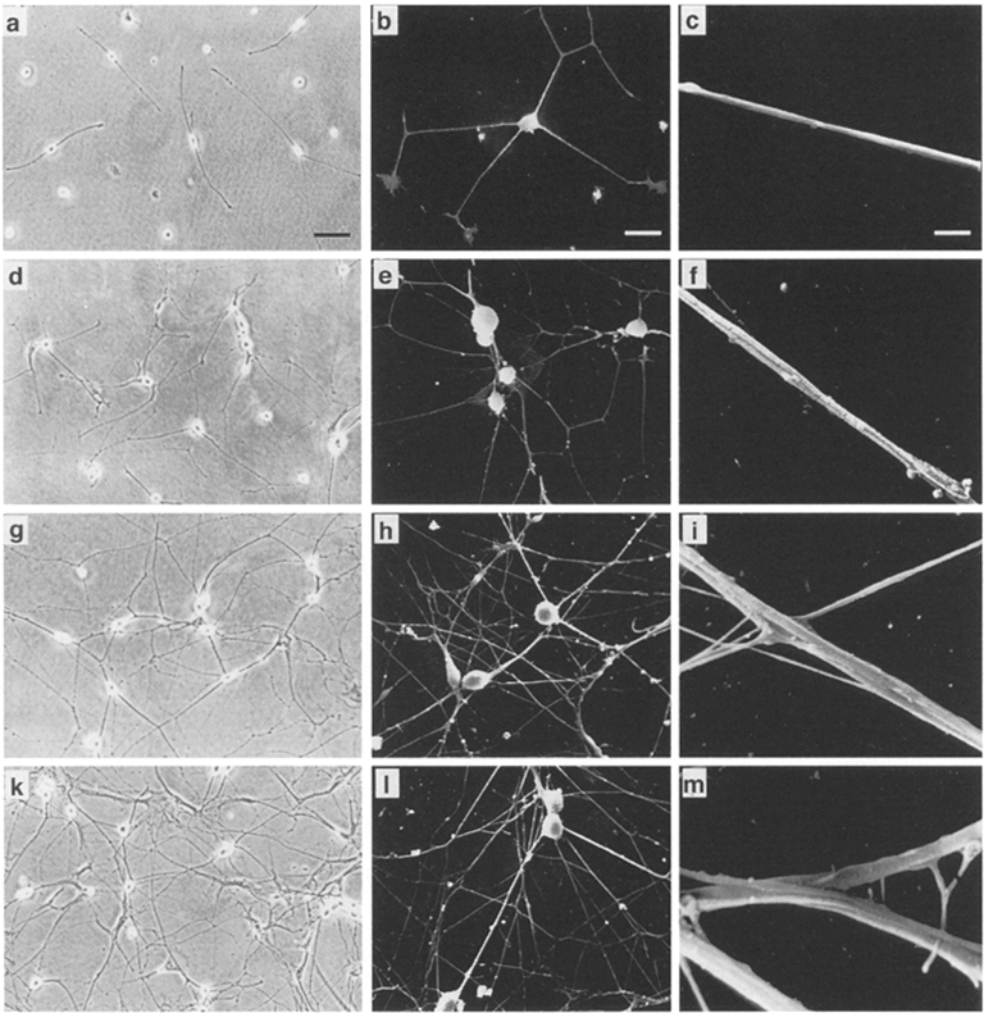

Figure 6. Morphology of dissociated chicken DRG neurons depending on time in culture. Dissociated chicken DRG neurons were cultivated in serum-free, defined medium for 15 h (*a–c*), 30 h (*d–f*), 45 h (*g–i*), and 60 h (*k–m*). (*a*, *d*, *g*, and *k*) Phase contrast; (*b*, *e*, *h*, and *l*) and (*c*, *f*, *i*, and *m*) scanning electron microscopy. Bars: (*a*, *d*, *g*, and *k*) 50 μm; (*b*, *e*, *h*, and *l*) 25 μm; (*c*, *f*, *i*, and *m*) 5 μm.

tion in the activity of the axonin-1–associated fyn kinase. The fyn/axonin-1 ratio at the protein level in the the axonin-1 immunocomplexes was reduced correspondingly (Fig. 7, *B* and *D*). Thus, the observed reduction of the axonin-1–associated fyn activity was mainly due to a reduction of fyn protein. In contrast to axonin-1–associated fyn, the amount and activity of total fyn in the Triton X-100–soluble fraction of total cellular protein increased with time in culture (Fig. 7 *E* and *F*). Therefore, the reduction of axonin-1–associated fyn with time in culture can not be attributed to a depletion of fyn in the cells.

Likewise, the kinase activities associated with NgCAM were investigated at the different stages of neurite outgrowth and fasciculation. NgCAM immunoprecipitates obtained from cells cultivated for the same time periods were subjected to in vitro kinase assays. The kinase reaction was carried out in the presence and in the absence of 100 μg/ml heparin, a concentration found to be sufficient for a complete inhibition of the heparin-sensitive NgCAM-associated kinase (Fig. 8, *A* and *B*). The ^{32}P labeling of the 80-kD fragment of NgCAM was normalized to the amount of NgCAM protein in the immunoprecipitates as determined on the Western blot (Fig. 8 *C*). In the absence of heparin, phosphorylation of NgCAM increased with ongoing fasciculation. In contrast, the phosphorylation of NgCAM by the heparin-insensitive S6 kinase-related activity remained unchanged with time in culture. The increased phosphorylation of NgCAM was, therefore, attributed to the heparin-sensitive casein kinase II–related activity.

In Vitro Differentiation Is Accompanied by Formation of Higher Molecular Mass Complexes of Axonin-1 and NgCAM

The question of whether the interactive pattern of axonin-1 undergoes changes during the formation of cellular contacts was addressed with chemical cross-linking performed on intact neurons in different stages of neurite outgrowth and fasciculation. DST, a hydrophilic reagent with a short spacer (0.64 nm) between the reactive *N*-hydroxysuccinimidyl ester groups, was chosen to restrict the chemical cross-linking to the extracellular moieties of proteins that are in close contact with axonin-1. Immunochemical visualization of axonin-1 on Western blots revealed a characteristic pattern of chemically cross-linked complexes of axonin-1 depending on the stage of fasciculation (Fig. 9). In the unfasciculated culture (after 15 h), cross-linking revealed a new component containing axonin-1 with an apparent molecular mass of 260 kD (Fig. 9 *A*). In the cultures

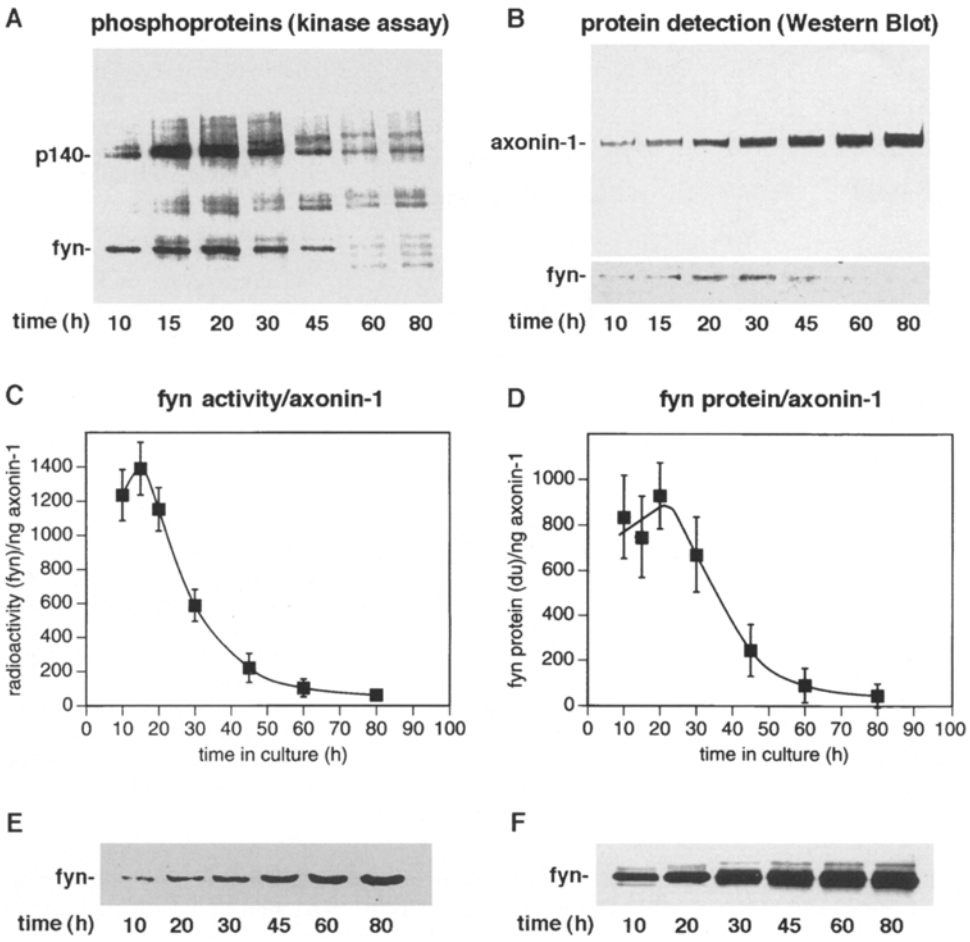

Figure 7. Dependence of the composition of the axonin-1 immunoprecipitates on time in culture. (*A*) The activity of fyn associated with axonin-1 was reduced with time in culture. Axonin-1 was immunoprecipitated from Triton X-100 lysates of DRG neurons kept in culture for the time periods indicated. The phosphoproteins obtained from in vitro kinase assays with axonin-1 immunocomplexes are represented. Autoradiography was for 10 h. One of three experiments is presented (amounts of axonin-1 not normalized). (*B*) Detection of axonin-1 and fyn in the axonin-1 immunoprecipitates. Axonin-1 and fyn were detected by Western blot analysis. Exposure times were 5 s for axonin-1 (*upper panel*) and 10 min for fyn (*lower panel*, same nitrocellulose). One of three experiments shown. (*C*) A strong reduction of axonin-1–associated fyn activity, relative to axonin-1 protein occurred with time in culture. ^{32}P incorporation in fyn kinase associated with axonin-1 (see *A*) was quantified using a phosphoimager, normalized to axonin-1 protein in the immunocomplexes as determined on Western blot (see *B*), and plotted against time in culture (mean ± SD; $n = 3$). (*D*) The protein ratio fyn/axonin-1 was also reduced with time in culture. Fyn protein was quantified by densitometric analysis of fyn Western blots (*B*), normalized to the amount of axonin-1 on the Western blot, and plotted against time in culture (mean ± SD; $n = 3$). (*E*) Fyn in the Triton X-100 soluble fraction of total cellular protein increased with time in culture. Fyn detected on Western blot in total protein of Triton X-100 lysate from cell cultures kept for the time periods indicated. (*F*) Fyn activity in the Triton X-100 soluble fraction of total cellular protein increased with time in culture. Fyn was immunoprecipitated from Triton X-100 lysates of cells kept in culture for the time indicated and subjected to a kinase assay. Autoradiography for 1 h.

grown for 45 h, exhibiting strong fasciculation, the 260-kD complex of axonin-1 was obtained in considerably higher quantity, and an additional complex with an approximate molecular mass of 500 kD (Fig. 9 *B*) appeared. The cross-linked complexes were immunochemically analyzed for the presence of NgCAM, F11, and NrCAM by consecutive immunodetection on the same nitrocellulose blot. The nitrocellulose was subsequently reprobed with anti–axonin-1 to rule out a degradation of the protein during the detection procedure. In both the 260 and 500-kD complex containing axonin-1, NgCAM was also found. Neither F11 nor NrCAM were detected (Fig. 9, *A* and *B*). At the end of the procedure, the signal intensity for axonin-1 corresponded to that of the initial axonin-1 detection. The NgCAM and the axonin-1 signals of the cross-linked complexes were precisely colocalized on the gels. Assuming a comparable sensitivity of the detection assays for axonin-1, F11, NgCAM, and NrCAM, these results indicate that in these neurons axonin-1 is exclusively cross-linked with NgCAM. The complex of 260 kD, therefore, represents a heterodimer consisting of axonin-1 and the 140-kD fragment of NgCAM. The cross-linked species of ~500 kD, which was detectable only in cultures with fasciculated neurites, corresponds to a complex of higher molecular weight containing axonin-1 and NgCAM.

Discussion

We have found that the interacting neural cell adhesion molecules axonin-1 and NgCAM are associated with distinct protein kinases. The glycosyl phosphatidylinositol (GPI)-anchored axonin-1 was demonstrated to be in a specific association with the src-related nonreceptor tyrosine kinase fyn. NgCAM was associated with two serine/threonine kinases, a kinase related to casein kinase II and a kinase related to S6 kinase. When neurites fasciculated by the formation of extensive cell–cell contacts, heterooligomeric complexes containing axonin-1 and NgCAM were formed. Concomitant with these processes, marked changes in the axonin-1– and NgCAM-associated kinases

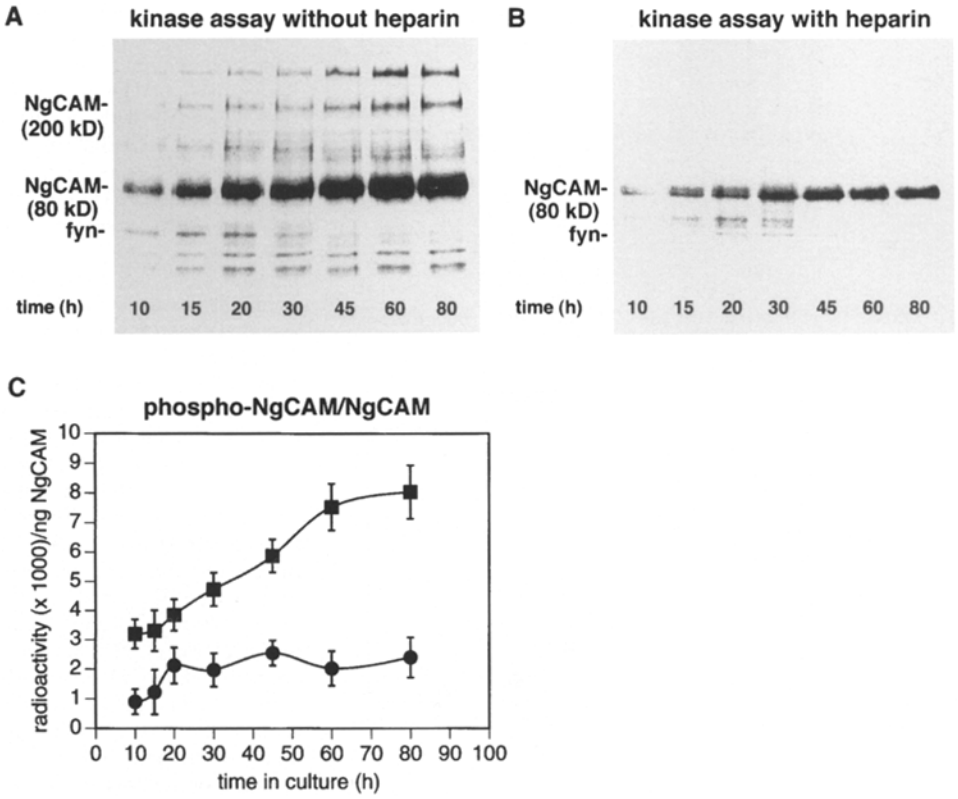

Figure 8. Dependence of the composition of the NgCAM immunoprecipitates on time in culture. (*A*) In vitro phosphorylation of NgCAM by its associated protein kinases increased with time in culture. Phosphoproteins of in vitro kinase assays with NgCAM immunoprecipitates of Triton X-100 lysates of cells kept in culture for the indicated time periods. The ATP concentration was 50 nM. Autoradiography was for 48 h. (*B*) Same as A, but in presence of 100 μg/ml heparin. (*C*) Total phosphorylation of NgCAM increased with time in culture, whereas NgCAM phosphorylation by the S6 kinase–related activity remained constant. Incorporation of [^{32}P] into NgCAM (see *A* and *B*) was quantified using a phosphoimager, normalized to the amount of NgCAM in the immunoprecipitates as determined on the Western blot, and plotted against time in culture (*Squares*, data from Fig. 7 *A*; *circles*, data from Fig. 7 *B*; mean ± SD, $n = 3$).

were observed. While the axonin-1–associated fyn kinase virtually disappeared, an increase of the NgCAM-associated casein kinase II–related activity was observed.

The Nonreceptor Tyrosine Kinase fyn Is Associated with Axonin-1 in DRG Neurons

Associations of src family kinases with GPI-anchored proteins have first been reported to be involved in cell recognition processes in the immune system (Robinson, 1991; Stefanova et al., 1991; Thomas and Samuelson, 1992; Draberova and Draber, 1993). The observation that src family kinases exhibit an expression pattern on developing axonal tracts resembling that of cell adhesion molecules throughout the nervous system has prompted research resulting in the identification of src family kinases, such as src and fyn, to be proximal components in neural cell adhesion molecule signaling (Maness et al., 1988; Ingraham et al., 1992; Bare et al., 1993; Bixby and Jhabvala, 1993; Zhao et al., 1993; Zisch et al., 1995). GPI-anchored proteins restricted to the outer leaflet of the cell membrane have been speculated to interact with src kinases that are linked to the cytoplasmic site by myristoic acid in membrane areas characterized by a particularly high content of glycolipids (Shenoy-Scaria et al., 1993; Rodgers et al., 1994). The selective instability of complexes between GPI-anchored proteins and src kinases in presence of β-octyl glucoside, a nonionic detergent structurally resembling glycolipids, supports this hypothesis. The axonin-1/fyn complex proved to be resistant to Triton X-100 but was unstable in β-octyl glucoside. Thus, the association between axonin-1 and fyn exhibits a detergent resistance pattern previously described as typical for associations between GPI-linked surface proteins and src family kinases (Thomas and Samuelson, 1992; Shenoy-Scaria et al., 1993). As an alternative to a binding involving mainly lipid–lipid interactions, as of yet, unidentified transmembrane proteins that act as docking elements between the extracellular GPI proteins and the cytoplasmic src kinases have been discussed (Casey, 1995). The transmembrane proteins NgCAM and NrCAM, previously identified as binding partners for axonin-1 (Kuhn et al., 1991; Suter et al., 1995) were ruled out as docking proteins because none of them was detected in the axonin-1/fyn complex. However, the in vitro kinase assay revealed a phosphoprotein of 140 kD (p140) in the axonin-1/fyn complex, which was found to be phosphorylated proportional to the activity of fyn in the complex. Because fyn has recently been identified as an essential component of the NCAM-signaling pathway in DRG neurons (Beggs et al., 1994), we considered that p140 could be identical with the 140-kD isoform of NCAM. However, neither the 140-kD nor other isoforms of NCAM were found in the axonin-1/fyn complex. An additional candidate for the phosphoprotein p140, the focal adhesion kinase p125 FAK, a tyrosine kinase involved in the formation of focal adhesion complexes on laminin (Schaller and Parsons, 1994; Clark and Brugge, 1995; Miyamoto et al., 1995) was also excluded.

Two Distinct Serine/Threonine Kinases Are Associated with NgCAM in DRG Neurons

In vitro kinase assays revealed a strong serine/threonine phosphorylation of the 80-kD fragment of NgCAM. Two serine/threonine kinases were identified. One resembled casein kinase II, a multifunctional serine/threonine kinase

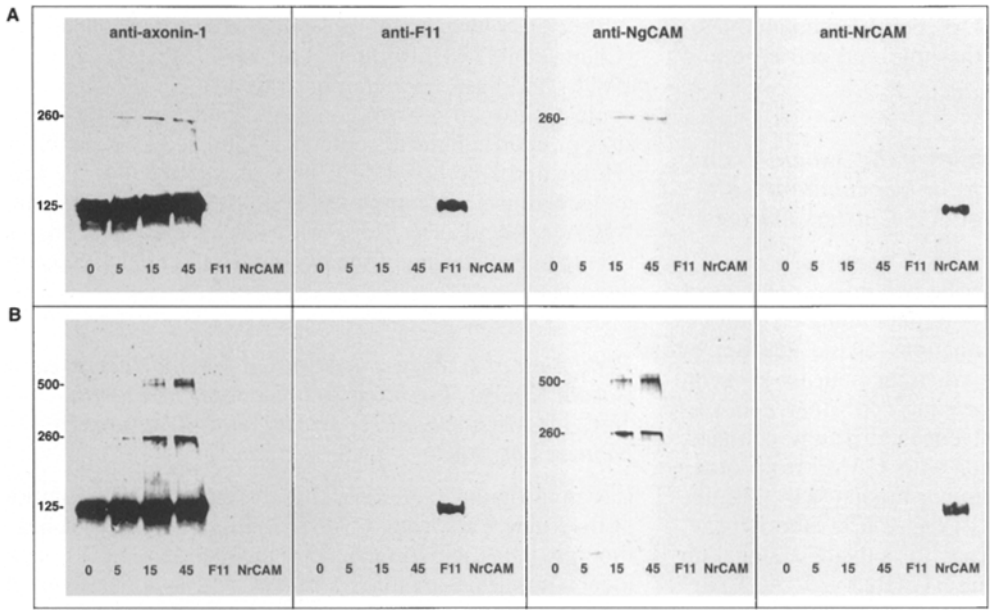

Figure 9. Chemical cross-linking on intact DRG neurons in different states of neurite outgrowth and fasciculation. Dissociated DRG neurons were cultivated for 15 h (*A*) and 45 h (*B*) on laminin/poly-D-lysine. Cross-linking was performed with 1 mM DST and the reaction was quenched after 0, 5, 15, and 45 min. Cells were lysed, axonin-1 immunoprecipitated and the isolated protein subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was probed serially with anti-axonin-1, anti-F11, anti-NgCAM, and an anti-NrCAM antibodies using enhanced chemiluminescence for detection. The location of axonin-1, F11, NgCAM, NrCAM, and the cross-linked complexes of 260 and 500 kD are indicated.

that can phosphorylate a variety of substrates (Hathaway and Traugh, 1982, 1983; Tuazon and Traugh, 1991). A second, heparin-insensitive kinase activity phosphorylated NgCAM but not casein. This activity was inhibited by the peptide RRRLSSLRA, a substrate for S6 kinase and protein kinase C (Pelech et al., 1986). No increase in phosphorylation of NgCAM was detected neither after addition of cAMP or cGMP, nor with Ca^{2+} in combination with either calmodulin or activators of protein kinase C. Histone H1, which was described as a good substrate for protein kinase A (Roskosi et al., 1983) and protein kinase C (Go et al., 1987), was phosphorylated only very weakly by the NgCAM-associated kinases. Based on these results protein kinase A, Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C were excluded as candidates for the NgCAM-associated kinases. Because of the specific inhibition by the substrate peptide for S6 kinase, the heparin-insensitive serine/threonine phosphorylation of NgCAM was ascribed to a S6 kinase-related activity. These results are in line with studies on the phosphorylation of L1, the putative mouse homologue of NgCAM, where casein kinase II and S6 kinase had been found involved (Sadoul et al., 1989; Wong et al., 1996*a*,*b*).

The coprecipitation of fyn and axonin-1 with NgCAM from DRG neurons suggests an interaction of NgCAM with the axonin-1/fyn complex. The association of fyn with NgCAM exhibited a detergent resistance profile very similar to that of the axonin-1/fyn complex. The coprecipitation of fyn with NgCAM depended on the presence of axonin-1, since the fyn protein was markedly decreased in NgCAM precipitates from axonin-1–reduced cells. Based on these results, we concluded that fyn was not directly associated with NgCAM but via axonin-1. The nonreceptor tyrosine kinase src has been implicated as a component of the signaling pathway of L1, the putative mouse homologue of NgCAM (Ignelzi et al., 1994). Cultured cerebellar neurons from src−/src−, but not fyn−/fyn−, mice were strongly impaired in their ability to extend neurites on L1. In our system, src was not found to coprecipitate with NgCAM, indicating the absence of a stable association between NgCAM and src in DRG neurons. Possibly, L1-related molecules undergo distinct associations with protein kinases of the src family in different neuronal cell types.

Doherty and coworkers have shown that neurite outgrowth mediated by L1 depends on Ca^{2+} influx (Williams et al., 1992) as well as tyrosine phosphorylation by a kinase sensitive to inhibition by erbstatin analogues (Williams et al., 1994*a*). It has been demonstrated that calcium influx induced by T cell receptor activation is compromised in fyn−/fyn− lymphocytes (Appleby et al., 1992; Stein et al., 1992), indicating that fyn can regulate calcium channels in these cells. In analogy, the association of NgCAM with the axonin-1/fyn complex during neurite outgrowth on NgCAM could result in fyn-mediated calcium influx. If so, axonin-1 would function as a costimulator of NgCAM. Recent results demonstrating that DRG neurons require axonin-1 for NgCAM-mediated neurite growth (Stoeckli et al., 1996) support this speculation.

In Doherty's system, the FGF receptor, a transmembrane receptor tyrosine kinase, has been identified as a proximal signal activator in L1-mediated neurite outgrowth (Williams et al., 1994*b*). Structural characteristics suggest a direct interaction between L1 (and other CAMs) and the extracellular moiety of the FGF rceptor. In several nonneuronal cell types, the FGF receptor FGFR-1 was implicated in the regulation of the src family kinases src, fyn, and yes (Landgren et al., 1995). However, it is presently not known whether nonreceptor tyrosine kinases, like fyn and src, are also elements of the neuronal signaling cascades involving the FGF receptor. In consideration of the

increasing number of observations of cross-talk between different signaling pathways, it is conceivable that different proximal signals eventually activate neurite outgrowth by converging into a common pathway. Then, different proximal signals would be used to elicit L1-mediated neurite outgrowth depending on the neuronal cell type and state of differentiation.

Opposite Changes of the Activities of the Axonin-1- and NgCAM-associated Kinases Occur Concomitant with the Formation of Axonin-1/NgCAM Clusters During In Vitro Differentiation

The results presented here describe two different states of signaling for axonin-1 and NgCAM, depending on their involvement in the interactive functions of the neurite. In the early period of the cultures, when the neurites grow on a laminin substratum without contacts to other neurites, axonin-1 and NgCAM are not engaged in any contacts, neither with the substratum nor with CAMs from other cells. Neurite extension on laminin is mediated by β1-integrin receptors (Tomaselli et al., 1986; Clarke and Brugge, 1995), which have been reported to activate a signaling pathway involving protein kinase C (Bixby, 1989; Bixby and Jhabvala, 1990). The cytoplasmic domains of β1 integrins involved in laminin binding were demonstrated to couple with cytoplasmic proteins that nucleate the formation of large protein complexes containing both cytoskeletal and catalytic signaling proteins (Clark and Brugge, 1995; Miyamoto et al., 1995). However, neurite outgrowth on laminin was demonstrated to be independent of axonin-1 or NgCAM (Kuhn et al., 1991), and the distribution of axonin-1 and NgCAM in the neuronal membrane was found to be homogeneous when neurites extended on laminin substratum (Stoeckli et al., 1996). Thus, an involvement of axonin-1 and NgCAM in laminin-mediated neurite ougrowth appears rather unlikely. On laminin and in the absence of cell–cell contacts, axonin-1 and NgCAM are predominantly monomeric. In this state, the axonin-1–associated fyn activity is at its peak, whereas the NgCAM-associated casein kinase II–related activity is at its lowest level. Some heterodimeric complexes of axonin-1 and NgCAM observed at this point may reflect a tendency of axonin-1 and NgCAM to bind each other in the plane of the same membrane without being involved in cell–cell interactions.

In the course of in vitro differentiation, extensive contacts between neurites of different cells become established and the formation of fascicles results. In these cultures, axonin-1 and NgCAM were found in complexes with an apparent molecular mass of 500 kD. Concomitantly, the amount and the activity of axonin-1–associated fyn decreased. In contrast, the NgCAM-associated casein kinase II–related activity increased during the same period. Because axonin-1 and NgCAM formed heterodimeric complexes of 260 kD already in the membrane of single neurons and in consideration of the well established homophilic binding capabilities of both NgCAM and axonin-1 exposed on the surface of distinct cells (Lemmon et al., 1992; Rader et al., 1993), it is most likely that the 500-kD complex is a tetramer composed of axonin-1 and NgCAM. This assumption is supported by the reported observation that neuronal CAMs with homophilic binding capacity tend to accumulate at sites of cell–cell contacts (Bloch, 1992; Kobayashi et al., 1992; Shiga et al., 1993; Woo et al., 1993). Axonin-1 and NgCAM belong to this group of molecules. They have been implicated in neurite fasciculation (Chang et al., 1987; Rathjen et al., 1987, 1991; Ruegg et al., 1989*b*) and have been demonstrated to cluster at sites of contact between growth cones and neurites as well as at sites of neurite/neurite contacts in cultured DRG neurons (Honig and Kuether, 1995). Thus, it is likely that the high molecular weight complexes composed of axonin-1 and NgCAM found in cultures with fasciculated neurites reflect the coclustering of the two molecules at cell–cell contact sites.

Reduction of Axonin-1–associated fyn and Increased Casein Kinase II–related Activity Associated with NgCAM Might Stabilize Cytoskeletal Structures in Neurite Fascicles

It is an intriguing possibility that the differential regulation of the kinases associated with axonin-1 and NgCAM has a function in fasciculation. Several independent pieces of evidence implicate axonin-1 and NgCAM as key players in neurite fasciculation. Antibody pertubation experiments indicated that the formation of contacts between neurite membranes during fasciculation of cultured DRG neurons involves NgCAM and axonin-1 (Rathjen et al., 1987, 1991; Ruegg et al., 1989*b*). Immunocytochemical analysis revealed that axonin-1 is concentrated in axon fascicles at sites of close contacts of the axonal membranes (Honig and Kuether, 1995). Furthermore, an interaction between axonin-1 and NgCAM was found to be crucial for the fasciculation of commissural neurites in vivo (Stoeckli and Landmesser, 1995). Thus, the reciprocal changes in the activities of the protein kinases associated with axonin-1 and NgCAM during neurite outgrowth and fasciculation could reflect a change in the signal transduction due to an altered interactive pattern of axonin-1 and NgCAM at newly formed cell–cell contact sites.

The changed activities of the kinases associated with axonin-1 and NgCAM concurring with the altered interaction patterns of the two molecules could be achieved by diverse mechanisms. A simple competition of fyn and NgCAM for binding sites on axonin-1 appears to be unlikely because of the membrane disposition of the molecules. One might speculate that specific interactions with the cytoplasmic domains of proteins included in these aggregates could dissociate fyn from axonin-1 via conformational changes. Alternatively, clustering of axonin-1 and NgCAM could result in a deactivation of fyn by protein kinases or phosphatases associated with NgCAM and the subsequent dissociation of fyn.

The observed change from fyn tyrosine kinase activity to the casein kinase II–related serine/threonine kinase activity during in vitro differentiation might play a role in the organization of cytoskeletal structures implicated in the establishment of the interactions between axonal surface molecules and the cytoskeleton. The decreased activity of the axonin-1–associated fyn kinase could result in a reduced tyrosine phosphorylation of cytoskeletal proteins subjacent to the axonin-1/NgCAM clusters at cell–cell

contact sites. The cytoskeletal components would thus acquire the phosphorylation patterns required for the consolidation of stable structures. Interestingly, throughout the developing brain a high expression of fyn was found restricted to developing axonal tracts exhibiting neurite outgrowth activity, and a characteristically low abundance was discovered in the consolidated structures characteristic of later stages (Bare et al., 1993). Furthermore, tyrosine phosphorylation of tubulin and other proteins enriched in nerve growth cones and neuritic shafts was inhibited by mimicking cell–cell interactions by the addition of purified extracellular domains of L1 and NCAM, as well as with specific antibodies reacting with L1 and NCAM (Atashi et al., 1992). In the case of L1, cross-linking on the cell surface was shown to be essential for the observed reduction of tyrosine phosphorylation. Due to their reported roles in organizing the cytoskeleton, src and fyn are candidates for kinases mediating these effects (Thomas et al., 1995). The increased activity of the NgCAM-associated casein kinase II–related kinase with time in culture could reflect a stabilization of cytoskeletal structures. Casein kinase II in developing neurons has been implicated in the promotion of neurite outgrowth by the phosphorylation of several cytoskeletal proteins including microtubular-associated protein MAP1B (Diaz-Nido et al., 1988, 1990*a*, 1992; Serrano et al., 1989; Crute and Van Buskirk, 1992) and the neuron-specific βIII tubulin (Serrano et al., 1987; Diaz-Nido et al., 1990*a*,*b*). Phosphorylation of MAP1B by casein kinase II was reported to be required for its efficient association with microtubules (Ulloa et al., 1993), which seems to be essential for the consolidation of neurite structures (Matus, 1991; Brugg et al., 1993). The increased activity of the NgCAM-associated casein kinase II during fasciculation could, therefore, be involved in the stabilization of cytoskeletal structures within fascicles.

A possible function of the NgCAM-associated S6 kinase–related kinase remains to be elucidated. Both forms of S6 kinase, p70 S6 kinase and p90Rsk, were detected in DRG neurons. Previous studies suggested that the p90Rsk enzymes are likely to function as mitogen-activated serine/threonine kinases with broad specificity that phosphorylate an array of substrates overlapping with those phosphorylated by Ca^{2+}/calmodulin kinase II, protein kinase A, and probably protein kinase C (Erikson and Maller, 1988; Chen et al., 1991). The pathway leading to p90Rsk activation involves the stimulation of a receptor tyrosine kinase followed by the sequential activation of Ras, Raf, MAP kinase kinase (MEK), and the MAP kinases erk-1 and erk-2 (Blenis, 1993). Though no direct function of p90Rsk in the nervous system has been demonstrated so far, it was implicated in the NGF-induced neuronal differentiation of PC12 pheochromocytoma cells (Scimeca et al., 1992). In comparison to the Rsk enzymes, the p70 S6 kinase has a narrow substrate specificity (Flotow and Thomas, 1992), and none of the proposed consensus sequences for substrates of p70 S6 kinase are present in the cytoplasmic domain of NgCAM (Burgoon et al., 1991). Because NgCAM phosphorylation by the S6 kinase–related activity remained constant during fasciculation, a function in the stabilization of fascicles is unlikely. However, other functions of L1, such as the promotion of neurite outgrowth may involve this kinase as a proximal signaling component.

Conclusion

The present study demonstrates that axonin-1 and NgCAM are predominantly in monomeric form when neurites grow without detectable cell–cell or cell–substratum contacts involving axonin-1 or NgCAM. In this form of neurite outgrowth, strong fyn kinase activity is associated with axonin-1, whereas NgCAM-associated casein kinase II–related activity is low. During neurite fasciculation, a 500-kD complex containing axonin-1 and NgCAM appears. This complex, which is probably located at the sites of contacts between axonal membranes, presumably represents a tetramer composed of two molecules each of axonin-1 and NgCAM. The appearance of the presumptive tetrameric complexes coincides with a reduction of the axonin-1–associated fyn tyrosine kinase activity and, conversely, an increase in the casein kinase II–related activity phosphorylating NgCAM is observed. Thus, complexes of cell adhesion molecules, such as axonin-1 and NgCAM, could represent functional units with the capability of triggering distinctive intracellular signal pathways depending on their state of aggregation. As a speculation, the observed change of the intracellular signaling might reflect a transition from a permissive function of axonin-1 and NgCAM in neurite outgrowth on laminin to a function in the establishment of stable contacts between axons.

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