Cell Adhesion Molecules NgCAM and Axonin-1 Form Heterodimers in the Neuronal Membrane and Cooperate in Neurite Outgrowth Promotion

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Abstract. The axonal surface glycoproteins neuron–glia cell adhesion molecule (NgCAM) and axonin-1 promote cell–cell adhesion, neurite outgrowth and fasciculation, and are involved in growth cone guidance. A direct binding between NgCAM and axonin-1 has been demonstrated using isolated molecules conjugated to the surface of fluorescent microspheres. By expressing NgCAM and axonin-1 in myeloma cells and performing cell aggregation assays, we found that NgCAM and axonin-1 cannot bind when present on the surface of different cells. In contrast, the cocapping of axonin-1 upon antibody-induced capping of NgCAM on the surface of CV-1 cells coexpressing NgCAM and axonin-1 and the selective chemical cross-linking of the two molecules in low density cultures of dorsal root ganglia neurons indicated a specific and direct binding of axonin-1 and NgCAM in the plane of the same membrane. Suppression of the axonin-1 translation by antisense oligonucleotides prevented neurite outgrowth in dissociated dorsal root ganglia neurons cultured on an NgCAM substratum, indicating that neurite outgrowth on NgCAM substratum requires axonin-1. Based on these and previous results, which implicated NgCAM as the neuronal receptor involved in neurite outgrowth on NgCAM substratum, we concluded that neurite outgrowth on an NgCAM substratum depends on two essential interactions of growth cone NgCAM: a *trans*-interaction with substratum NgCAM and a *cis*-interaction with axonin-1 residing in the same growth cone membrane.

THE function of the nervous system is based on a highly intricate pattern of connections between neurons. To reach distant targets, neurons form axons that elongate along prespecified pathways to the appropriate target area. Cell surface molecules and components of the extracellular matrix serve as cues for directional growth of the growth cone (Dodd and Jessell, 1988; Hynes and Lander, 1992). Sensor molecules on the growth cone that recognize and transduce environmental growth signals include molecules of the Ig superfamily (Rathjen and Jessell, 1991). A well-characterized representative of these is the neuron–glia cell adhesion molecule (NgCAM)[1] (Grumet and Edelman, 1984; Burgoon et al., 1991), which is the presumptive avian homologue of L1 (Rathjen and Schachner, 1984; Moos et al., 1988). NgCAM on one cell binds homophilically to NgCAM on another cell (Grumet and Edelman, 1988). Heterophilic interactions of NgCAM have been reported with the cell adhesion molecules axonin-1 (Kuhn et al., 1991), F11 (Brümmendorf et al., 1993), the proteoglycans 3F8 (Grumet et al., 1993*a*), neurocan (Friedlander et al., 1994), phosphacan (Milev et al., 1994), and the extracellular matrix glycoprotein laminin (Grumet et al., 1993*b*).

The heterophilic interaction between NgCAM and axonin-1 had initially been discovered with isolated NgCAM and axonin-1 conjugated to the surface of fluorescent polystyrene beads (Kuhn et al., 1991). Studies at the single cell level had revealed that NgCAM and axonin-1 are colocalized on growth cones of several neuronal populations including dorsal root ganglia (DRG) neurons (Kuhn et al., 1991; Stoeckli et al., 1996), cutaneous and muscle sensory neurons (Honig and Kueter, 1995), and commissural neurons of the spinal cord (Stoeckli and Landmesser, 1995). Both molecules might therefore have a role as receptors mediating neurite outgrowth (Sonderegger and Rathjen, 1992). Observations with cultivated DRG neurons growing on NgCAM substratum indicated that axonin-1 is required in growth cones for neurite outgrowth (Stoeckli et al., 1996), although NgCAM is generally accepted as the major neuronal receptor involved in the in-

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1. *Abbreviations used in this paper*: DRG, dorsal root ganglia; DST, disuccimidyl tartarate; E, embryonic day; FnIII, fibronectin type III; GPI, glycosylphosphatidylinositol; NgCAM, neuron-glia cell adhesion molecule; NrCAM, NgCAM-related cell adhesion molecule; RT, reverse transcription.

teraction with substratum NgCAM (Lemmon et al., 1989). Moreover, on NgCAM but not on laminin substratum, axonin-1 and NgCAM were redistributed to the substratum-facing membrane of the growth cone. Since a direct interaction of growth cone axonin-1 with substratum NgCAM was not required, an interaction of axonin-1 and NgCAM in the plane of the growth cone membrane (*cis*-interaction) was suggested (Stoeckli et al., 1996).

The studies presented here provide direct evidence for the existence of a *cis*-interaction between NgCAM and axonin-1. Antibody-induced capping of NgCAM on CV-1 cells expressing both molecules resulted in cocapping of axonin-1. Chemical cross-linking experiments with DRG neurons cultivated on a laminin substratum without cell–cell contacts revealed the existence of NgCAM/axonin-1 heterodimers in the neuronal membrane. Conversely, NgCAM and axonin-1 expressed in myeloma cells did not induce mixed aggregates, indicating that a binding between NgCAM and axonin-1 residing in the membranes of different cells (*trans*-interaction) was not formed. Since the suppression of axonin-1 translation via antisense oligonucleotides resulted in an inhibition of neurite outgrowth on NgCAM substratum, we suggest that NgCAM/axonin-1 dimers in the growth cone membrane play an essential role in the mechanism of neurite outgrowth promotion.

Materials and Methods

Proteins, Antibodies, and Reagents

Axonin-1 was purified from the ocular vitreous fluid of embryonic day (E) 14 chicken embryos as described (Ruegg et al., 1989*a*). NgCAM was immunoaffinity purified from detergent-solubilized brain membranes of E14 chicken (Stoeckli et al., 1991), using the mAb 12-I-14E-311, provided by Dr. Fritz G. Rathjen (Max Delbrück-Centrum für Molekulare Medizin, Berlin, Germany). Purified NrCAM was provided by Dr. G. Elisabeth Pollerberg (Max-Planck-Institute for Developmental Biology, Tübingen, Germany). Laminin was purchased from GIBCO BRL (Gaithersburg, MD). Antisera against axonin-1 and NgCAM were raised in rabbits and goats (Ruegg et al., 1989*a*), and Fab fragments were prepared as described previously (Stoeckli et al., 1991). Polyclonal antiserum against F11 was provided by Dr. Fritz G. Rathjen, polyclonal antiserum against NrCAM(Bravo) by Dr. G. Elisabeth Pollerberg, and the mAb 2B3-CA against NrCAM by Dr. William J. Dreyer (California Institute of Technology, Pasadena, CA). Peroxidase-conjugated anti–mouse Fab and goat anti–rabbit IgG, and the secondary antibodies for enhanced chemiluminescence detection, were from Boehringer Mannheim (Mannheim, Germany). Affinity-purified rabbit anti–mouse IgG, FITC-labeled donkey anti–goat IgG, and Texas red–conjugated donkey anti–rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-labeled rabbit anti–goat IgG were from Zymed Laboratories, Inc. (South San Francisco, CA). Disuccinimidyl tartarate (DST) was purchased from Pierce Chemical Co. (Rockford, IL). Dimethylsulfoxide was from Merck (Dietikon, Switzerland). PMSF, pepstatin A, leupeptin, aprotinin, and CHAPS were from Sigma Chemical Co. (St. Louis, MO). Triton X-100, NP-40, and β-octylglucoside were from Fluka (Buchs, Switzerland); DAB was from Serva (Heidelberg, Germany); and CNBr-activated Sepharose 4B from Pharmacia (Dübendorf, Switzerland). Reagents for enhanced chemiluminescence detection were from Boehringer Mannheim.

PCR Cloning and Sequencing of the cDNA of NgCAM

Total RNA was prepared from E14 chicken brain and retina (Han et al., 1987). Poly(A)-rich RNA was isolated by a passage over an oligo(dT)-cellulose column (type 3; Collaborative Research, Inc., Bedford, MA). First-strand cDNA was transcribed with SuperscriptRNaseH$^-$ reverse transcriptase (GIBCO BRL). 20 μg poly(A)-rich RNA was dissolved in 80 μl diethylpyrocarbonate-treated H_2O, containing 5.5 mM methylmercuri(II)hydroxide (Johnson Mathey Alfa Products, Karlsruhe, Germany), and heated for 4 min at 94°C to prevent formation of secondary RNA structures. After cooling on ice, the methylmercurihydroxide was neutralized by addition of 2-mercaptoethanol to a final concentration of 8 mM. Transcription was then performed for 1.5 h at 45°C in 50 mM Tris-HCl, pH 8.3, with 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, 500 μM dATP, dCTP, dGTP, and dTTP, 1 U/μl RNAsin (Promega, Catalys AG, Wallisellen, Switzerland), 1 μM random hexamer primer (Promega) or 100 μg/ml oligo-$dT_{12\text{-}16}$- primer (Promega), and 20 U/μl reverse transcriptase (GIBCO BRL). The cDNA fragments, termed AB1FC-AB16B, AB15F-AB10B, AB9F-AB4B, and AB5F-AB2B, were amplified with PCR using the oligo-(dT)– or random hexamer–primed first-strand cDNA as a template and conditions that favored strand denaturation. A typical reaction mixture contained 0.5 U/ml AmpliTaq DNA Polymerase (Perkin-Elmer Corp., Norwalk, CT), 200 μM of dATP, dGTP, dCTP, and dTTP, 1.0 mM of Mg^{2+}, 200 nM of each primer, 1/10 of the transcription pool, and 2% formamide in a vol of 50 μl. In a 35-cycle amplification, denaturation was performed for 1 min at 95°C, annealing for 2 min at 60–70°C (depending on the primer pair), and elongation for 3 min at 72°C.

Primers were synthesized by Microsynth (Balgach, Switzerland). Each PCR fragment was amplified in three parallel reactions from first-strand cDNA originating from three independent RNA pools. The following oligonucleotides were used: AB1FC: 5′-GCTGAAGCTTCCGCC**ATG**-GCTCTGCCCCATG-3′ (HindIII restriction site underlined, start codon bold-typed); AB16B: 5′-GGATCGATCACGGTGACGCTGTGGGT-GCC-3′ (ClaI underlined); AB15F: 5′-CGCCTGCTGCTGCCTCGAG ACC-3′ (XhoI underlined); AB10B: 5′-GAAGGATCGATCGTCCTG-CAGAGC-3′ (ClaI underlined); AB9F: 5′-CTCTGCAGGACGATC-GATCCTTCG-3′ (ClaI underlined); AB4B: GGAAGCTTACTTAC-CTATTGTCGACGACTGCGCTCCCCCAC (SalI underlined); AB5F: TGGGGGAGCGCAGTCGTCGACCAAGC (SalI underlined); AB2B: CCAAGCTT*ACTTAC***CTA**ATCCAGGGGGGGCCCAGCAC (HindIII underlined, termination codon bold-typed, splice donor consensus sequence in italics).

To generate single-strand DNA, each PCR fragment was subcloned separately into the pBluescript vector (Stratagene, La Jolla, CA). Each fragment was sequenced with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH) using the dideoxy chain-termination method (Sanger et al., 1977). For sequence analysis we used GCG (version 7, Unix; Silicon Graphics Inc., Schlieren, Switzerland).

Heterologous Expression of NgCAM by Myeloma Cells and Analysis of Myeloma Cell Aggregation

To express NgCAM on the surface of nonadherent cells, we used a myeloma-based expression system that has been applied successfully to the production of membrane-bound axonin-1 (Rader et al., 1993). As we have reported previously, these cells do not express detectable quantities of L1 and TAG-1, the presumptive mammalian homologues of NgCAM and axonin-1. A full-length cDNA clone of NgCAM was constructed with (*a*) the 986-bp AB1FC-AB16B fragment using the primer-derived HindIII and an internal XmaI site at position 845; (*b*) the 678-bp AB15F-AB10B fragment using the same XmaI and a backward primer-derived silent ClaI site at position 1403; (*c*) the 1,188-bp AB9F-AB4B fragment with the same silent ClaI and a silent SalI site at position 2591 in the backward primer; and (*d*) the 1,260-bp AB5F-AB2B fragment by means of the SalI silent site and a HindIII site on AB2B at the very 3′ end of the molecule (see Fig. 1). The full-length cDNA was then digested with HindIII and cloned into the vector pCD4-FvCD3-c_k (Traunecker et al., 1991; Rader et al., 1993) to generate the vector pNgCAM for NgCAM expression in mouse myeloma cells. Stable transfectants were obtained by protoplast fusion and selection with L-histidinol. After 10 d, the cells were screened for surface NgCAM by indirect immunofluorescence. Colonies with a high NgCAM expression were expanded and subcloned.

To analyze the aggregation of NgCAM- and axonin-1–expressing myeloma cells, fluorescent intracellular dyes were used. Myeloma cells were incubated in PBS with either 1 mM 2′,7′-bis-(2-carboxyethyl)-5-(and 6-) carboxyfluorescein acetoxymethyl ester (B-1150; Molecular Probes, Eugene, OR) or 7.5 mM 5-(and 6-) carboxynaphtofluorescein diacetate (C-654; Molecular Probes) for 30 min at 37°C. Differently stained cells were mixed, dissociated by repeated pipetting through a 22-gauge cannula, incubated for 1 h at 37°C, and then examined with a fluorescence microscope (Leitz DMRXE; Leica AG, Glattbrugg, Switzerland) using a combined FITC/TRITC filter. For controls, the myeloma cells were preincubated for 1 h at 4°C with 250 μg/ml goat anti–NgCAM Fab.

Transient Transfection of COS-7 Cells by Electroporation and Analysis of Covaspheres Binding

For expression of NgCAM and axonin-1 in CV-1(CCL-70; American Type Culture Collection, Rockville, MD) and COS-7 (CRL-1651; American Type Cultutre Collection) cells, full-length cDNAs of NgCAM and axonin-1 (Zuellig et al., 1992) were cloned into the vector pSCT-1 (Rusconi et al., 1990) to generate the vectors pSCT-NgCAM and pSCT-axonin-1 (Rader et al., 1996), respectively. Transient transfectants were obtained by electroporation.

Binding assays were performed 2 d after transfection (Rader et al., 1996). TRITC-labeled fluorescent polystyrene microspheres (Covaspheres, nominal diam 0.5 μm; Duke Scientific Corp., Palo Alto, CA) were conjugated with NgCAM, axonin-1, and recombinant axonin-1, as previously described (Kuhn et al., 1991). The cells were incubated with NgCAM- or axonin-1–conjugated Covaspheres for 1 h at 37°C, washed twice with DME containing 5 mg/ml BSA, and fixed in 2% formaldehyde (Fluka) for 1 h at 37°C. NgCAM- or axonin-1–expressing cells were visualized by indirect immunofluorescence using goat antisera against NgCAM or axonin-1, respectively, and FITC-conjugated rabbit anti–goat IgG. For quantification, NgCAM- or axonin-1–expressing cells that bound Covaspheres fivefold above background were scored as binding cells. A total of 100 NgCAM- or axonin-1–expressing cells were counted. Background bindings by nonexpressing cells were 2 for NgCAM– and 1 for axonin-1–Covaspheres.

For antibody perturbation of Covaspheres binding, cells were preincubated for 2 h at 37°C with 250 μg/ml goat anti–NgCAM or anti–axonin-1 Fab.

Transfection, Selection, and Screening of CV-1 Cells

CV-1 cells were transfected with pSCT-axonin-1 using [*N*-1-(2,3-dioleyloxy)propyl]- *N,N,N,*-trimethylammonium chloride (synthesized by P. Rüedi, Institute of Organic Chemistry, University of Zurich, Switzerland). Axonin-1–expressing clones were selected by incubating the transfected cells with Dynabeads (DYNAL A.S., Oslo, Norway) coated with polyclonal goat anti-axonin–1 antibodies. NgCAM-expressing clones and NgCAM/axonin-1–expressing clones were obtained by cotransfecting wild-type– and axonin-1–expressing CV-1 cells, respectively, with pSCT-NgCAM and pSVneo2. After the selection with the antibiotic G418 (GIBCO BRL), NgCAM–expressing cells were isolated with Dynabeads coated with polyclonal anti-NgCAM antibodies. The transfected cells were then analyzed for NgCAM and/or axonin-1 expression by indirect immunofluorescence.

Neurite Outgrowth Assays on NgCAM-expressing CV-1 Cells

CV-1 cells were cultured in dishes coated with 500 μg/ml poly-D-lysine in DME supplemented with 2.5% FCS and 2.5% newborn calf serum and grown to confluency. DRG were dissected from E10 chick embryos, dissociated by trypsinization and trituration (Sonderegger et al., 1985; Stoeckli et al., 1991). Cultures were enriched for neurons by preplating on plastic for 1 h. The enriched DRG neurons were then plated at low density on the CV-1 cells. The cocultures were maintained in DME supplemented with 100 μg/ml holo-transferrin (human; Sigma Chemical Co.), 20 ng/ml NGF, 10 μg/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 200 ng/ml corticosterone, 200 μM putrescine and 60 nM sodium selenite, 10% horse serum, and 5% chicken embryo extract. Where indicated, the medium contained 250 μg/ml anti-NgCAM or anti–axonin-1 Fab. After 20 h at 37°C, the cells were fixed in 2% formaldehyde, 0.1% glutaraldehyde, 12.5 mM sodium cacodylate, pH 7.2, for 1 h. Neurons were stained with the rabbit anti-F11 serum and Texas red–conjugated donkey anti–rabbit IgG. Neuronal processes longer than two cell diameters were defined as neurites (Venstrom and Reichardt, 1995).

Cocapping Experiments

CV-1 cells stably transfected with axonin-1 and NgCAM were cultivated to a confluency of 25%. Cells were washed in cold PBS and incubated for 30 min at 4°C with 10 μg/ml monoclonal anti-NgCAM (12-I-14-E 311) or anti–axonin-1 (V1F1) antibodies, respectively. To induce capping of NgCAM or axonin-1, the cells were incubated with affinity-purified rabbit anti–mouse IgG (20 μg/ml) for 30 min at 4°C, washed twice in HBSS, and incubated for 30 min at 37°C in DME. Thereafter, the cells were washed with HBSS containing 0.1% sodium azide and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde, 12.5 mM sodium cacodylate, pH 7.2, for 1 h at 4°C. The antibody-induced caps were stained with Texas red–conjugated donkey anti–rabbit IgG (5 μg/ml) in 10% (vol/vol) FCS/PBS for 1 h. In NgCAM-capped cells, axonin-1 was detected using goat anti–axonin-1 Fab (20 μg/ml). In axonin-1–capped cells, NgCAM was detected with goat anti–NgCAM Fab (10 μg/ml). Goat Fab were then detected by FITC-conjugated donkey anti–goat IgG (10 μg/ml). FITC and Texas red fluorescence was detected with a confocal laser scanning microscope (Multi Probe 2001™ CLSM; Molecular Dynamics, Sunnyvale, CA), using an argon/krypton laser and the single scanning mode. For the detection of FITC, the band-pass excitation filter 488DF10 was used in combination with a 510DRLP primary beam splitter and a long-pass barrier filter 510EPLP. For the detection of Texas red, the band-pass excitation filter 568DF10 was used in combination with a 488/568 dual dichroic filter as a primary beam splitter and a 590EFLP long-pass barrier filter.

Chemical Cross-linking

Chemical cross-linking was performed on DRG neurons cultivated in 100-mm culture dishes coated with laminin. The culture, containing 1×10^6 neurons, was washed in PBS and placed on ice. All subsequent steps were performed on ice. The cross-linking reagent DST dissolved in DMSO was added to a final concentration of 1 mM. The reaction was allowed to proceed for 0, 20, and 60 min, and then stopped by the addition of glycine (pH 8.0) to a final concentration of 50 mM. After quenching for 5 min, the cells were lysed for 1 h in 1 ml 50 mM Tris, pH 7.6, containing 1% (wt/vol) CHAPS, 0.1% (wt/vol) SDS, 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 centrifuged at 12 000 *g* for 15 min at 4°C. The supernatant was transferred to a vial containing 5 μl monoclonal anti–axonin-1 antibody coupled to Sepharose 4B (5 μg IgG per μl Sepharose) and incubated at 4°C for 90 min. Immunoprecipitates were washed four times in the lysis buffer, twice in 50 mM Tris (pH 7.6), 0.1% (wt/vol) CHAPS, 0.1% (wt/vol) SDS, and 150 mM NaCl, and once in 50 mM Tris (pH 7.6), 150 mM NaCl. The protein was eluted in reducing buffer and separated by SDS-PAGE using a 5% polyacrylamide gel (Laemmli, 1970).

Western Blotting

For Western blot analysis of total cellular proteins, $\sim 10^6$ transfected myeloma, CV-1, or COS-7 cells, 10^5 DRG neurons, or 250 μg E14 chicken brain protein were washed twice and resuspended in 500 μl ice-cold PBS. The cells were centrifuged at 4°C in an Eppendorf centrifuge at 1,000 rpm, resuspended in 500 μl lysis buffer, and shaken for 1 h at 4°C. Cell debris was removed by centrifugation for 10 min at 4°C with 14,000 rpm in an Eppendorf centrifuge. The proteins of the supernatant were precipitated (Wessel and Fluegge, 1984), resuspended in 50 μl SDS-containing sample buffer, subjected to electrophoresis on a 7.5% acrylamide gel, and then transferred to nitrocellulose. For detection we used: rabbit anti–axonin-1, anti-NrCAM, anti-F11, goat anti–NgCAM, and monoclonal anti-NgCAM antibody (1 μg/ml of purified IgG). Secondary antibodies were 0.2 μg/ml anti–rabbit IgG, 0.4 μg/ml anti–goat Fab, or 0.4 μg/ml anti–mouse Fab fragments, all coupled to peroxidase. The chemiluminescence detection system from Boehringer Mannheim was used according to the manufacturer's recommendation. Exposure times ranged from 5–30 s. For quantification of axonin-1 and NgCAM, 50, 100, 250, 500, 750, and 1,000 pg of purified protein were used as standards. Densitometric analysis was carried out with Image Quant software (version 4.1; Molecular Dynamics).

For sequential immunodetection with different antibodies on the same nitrocellulose, the antibodies bound to the nitrocellulose were removed by incubation in TBS containing 50 mM 2-mercaptoethanol and 1% (wt/vol) SDS for 30 min at 25°C. The membrane was washed four times for 15 min each in TBS, blocked for at least 1 h in 1% blocking reagent, and reprobed with another antibody. This procedure allowed the serial application of up to five different antibodies.

Suppression of Axonin-1 by Antisense Oligonucleotides

The 5′ proximal axonin-1 antisense phosphorothionate oligonucleotide with the sequence 5′-CAGTGCCTCCCATCCTGTGGCTC 3′ spanned from the nucleotides −10 to +12, referring to +1 being the first nucleotide of the translation initiation codon. The 3′ proximal antisense phosphorothionate oligonucleotide including the natural stop codon had the sequence 5′-GCTACCTGGAGCTCTGATGG-3′ (Zuellig et al., 1992).

These oligonucleotides did not match any nucleotide sequence of known genes. After purification by ion exchange chromatography and gel filtration, the oligonucleotides were dissolved in quartz-distilled water in a concentration of 2 mg/ml. Trituration was used to introduce oligonucleotides into DRG neurons (Aigner and Caroni, 1995). Chick DRGs were trypsinized for 15 min at 37°C and subjected to 15–20 cycles of trituration through a yellow tip (Gilson, Synmedic, Zurich, Switzerland) in 50 μl serum-free defined medium containing the oligonucleotides in the concentrations indicated. The cells were pelleted by centrifugation for 2 min at 320 *g*, washed once in serum-free medium, centrifuged again, resuspended, and plated. Within a range of 25–100 μM of axonin-1 antisense oligonucleotide, a dose-dependent reduction of axonin-1 was observed. In a typical reaction, 100 μM of each oligonucleotide were applied. The axonin-1 expression remained reduced for ~24 h, but it was restored thereafter. The administration of sense oligonucleotides resulted in a slight reduction of the measured axonin-1 to 92 ± 6% (mean ± SD, $n = 3$) as compared with the amounts found in untreated cultures. Similar differences between sense oligonucleotide–treated and untreated control cultures were also found for F11 and NgCAM. Therefore, we concluded that the oligonucleotides were not toxic at the concentrations we used. Cell viability was assessed by dye exclusion with trypan blue. Neurons were identified by immunocytochemical detection using the neuronal marker F11.

Neurite lengths were measured 15 h after plating the cells, as described previously in detail (Stoeckli et al., 1991) using an inverted microscope equipped with phase-contrast optics. A neurite was defined as a process extending from the neuronal cell soma by >20 μm. Only neurites that emerged from an isolated neuron (not a clump of cells) and did not make contact with other neurites or cells were measured. The longest of all neurite branches elaborated by a neuron was measured. The percentage of neurons with a neurite longer than a given length was plotted vs neurite length (Chang et al., 1987). As a characteristic for neurite outgrowth under a given condition, the neurite length developed by 50% of the neurite-bearing neurons (NL_{50}) was determined (Stoeckli et al., 1991).

Results

Reverse Transcription (RT)–PCR Reveals NgCAM with a Sequence Different from Published NgCAM

Reverse transcription (RT)–PCR cloning of the cDNA of NgCAM was performed based on three different poly(A)-rich RNA pools prepared from E14 chicken brain and retina. PCR primers were designed according to the published sequence (Burgoon et al., 1991, 1995). PCR fragments were subcloned and then sequenced. Where deviations from the published sequence were found, they were confirmed in at least one additional RT-PCR fragment derived from a different mRNA pool. Reproducible deviations from the published sequence were confirmed by backward sequencing. The sequences of the primer regions were checked by RT-PCR amplification and sequencing of fragments spanning over the primer regions.

The sequence of the coding region of the PCR-cloned NgCAM presented here (Fig. 1) was compared with the published NgCAM sequence (Burgoon et al., 1991, 1995) using the program GAP (Needleman and Wunsch, 1970), which maximizes the number of matches and minimizes the number of gaps in an alignment of two complete sequences. The identity of the nucleotide sequences was 98.5%. Several insertions and deletions of single basepairs at various locations throughout the length of the molecule resulted in frameshifts over extended segments (underlined in Fig. 1). Thus, the identity between PCR-cloned and published NgCAM at the level of the deduced amino acid sequence was 93%. A domain-wise comparison demonstrated that the largest deviations were in the fourth and fifth fibronectin type III (FnIII)–like domains that exhibited an identity of 67.8% and 74.8%, respectively. Tryptophan residues, which are considered characteristic of FnIII domains, but were not present in the published sequence, are located at positions 958 and 1056 of PCR-cloned NgCAM. PCR-cloned NgCAM exhibits an amino acid identity of 39.7% with NgCAM-related cell adhesion molecule (NrCAM) (Grumet et al., 1991; Kayyem et al., 1992), the closest relative of NgCAM in the chicken. The sequence identity between PCR-cloned NgCAM and L1 (Moos et al., 1988), the putative species homologue of the mouse, is 49.3%. Pairwise alignment of colinear domains showed that PCR-cloned NgCAM exhibits a markedly higher sequence similarity to L1 than the published NgCAM sequence in the region of the FnIII-like domains 4 and 5 (data not shown). This region had been previously discussed to be unusually divergent in the published NgCAM sequence from the colinear domains of other members of the L1 subgroup (Brümmendorf and Rathjen, 1993).

The relationship between the PCR-cloned NgCAM presented here and the published NgCAM (Burgoon et al., 1991, 1995) cannot be determined based on the presently available data. The two sequences have been derived from mRNA of outbred chickens, and a detailed analysis of the genomic representation of NgCAM has not been published so far. Several related Ig superfamily cell adhesion molecules have been found to be expressed in multiple splice variants. The most extensive alternative RNA splicing has been reported for NCAM (Cunningham et al., 1987), where variants differing by as little as one codon have been found (Moolenaar et al., 1992) and, based on RNA diversity, as many as 192 possibilites for different proteins have been calculated (Bartels et al., 1992). Intriguingly, multiple splice variants have also been reported for NrCAM(Bravo), the closest relative of NgCAM (Kayyem et al., 1992). We have found evidence for at least one variant form of NgCAM. Amplification of the cytoplasmic domain provided one clone lacking the RSLE sequence. This sequence has been previously reported to be variably expressed also in human L1 (Hlavin et al., 1991; Reid and Hemperly, 1992) and in NrCAM(Bravo) (Kayyem et al., 1992). Further experimental work, e.g., at the genomic or at the mRNA level, will be necessary to clarify the issue of possible additional variants of NgCAM.

NgCAM Expressed by CV-1 Cells Promotes Neurite Outgrowth in the Presence of Anti–Axonin-1 Antibodies

Published results demonstrated that the neurite outgrowth–promoting effect of brain-derived, affinity-purified NgCAM adsorbed to tissue-culture plastic is not affected by the presence of polyclonal Fab against axonin-1 (Kuhn et al., 1991; Stoeckli et al., 1996), indicating that a heterophilic *trans*-interaction between NgCAM substratum and growth cone axonin-1 may not be required for neurite outgrowth. To investigate the neurite outgrowth–promoting activity of NgCAM expressed on the surface of a cell rather than adsorbed to tissue-culture plastic, dissociated DRG neurons were cultivated on a confluent monolayer of CV-1 cells that were stably transfected with a full-length cDNA encoding NgCAM. As revealed by

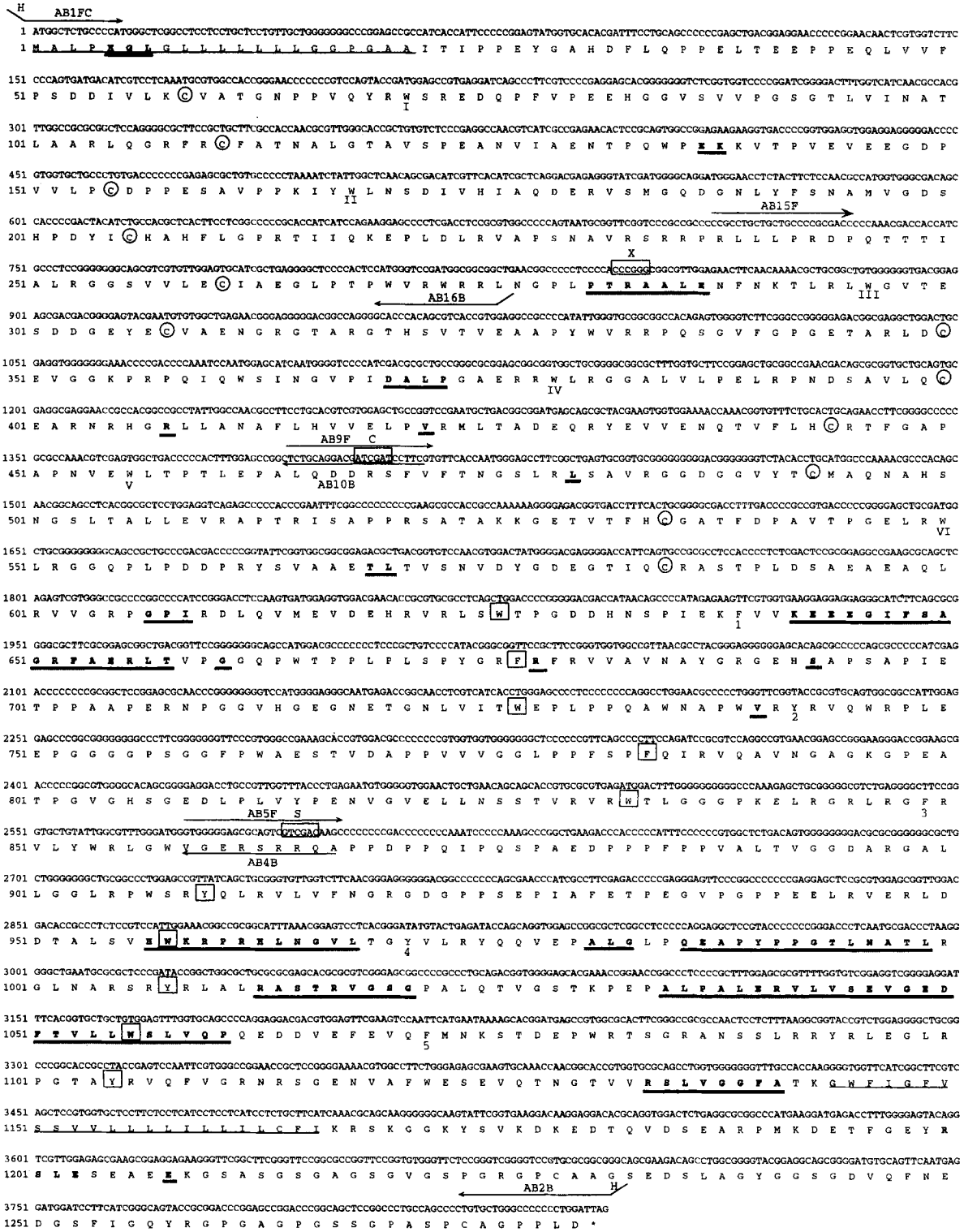


Figure 1. Nucleotide and deduced amino acid sequence of RT-PCR–NgCAM. The open reading frame contains 1,280 amino acids. The restriction sites of the cDNA that were used for cloning are boxed. Restriction sites on PCR primers are indicated only when used for cloning. Abbreviations for restriction sites are: *H*, HindIII; *X*, XmaI; *C*, ClaI; *S*, SalI. The signal peptide (1–21) and the transmembrane region (1,144–1,166) are underlined by a thin bar. The sequences used in the PCR primers are marked by arrows. The Ig-like domains are numbered from I to VI under the conserved tryptophans, and the characteristic cysteines are circled. The FnIII-like domains are numbered 1–5, and the conserved tryptophans, phenylalanines, and tyrosines are boxed. Amino acids that differ from the published sequence (Burgoon et al., 1991, 1995) are marked with bold letters and underlined with a thick bar. Additional conserved tryptophans that were not in the published sequence were found at the amino acid positions 958 and 1056. The RSLE peptide in the cytoplasmic domain (*bold*) was not present in one of the clones found in this study. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z75013.

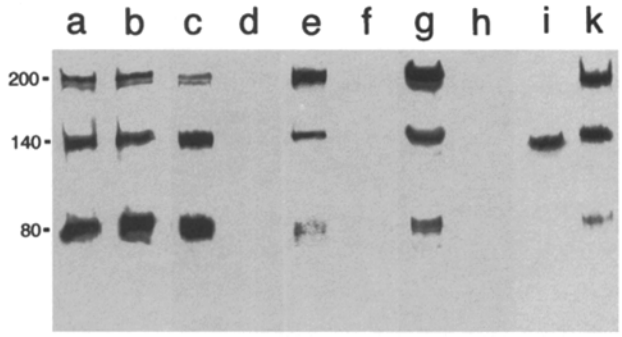


Figure 2. Western blot analysis of recombinant NgCAM originating from expression in different cell lines. Extracts from chicken brain membranes (*a*), chicken DRG neurons (*b*), stably transfected myeloma (*c*), transiently transfected COS-7 (*e*), and stably transfected CV-1 (*g*) cells, as well as from untransfected myeloma (*d*), COS-7 (*f*), and CV-1 (*h*) cells were subjected to Western blotting. NgCAM was visualized using a polyclonal antibody. Each lane contains ~250 μg protein. Untransfected myeloma (*d*), COS-7 (*f*), and CV-1 (*h*) cells do not express NgCAM. Double-transfected CV-1 cells express axonin-1 (*i*) and NgCAM (*k*) at a ratio of ~1:2.

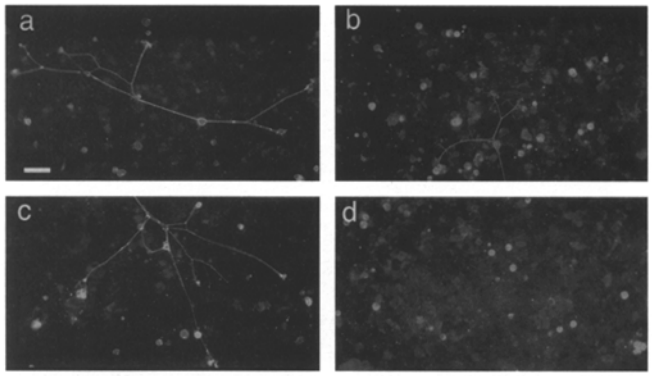


Figure 3. Neurite outgrowth of dorsal root ganglia neurons on NgCAM-expressing CV-1 cells. DRG neurons cultured on CV-1 cells were stained with indirect immunofluorescence using anti-F11 antibodies. NgCAM-expressing CV-1 cells promote neurite outgrowth (*a*). The presence of polyclonal anti-NgCAM Fab strongly reduced neurite outgrowth (*b*), whereas the presence of polyclonal anti–axonin-1 Fab was without effect (*c*). Parental CV-1 cells did not promote neurite outgrowth (*d*). Bar, 50 μm.

Western blot analysis, the transfected cells expressed recombinant NgCAM with the characteristic band pattern (Fig. 2 *g*). 15 h after plating, the cultures were fixed and the neurons were stained for the neuronal marker F11 (Fig. 3). Neurite outgrowth was quantified by counting the neurons with neurites longer than two cell diameters (Venstrom and Reichardt, 1995). NgCAM-transfected cells, but not the parental untransfected CV-1 cells, stimulated neurite outgrowth of DRG neurons (Fig. 3; Table I). Neurite outgrowth was inhibited by anti-NgCAM but not by anti–axonin-1 Fab. The polyclonal anti-NgCAM Fab did not perturb neurite outgrowth on a laminin substratum at concentrations up to 500 μg/ml (Kuhn et al., 1991), and thus we concluded a toxic effect of this preparation for neurites growing on CV-1 cells to be unlikely. The polyclonal anti–axonin-1 Fab had been previously demonstrated to completely prevent the heterophilic binding between NgCAM and axonin-1 conjugated to fluorescent microspheres (Kuhn et al., 1991), as well as the homophilic *trans*-binding of axonin-1 expressed by myeloma cells (Rader et al., 1993). Based on these characteristics, we assumed that in the presence of these antibodies, a heterophilic *trans*-interaction between NgCAM on the CV-1 cells and axonin-1 on the growth cones could not be formed. Thus, the result suggests that recombinant NgCAM expressed on the surface of CV-1 cells stimulates neurite outgrowth without a heterophilic *trans*-interaction with growth cone axonin-1.

Axonin-1– and NgCAM-expressing Myeloma Cells Do Not Form Mixed Aggregates

To test whether NgCAM and axonin-1 could mediate cell aggregation via binding across the intercellular space (*trans*-binding), NgCAM and axonin-1 were stably expressed in myeloma cells, and the NgCAM- and axonin-1–expressing myeloma cells were stained with different fluorescent intracellular dyes and subjected to a cell aggregation assay. Microscopic inspection revealed that only monocolored aggregates were formed (Fig. 4, *i* and *k*). The exclusion of axonin-1–expressing cells from aggregates of NgCAM-expressing cells and the exclusion of NgCAM-expressing cells from aggregates of axonin-1–expressing cells was nearly complete. In >100 aggregates of NgCAM-expressing cells, <10% contained an axonin-1–expressing cell and vice versa. Thus, a heterophilic NgCAM/axonin-1 binding between molecules located on different cells was not formed.

Several independent pieces of evidence confirmed that axonin-1 and NgCAM expressed heterologously in the myeloma cells were structurally intact and functionally competent. Axonin-1–expressing myeloma cells were described in detail previously (Rader et al., 1993). The solubilized membrane fraction of NgCAM-transfected cells contained recombinant NgCAM with the characteristic pattern of bands at 210/190, 140, and 80 kD (Fig. 2 *c*), as described for wild-type NgCAM purified from chicken brain (Fig 2 *a*, Grumet et al., 1984) and chicken DRG neurons (Fig. 2 *b*). This band pattern had been demonstrated to originate from proteolytic cleavage of the molecule in the third FnIII domain, and it is thought to occur in the native conformation of the protein (Burgoon et al., 1991; Grumet, 1992). Indirect immunofluorescence staining demonstrated that recombinant NgCAM is exposed on the surface of the myeloma cells (Fig. 4, *a* and *b*). These Ng-

Table I. CV-1 Cells Expressing NgCAM Promote Neurite Outgrowth from DRG Neurons

Cell	Without Fab	Anti-ax-1	Anti-Ng
CV-1–NgCAM	34 ± 5	28 ± 4	6 ± 3
CV-1 (parental)	2 ± 2	1 ± 1	7 ± 3

DRG neurons were cultured on parental and NgCAM-expressing CV-1 cells in the absence and presence of anti–axonin-1 or anti-NgCAM Fab. After 20 h, the cultures were fixed and stained for F11. In each condition, 100 neurons were inspected. The percentage of neurons with neurites longer than two cell diameters was determined. The numbers given represent mean ± SEM of three independent experiments.

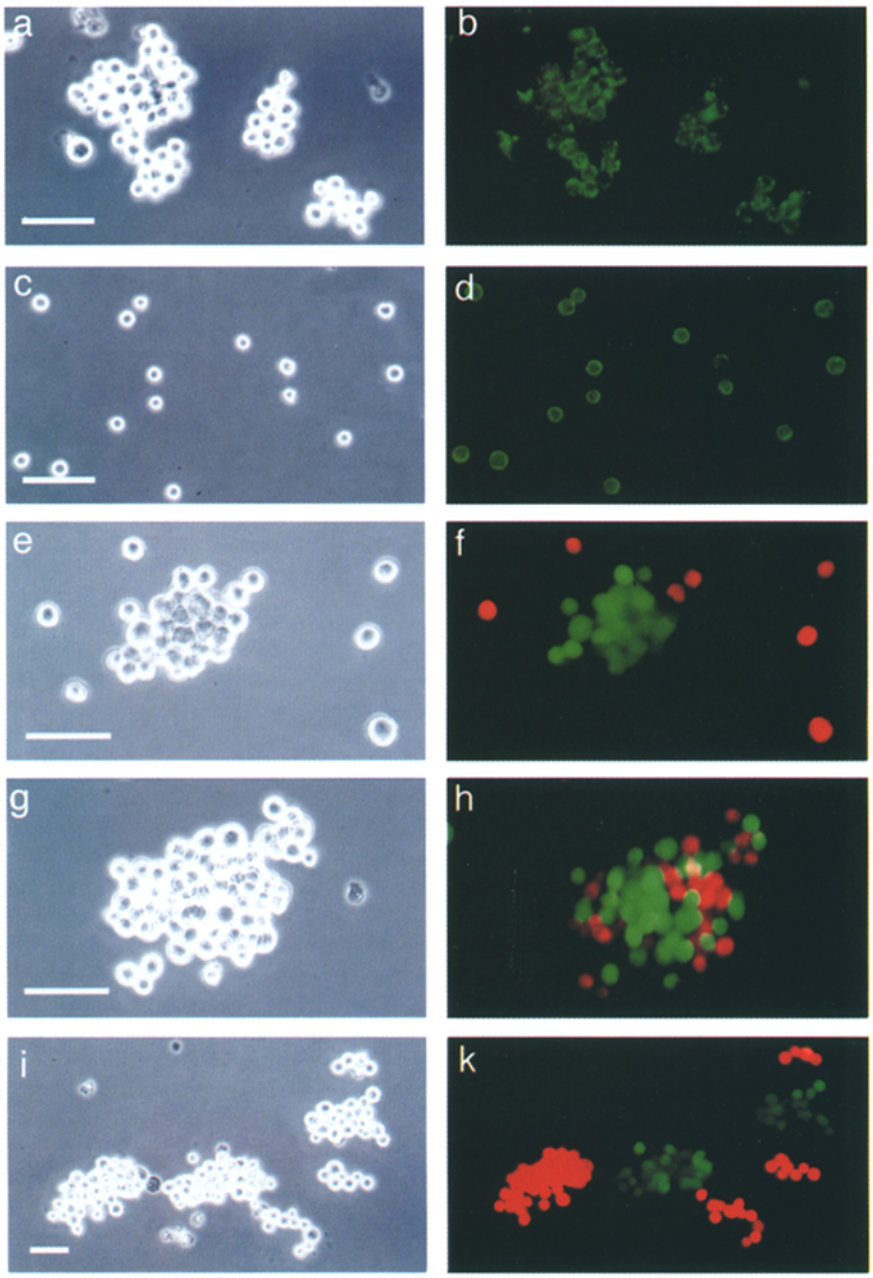


Figure 4. NgCAM- and axonin-1–mediated myeloma cell aggregation. NgCAM-expressing myeloma cells formed large aggregates (*a* and *b*). Indirect immunofluorescence demonstrates that NgCAM is expressed on the cell surface (*b*). Aggregate formation was prevented by preincubation with anti-NgCAM Fab (*c* and *d*). Only NgCAM-expressing cells participated in aggregates. Parental myeloma cells (*red*) were not included in the aggregates of NgCAM-expressing cells (*e* and *f*). NgCAM-expressing myeloma cells marked with red and green fluorescent dyes formed mixed aggregates (*g* and *h*). NgCAM-expressing (*green*) and axonin-1–expressing (*red*) myeloma cells formed monocolored, but not mixed-colored, cell aggregates (*i* and *k*). Phase-contrast (*a*, *c*, *e*, *g*, and *i*) and fluorescence photographs (*b*, *d*, *f*, *h*, and *k*). Bar, 50 μm.

CAM-expressing myeloma cells formed aggregates consisting of up to 50 cells. Aggregation was prevented by incubation with anti-NgCAM Fab (Fig. 4, *c* and *d*). To test whether the aggregation of the NgCAM-expressing cells was due to a heterophilic binding between NgCAM and an undetermined surface protein of the myeloma cells, we investigated cell aggregation in mixed populations of NgCAM-expressing and wild-type myeloma cells. NgCAM-expressing myeloma cells were stained with a fluorescent intracellular dye and mixed with nontransfected cells stained with a different dye. Under this condition, only NgCAM transfectants participated in aggregates, whereas the vast majority of single cells were parental J558L cells (Fig. 4, *e* and *f*). In contrast, different populations of NgCAM-expressing cells formed mixed aggregates (Fig. 4, *g* and *h*). These results indicate that recombinant NgCAM on the surface of myeloma cells mediates cell aggregation via homophilic binding across the intercellular space.

NgCAM-expressing COS Cells Do Bind Axonin-1–Conjugated Covaspheres

To test whether cell surface–expressed recombinant NgCAM was able to bind axonin-1 presented on Covaspheres rather than on the surface of a cell, COS-7 cells were transiently transfected by electroporation with the expression vector pSCT-NgCAM. Immunocytochemical staining revealed a transfection efficiency of 15% and demonstrated that heterologously expressed NgCAM was exposed on the cell surface (Fig. 5). Analysis of membrane fractions by SDS-PAGE and immunoblotting showed that

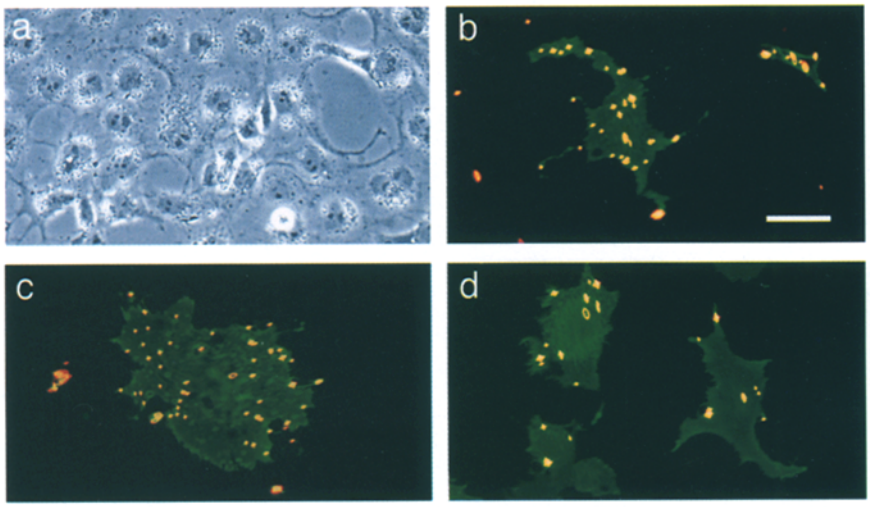


Figure 5. Binding of NgCAM- and axonin-1–conjugated Covaspheres to NgCAM- and axonin-1–expressing COS cells. NgCAM- and axonin-1–expressing cells were visualized by indirect immunofluorescence. Covaspheres conjugated with axonin-1 (*b*) or NgCAM (*c*) bound strongly to NgCAM-expressing cells. Axonin-1–expressing cells bound Covaspheres conjugated with NgCAM poorly (*d*; see also Rader et al., 1996). Phase-contrast (*a*) and fluorescence optics (*b–d*). Bar, 25 μm.

transfected COS cells produced NgCAM with the same electrophoretic band pattern as brain-derived NgCAM (Fig. 2 *e*). Cells expressing NgCAM were capable of binding Covaspheres conjugated with axonin-1 (Fig. 5, *a* and *b*) and NgCAM (Fig. 5 *c*). This binding was abolished completely after preincubation of the cells with anti-NgCAM Fab (data not shown). If the heterophilic interaction between axonin-1 and NgCAM was studied in the reversed situation, i.e., with axonin-1 presented on the surface of COS cells and NgCAM conjugated to Covaspheres, a binding was observed as well. However, the cells with bound Covaspheres represented a relatively minor fraction of the axonin-1–expressing cells (Fig. 5 *d*). Quantification of the binding of Covaspheres to NgCAM-expressing cells revealed that 61 ± 3% (mean ± SD; $n = 3$) of the cells bound NgCAM–Covaspheres and 53 ± 5% ($n = 5$) bound axonin-1–Covaspheres. In contrast, only 15 ± 6% ($n = 5$) of the axonin-1–expressing cells bound NgCAM–Covaspheres.

In summary, the results of the COS cell–Covaspheres binding studies demonstrated that membrane-bound NgCAM can engage in a heterophilic binding with axonin-1 that is presented in the Covaspheres-conjugated form rather than on the surface of another cell. Intriguingly, a considerably weaker binding was found when axonin-1 was presented in the cell membrane-bound form and NgCAM was on the Covaspheres (see also Rader et al., 1996).

Antibody-induced Capping of NgCAM Results in Cocapping of Axonin-1

To test whether NgCAM and axonin-1 interact when they are present on the surface of the same cell, cocapping experiments were performed on CV-1 cells that were stably transfected with both NgCAM and axonin-1. These cells are well suited for such studies because of their large size and smooth surface. As shown by Western blot analysis (Fig 2, *i* and *k*), they expressed axonin-1 and NgCAM at a ratio of ~1:2. For the cocapping assays, cells were incubated with monoclonal anti-NgCAM IgG. To induce capping, the first antibodies were cross-linked with rabbit anti–mouse IgG. After the capping procedure, NgCAM exhibited a patchy distribution in >90% of the cells, as demonstrated by immunocytochemical staining with a Texas red–labeled donkey anti–rabbit antibody (Fig. 6 *a*). Staining of axonin-1 with a FITC- labeled second antibody revealed a striking colocalization of axonin-1 with NgCAM patches (Fig. 6 *c*). Possible cross talk between FITC and Texas red fluorescence was ruled out as an explanation for the colocalized fluorescence signal because, under the conditions used, patches labeled with only one fluorochrome did not elicit a signal detectable with the filter set for the other fluorochrome. Axonin-1 colocalized with almost all of the NgCAM patches on NgCAM-capped cells. In rare instances, however, clusters composed exclusively of axonin-1 were observed. No capping and cocapping was observed when the primary antibody was omitted (Fig. 6, *b* and *d*). Cocapping of axonin-1 and NgCAM was also found when capping was induced with a monoclonal anti–axonin-1 IgG. However, clusters exclusively composed of the antibody-capped molecule, i.e., axonin-1, were more frequent than when anti-NgCAM IgG was used for capping, and randomly distributed NgCAM that had not accompanied axonin-1 into clusters was relatively abundant in this situation. A possible explanation for this observation may be that the force pulling NgCAM along with the capping axonin-1 molecule as a result of a *cis*-interaction may be opposed by contact of the cytoplasmic domain with cytoskeletal components (see Davis and Bennett, 1994).

Heterodimeric Complexes of Axonin-1 and NgCAM Are Detected by Chemical Cross-linking

For the confirmation of a direct molecular interaction (*cis*-interaction) of axonin-1 with NgCAM in the membrane of intact neurons, chemical cross-linking was performed with dissociated DRG neurons. To minimize cell–cell contacts, the cells were cultured at low density (Fig. 7 *d*). The homobifunctional cross-linking reagent DST was chosen because of its short spacer, to obtain a high degree of selectivity. During the cross-linking procedure, no obvious morphological changes of the cultures were observed. At the end of the reaction, membrane proteins were extracted from the cells with detergent, and axonin-1 was immuno-

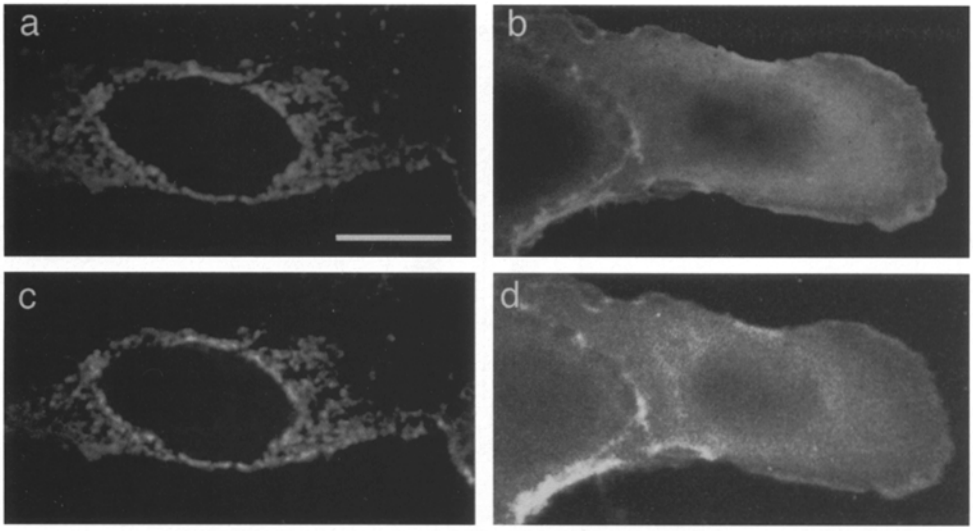


Figure 6. Cocapping of axonin-1 with NgCAM on CV-1 cells. Capping of NgCAM was induced with a monoclonal anti-NgCAM antibody and rabbit anti–mouse IgG. NgCAM caps were visualized by immunofluorescence staining using Texas red–labeled donkey anti–rabbit IgG (*a*). Cocapping of axonin-1 with NgCAM was determined using goat anti–axonin-1 Fab and FITC-labeled donkey anti–goat IgG (*c*). For a control, the mAb against NgCAM was omitted (*b* and *d*). For inspection of the cells, a confocal microscope was used. (*a* and *b*) Texas red filter set. (*c* and *d*) FITC filter set. Bar, 10 μm.

precipitated, separated by SDS-PAGE, and blotted onto nitrocellulose. Immunochemical detection localized axonin-1 in the monomeric form (125 kD) and additionally in a band with an apparent molecular mass of 260 kD (Fig. 7 *a*). In the analysis of the 260-kD component by serial immunodetection with anti–axonin-1, anti-NgCAM, and anti-NrCAM antibodies on the same nitrocellulose, NgCAM, but not NrCAM, was found (Fig. 7, *b* and *c*, respectively). Superposition of the chemiluminescence images allowed a precise colocalization of the NgCAM signal with the axonin-1 signal. Thus, the 260-kD component represents a heterodimeric complex consisting of axonin-1 and the extracellular fragment of NgCAM with their apparent molecular masses of 125 and 140 kD, respectively.

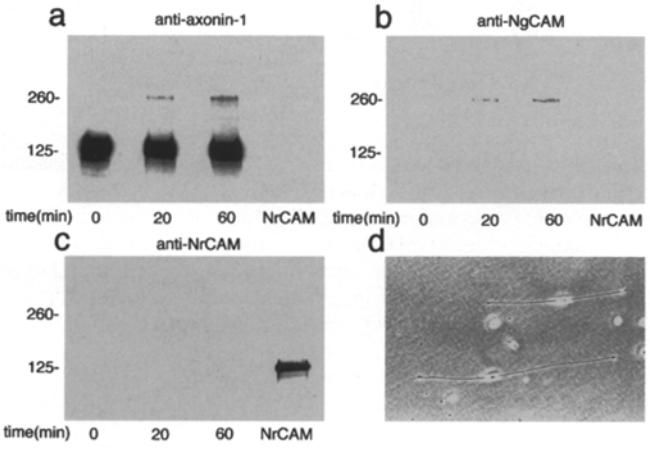


Figure 7. Chemical cross-linking of NgCAM and axonin-1 on intact DRG neurons. Dissociated DRG neurons were cultivated at low density on laminin for 15 h (*d*). Cross-linking was performed with 1 mM DST. The reaction was stopped after 0, 20, and 60 min. Axonin-1 and complexes containing axonin-1 were immunoprecipitated with a monoclonal anti–axonin-1 antibody, subjected to SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose was probed serially with anti–axonin-1, anti-NgCAM, and anti-NrCAM antibody using enhanced chemiluminescence detection. Axonin-1 appeared as a monomer (125 kD) and as a cross-linked product (band at 260 kD), as visualized by anti–axonin-1 staining (*a*). The 260-kD band contained NgCAM cross-linked to axonin-1 (*b*). The 260-kD band does not contain NrCAM (*c*).

Antisense Oligonucleotide-mediated Suppression of Axonin-1 Translation Prevents Neurite Outgrowth on NgCAM Substratum

To investigate the role of the *cis*-interaction of axonin-1 and NgCAM in neurite outgrowth, we suppressed the expression of axonin-1 in dissociated DRG neurons by antisense oligonucleotides. A combination of two antisense oligonucleotides complementary to the regions comprising the translation initiation site and the translation stop codon, respectively, were used. The corresponding sense oligonucleotides served as controls. After application of the oligonucleotides, the neurons were counted and plated onto NgCAM- or laminin-coated culture dishes. Immunocytochemistry for the neuronal marker F11 was used to distinguish neurons from nonneuronal cells, and with Trypan blue dye exclusion, we identified live cells. After 15 h in culture, the effects of the antisense treatment were investigated by cell counts, Western blot analysis, and an assessment of neurite outgrowth using the method described by Chang et al. (1987). When counted 4 h after plating, the antisense oligonucleotide–treated cultures of dissociated DRG cultures revealed a reduction in the plating efficiency of ~15% on NgCAM substratum, whereas on laminin, the cultures treated with antisense oligonucleotides had the same plating efficiency as the sense oligonucleotide–treated cultures.

Immunoblot analysis of cultures grown on laminin for 15 h showed that axonin-1 antisense oligonucleotides reduced the total amount of axonin-1 by ~95 % compared with sense oligonucleotide–treated cultures (Fig. 8 *a*). Related neural cell adhesion molecules, such as F11 and NgCAM, were only slightly reduced in the antisense oligonucleotide–treated cells. Therefore, we concluded that the antisense oligonucleotide treatment specifically and almost completely suppressed the translation of axonin-1.

When inspected 15 h after plating, no difference in neurite outgrowth on laminin was observed between the anti-

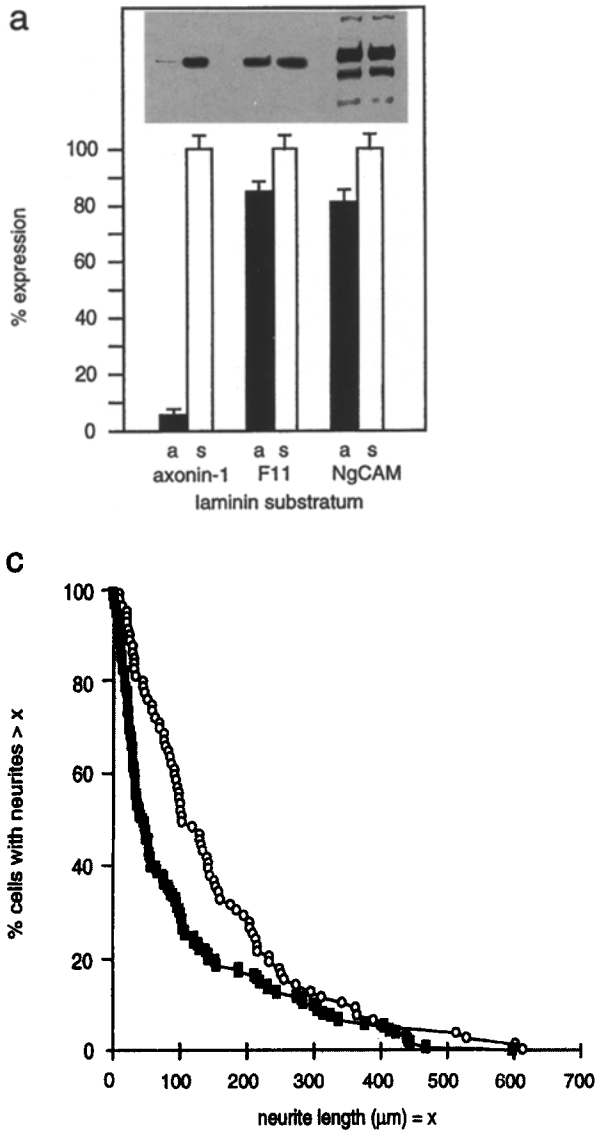


Figure 8. Suppression of axonin-1 translation by antisense oligonucleotides. (*a*) Western blot analysis of DRG cultures on laminin substratum using chemiluminiscence detection. Densitometric analyses were carried out with triplicate blots of independent experiments. Densitometric results of the sense oligonucleotide–treated cultures are given as 100%. Mean and SD are given. Axonin-1 expression was strongly reduced in antisense-treated cultures, whereas F11 and NgCAM expression was only slightly affected. (*b*) Quantification of neurite formation in cultures treated with antisense and sense oligonucleotides and plated on either NgCAM or laminin substratum. Neurites were defined as processes that extend from the neuronal soma by a distance longer than 20 μm. Each condition was carried out in triplicate, and at least 150 neurons were counted for each experiment. Mean and SD are given. (*c*) Measurements of neurite lengths and determination of the median values of the neurite lengths (NL_{50}). Processes extending from the cell soma for a distance longer than 20 μm were defined as neurites. For each cell, the longest neurite was measured, and measurements of at least 80 cells with neurites were plotted as percentage of neurons with neurites longer than a given length (y-axis) vs the neurite length (x-axis), as introduced by Chang et al. (1987). The neurite length reached by 50% of the neurites (NL_{50}) was determined as the median neurite length. The example presented shows measurements obtained with one pair of sense and antisense oligonucleotide–treated cultures on NgCAM substratum. (*Filled squares*) Cultures treated with antisense oligonucleotides. (*Open circles*) Cultures treated with sense oligonucleotides. (*d*) Median values of neurite lengths (NL_{50}) in cultures treated with antisense and sense oligonucleotides and plated on either NgCAM or laminin substratum. Each NL_{50} value represents the mean and the SD of four independent cultures on NgCAM and three cultures on laminin. In the cultures on NgCAM substratum, 79–138 neurites were measured, while in the cultures on laminin, 36–76 neurites were measured.

sense and the sense oligonucleotide–treated cultures (Fig. 8 *b*). Under both conditions, ~70% of the neurons developed neurites. On NgCAM substratum, 52% of the neurons of the sense oligonucleotide–treated cultures developed neurites. In contrast, only 34% of the neurons of the antisense oligonucleotide–treated cultures had neurites. The neurite lengths in sense and antisense oligonucleotide–treated cultures were measured on NgCAM and on laminin (Fig. 8, *c* and *d*). Of each condition, triplicate cultures were made and the results of each culture were plotted separately (e.g., see Fig. 8 *c*). From each culture, the median value of the neurite lengths (NL_{50}) was determined as the neurite length developed by 50% of the neurite-bearing neurons (NL_{50}). In Fig. 8 *d*, the means of the NL_{50} values, as determined from triplicates of each condition, are shown. On laminin, no significant difference in the median neurite lengths in cultures treated with sense or antisense oligonucleotides were found. In contrast, the median neurite length on NgCAM substratum was markedly reduced in the cultures treated with antisense oligonucleotides; in the cultures treated with sense oligonucleotides, the mean NL_{50} value was 97 μm, whereas a mean NL_{50} of 56 μm was measured in antisense cultures.

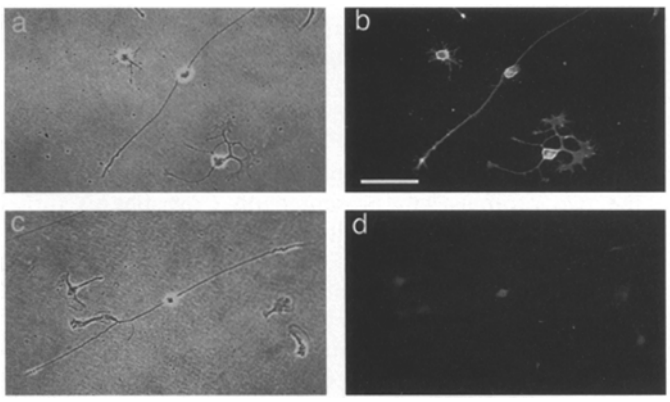
Comparison of the neurite length distributions in the sense and antisense oligonucleotide–treated cultures on NgCAM (Fig. 8 *c*) revealed a remakable left-shift over the

major fraction of the neurites in the antisense oligonucleotide–treated cultures. In contrast, in the region of the longest neurites, the curves were almost superimposable. Approximately 10% of the neurons had neurites longer than 300 μm, regardless whether the cultures were treated with sense or antisense oligonucleotides (Fig. 8 *c*). One possible explanation for this observation was that the long neurites in the antisense oligonucleotide–treated cultures were derived from neurons that had resisted oligonucleotide-mediated suppression of axonin-1 completely. Thus, we carried out immunocytochemical staining for axonin-1 at the end of the experiments and determined the percentage of neurites with detectable axonin-1. The results are presented in Fig. 9 *e*. On a laminin substratum, the number of neurons with neurites carrying axonin-1 on their surface was ~14% of the total number of the neurons with neurites (for an illustration, see Fig. 9, *c* and *d*). This result indicates that synthesis of axonin-1 was efficiently inhibited in the major proportion of neurons by the combination of antisense oligonucleotides used. In contrast, in the antisense oligonucleotide–treated cultures on NgCAM substratum, almost all neurites exhibited axonin-1 on their surface. Together, these results indicate that on NgCAM substratum only neurons expressing axonin-1 were capable of developing neurites, whereas neurons on laminin developed neurites regardless whether or not they expressed axonin-1.

When analyzed with regard to quantitative aspects, we found that on laminin substratum, ~14% of the neurites exhibited axonin-1 on their surface. Because, on laminin, ~70% of the neurons made neurites, and assuming that the proportion of axonin-1–expressing cells among the neurons without neurites was approximately equal to the proportion found among neurons with neurites, we concluded that antisense oligonucleotide treatment was partially or completely ineffective in ~20% of all neurons. In contrast, on NgCAM substratum, >90% of the neurites of antisense-treated cultures exhibited immunodetectable axonin-1 on their surface. Since, in this experimental condition, ~34% of the neurons produced neurites, ~1.5 times as many neurons with axonin-1–bearing neurites than those in antisense oligonucleotide-treated cultures on laminin were observed.

An explanation for this apparent discrepancy may be that the axonin-1–bearing neurites in the antisense oligonucleotide–treated cultures on NgCAM are not only found on neurons that are capable of synthesizing new axonin-1. Short neurites may also be formed with residual axonin-1 from the neuronal soma. As expected in consideration of a relatively slow turnover of axonin-1 with a half-life of >100 h in dissociated DRG cultures (Ruegg et al., 1989*b*), we found indeed that axonin-1 was not reduced on the neuronal somas of antisense oligonucleotide–treated cultures (Fig. 9 *b*). It is thus conceivable that residual axonin-1 of the neuronal somas can deliver a critical concentration of axonin-1 to forming neurites by anterograde diffusion. However, in the absence of axonin-1 synthesis, the supply of axonin-1 to the forming neurite is limited by the initial content of the cell soma. Diffusional spread of axonin-1 into newly formed neurites results in a decrease of the axonin-1 concentration per unit of membrane area. Eventually, the axonin-1 concentration will fall below a given threshold value necessary to support neurite growth, and neurite growth will stop. The markedly weaker axonin-1 staining of the somas of antisense oligonucleotide–treated neurons on laminin (Fig. 9 *d*), where the median neurite length was approximately three times as long as that in the antisense oligonucleotide–treated cultures on NgCAM, may be interpreted as evidence for a thinning out of axonin-1 by diffusional spreading from the neuronal soma into newly formed membrane that accompanies neurite elongation.

Residual axonin-1 of the cell somas cannot be considered as the source of the axonin-1 found on longer neurites. As evident in antisense oligonucleotide–treated cultures on laminin, residual axonin-1 from the cell soma cannot invade longer neurites in detectable concentrations. Thus, axonin-1 on the long neurites in the antisense oligonucleotide–treated cultures on NgCAM must be derived from continued protein synthesis while the neurites were growing. In support of this conclusion, we have recently found that axonin-1 on growth cones of long DRG



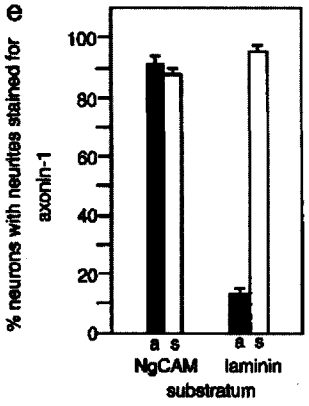


Figure 9. Morphological and immunocytochemical analysis of antisense oligonucleotide–treated DRG cultures. Cells treated with antisense oligonucleotides were cultured on NgCAM (*a* and *b*) or laminin (*c* and *d*). After 15 h the cells were stained with anti–axonin-1. Over 90% of the neurites on NgCAM substratum stained for axonin-1 (*a* and *b*). Antisense oligonucleotide–treated DRG neurons grew on laminin substratum regardless whether or not they expressed axonin-1 (*c* and *d*). Phase-contrast (*a* and *c*) and fluorescence optics (*b* and *d*). Bar, 75 μm. (*e*) Immunocytochemical staining of neurites for axonin-1. On NgCAM, ~90% of the neurites stained for axonin-1 regardless whether the cultures had been treated with sense or antisense oligonucleotides. On laminin, ~95% of the neurites of the sense oligonucleotide–treated cultures stained for axonin-1, whereas in antisense oligonucleotide–treated cultures, only ~14% of the cells were axonin-1 positive. Each value represents the mean and the SD of triplicate cultures. In each culture, at least 60 neurites were evaluated.

neurites originates from newly synthesized axonin-1 that is directly inserted into the growth cone membrane (Vogt et al., 1996). Thus, long neurites have to be interpreted as neurites of neurons that had resisted oligonucleotide-mediated suppression of axonin-1 translation.

In summary, the quantitative considerations of the oligonucleotide inhibition experiments, together with the immunocytochemical stainings of the neurites, suggest that the neurite length distribution curve of the antisense oligonucleotide–treated cultures on NgCAM is composed of three segments, (*a*) a segment with long neurites (>300 μm) most likely formed by neurons in which the antisense oligonucleotide treatment was completely ineffective; (*b*) a segment including neurites with reduced neurite lengths, comprising neurites of neurons in which the antisense oligonucleotide treatment had reduced but not completely suppressed the axonin-1 expression (middle segment of curve); and (*c*) a segment with short neurites, which could be formed without de novo synthesis of axonin-1, using residual axonin-1 of the neuronal soma (approximately one-third of the neurites). A common feature of virtually all neurites observed on NgCAM substratum was that they exhibit axonin-1 on the surface. Thus, we conclude that without axonin-1, DRG neurites cannot be formed on an NgCAM substratum.

Discussion

Cocapping and chemical cross-linking demonstrated that the cell surface molecules axonin-1 and NgCAM interact in the plane of the neuronal membrane. The suppression of axonin-1 translation by antisense oligonucleotides rendered chicken DRG neurons incapable of extending neurites on an NgCAM substratum. These results suggest that neurite outgrowth from DRG neurons on NgCAM substratum requires the formation of an NgCAM/axonin-1 heterodimers in the growth cone membrane in addition to the established homophilic *trans*-interaction between growth cone NgCAM and substratum NgCAM (Lemmon et al., 1989).

NgCAM and Axonin-1 Form Heterodimers in the Plane of the Neuronal Membrane

Speculations on the possibility that an interaction between NgCAM and axonin-1 may occur in the plane of the neuronal membrane found a structural basis in results of a recent study in which the NgCAM-binding site of axonin-1 was mapped (Rader et al., 1996). Domain deletion mutants and EM pictures of soluble wild-type axonin-1 were used as a basis for the proposal of a structural model of membrane-anchored axonin-1 in which the NH_2-terminal moiety of the molecule (containing the NgCAM-binding conglomerate composed of the Ig domains 1–4) was bent backwards toward the membrane in a horseshoe-like form. The localization of the NgCAM-binding domain conglomerate close to the membrane favored the idea that binding with an NgCAM of the same membrane might be more likely established than binding with an NgCAM of another cell. In the work presented here, we investigated whether axonin-1 and NgCAM are associated in the plane of the same membrane by two independent approaches, namely antibody-induced capping/cocapping and chemical cross-linking. Antibody-induced capping of NgCAM resulted in extensive cocapping of axonin-1. Since axonin-1 is glycosylphosphatidylinositol (GPI)–anchored to the cell membrane, it is localized exclusively extracellularly. Therefore, we considered a direct binding between the extracellular segment of NgCAM and axonin-1 as the most likely explanation. However, an association mediated by a linker protein could not be excluded. Thus, cross-linking studies with low density cultures of dissociated neurons were carried out. DRG neurons express both NgCAM and axonin-1 on their growth cones, axons, and cell somas (Kuhn et al., 1991; Stoeckli et al., 1996). Using a short bifunctional reagent, we found axonin-1 and NgCAM in an SDS- and β-mercaptoethanol–stable complex with an apparent molecular mass of 260 kD. The size of this complex corresponded closely to the sum of the molecular masses of axonin-1 and the 140-kD extracellular fragment of NgCAM. Known ligands of NgCAM and axonin-1, such as F11 (Brümmendorf et al., 1993) and NrCAM (Suter et al., 1995), respectively, were not found in this complex. Since cultures were grown on a laminin substratum in low density and collected before contacts between neurites occurred, encounters between NgCAM and axonin-1 were possible only in the plane of the neuronal membrane. Hence, these results demonstrated unequivocally that NgCAM and axonin-1 bind each other selectively in the plane of the neuronal membrane (*cis*-binding).

NgCAM and Axonin-1 on Different Myeloma Cells Are Not Capable of Mediating Cell–Cell Aggregation

The results of binding studies with myeloma cells expressing axonin-1 or NgCAM indicated that NgCAM and axonin-1 are not capable of mediating cell aggregation by binding each other across the extracellular space (*trans*-binding). Interestingly, TAG-1 (Furley et al., 1990) and L1 (Moos et al., 1988), the presumptive rodent homologues of axonin-1 and NgCAM, respectively, were also reported to be unable to mediate cell aggregation when expressed in *Drosophila* Schneider 2 cells (Felsenfeld et al., 1994). The lack of binding between NgCAM and axonin-1 expressed by myeloma cells cannot be explained as a deficiency of the recombinant proteins since both recombinant NgCAM and axonin-1 were structurally intact and functionally competent.

In contrast with studies involving NgCAM and axonin-1 exposed on the surface of different cells, a heterophilic NgCAM/axonin-1 interaction was established when one of the two proteins was presented on Covaspheres rather than on the surface of cells. Recombinant NgCAM expressed on the surface of COS cells was capable of binding axonin-1 (either wild-type or recombinant) when axonin-1 was presented on Covaspheres. Interestingly, NgCAM/axonin-1 binding in the reversed situation, when recombinant axonin-1 was expressed on the surface of COS cells and NgCAM was on Covaspheres, was considerably weaker. This result is in line with the notion that cell surface–bound axonin-1 is in a conformation and orientation that is unfavorable for NgCAM binding (Rader et al., 1996). Studies on the NgCAM-binding domain of axonin-1 had revealed a horseshoe-shaped structure of axonin-1 in

which the NgCAM-binding site (assigned to the conglomerate of the four amino-terminal Ig domains) was oriented toward the membrane that restricted its accessibility for macromolecules residing on the surface of another cell. Based on these results, we propose that axonin-1-Covaspheres bind to NgCAM-expressing cells better than NgCAM–Covaspheres bind to axonin-1–expressing cells, because at least a fraction of the axonin-1 molecules on Covaspheres is bound in an orientation and conformation appropriate for binding cell surface–exposed NgCAM, whereas membrane-anchored axonin-1 is present only in one (unfavorable) form. We suggest that the fraction of axonin-1 molecules on the Covaspheres that become engaged in a binding with membrane-bound NgCAM are oriented on the beads such that they approach NgCAM in the same relative orientation as an axonin-1 bound to the same membrane.

NgCAM/Axonin-1 Trans-Binding Is Not Required for Neurite Outgrowth on NgCAM Substratum, but Neurite Outgrowth Mediated by NgCAM Receptor Requires a Cooperation with Axonin-1 in the Neurite Membrane

Our observation that a heterophilic *trans*-interaction between axonin-1 and NgCAM expressed in different myeloma cells was not formed is in line with the observations that anti–axonin-1 antibodies did not perturb outgrowth of DRG neurons on NgCAM adsorbed to tissue-culture plastic (Kuhn et al., 1991; Stoeckli et al., 1996) or on NgCAM expressed on the surface of CV-1 cells (this paper). Together, these results support the hypothesis that in growth cones growing on an NgCAM substratum, only NgCAM, but not axonin-1, is required for substratum contact. However, axonin-1 is required in the growth cones for neurite outgrowth on NgCAM substratum. When we suppressed the translation of axonin-1 in DRG neurons and thus its insertion into the growth cone membrane, we found that neurite outgrowth on NgCAM substratum was strongly reduced. In contrast, on laminin substratum, on which neurite outgrowth occurs by a mechanism involving integrin receptors (Tomaselli et al., 1986), a lack of axonin-1 in growth cones did not affect neurite outgrowth. These results implicate axonin-1 of the growth cone as a cofactor in the promotion of neurite outgrowth on NgCAM substratum, acting through a *cis*-binding with growth cone NgCAM without being involved in a binding with the substratum.

The function of axonin-1 via a *cis*-interaction with NgCAM, rather than a *trans*-interacion with a ligand in the substratum, could explain why neurite outgrowth on an NgCAM substratum was not affected by anti–axonin-1 antibodies. Although we do not understand in detail the connection between NgCAM and axonin-1 in the plane of the neuronal membrane are in accordance with the recently determined domain organization of axonin-1, indicating that the Ig and FnIII domains of axonin-1 are arranged in a horseshoe-like form and that the NgCAM-binding site of axonin-1 is located on a conglomerate of the four amino-terminal Ig domains (Rader et al., 1996). As a result of the horseshoe-like form of axonin-1, the NgCAM-binding site is located in the proximity of the membrane. In this conformation, a binding to NgCAM of the same membrane rather than to NgCAM of another cell may be favored. Alternative models for an activation of neurite outgrowth on NgCAM substratum are conceivable. In both mechanisms proposed, a *trans*-binding of growth cone NgCAM to substratum NgCAM combined with a *cis*-binding to axonin-1 in the plane of the growth cone membrane is required. In the *Dual Activation Model* (*b*), a neurite outgrowth signal is emitted only from NgCAM. An activated form of NgCAM (*asterisk*) results from a combination of a *trans*-binding with substratum NgCAM and a *cis*-binding with growth cone axonin-1. In the *Signal Cooperation Model* (*c*), both NgCAM and axonin-1 emit signals. As a result of its binding to the substratum growth cone, NgCAM is concentrated in the substratum-apposed growth cone membrane. The *cis*-binding of axonin-1 in turn results in a concomitant accumulation of axonin-1. Thus, two initially independent signals are concentrated and brought into proximity in the substratum-facing area of the growth cone and thus may cooperate to generate a neurite outgrowth–promoting signal.

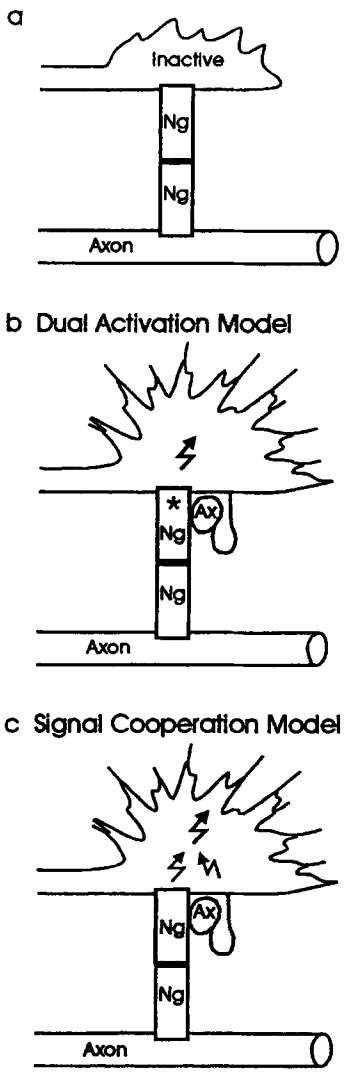


Figure 10. Molecular models for neurite outgrowth promotion mediated by interactions of NgCAM and axonin-1. (*a*) In the absence of axonin-1, when only a *trans*-binding contact with substratum NgCAM is established, growth cone NgCAM is in a nonsignaling state and no neurite outgrowth is observed. (*b* and *c*) In the presence of axonin-1 on growth cones, growth cone NgCAM establishes a *cis*-binding with growth cone axonin-1 in addition to the *trans*-binding with substratum NgCAM. In this case, neurite outgrowth is activated. Results demonstrating an interaction be-

tributions of the different interactions between epitopes and their corresponding antibodies in a reaction with a polyclonal Fab, it is conceivable that a *trans*-interaction may be more susceptible to perturbation by antibodies than a *cis*-interaction. In support of this speculation, one could argue that binding sites of *trans*-interactions may be more accessible for antibodies than binding sites for *cis*-interactions, which may be buried in the interior of the membrane.

Binding studies with myeloma or COS cells expressing NgCAM have demonstrated that NgCAM alone can mediate cell–cell or cell–Covaspheres binding by its homophilic-binding capability. These results do not contradict the observation that axonin-1 is required for the outgrowth of neurites from DRG neurons on an NgCAM substratum, as concluded from the experiments with the antisense oligonucleotides. Results of studies of the mechanisms of neurite outgrowth on a variety of substrata indicate that binding between growth cone surface receptors and substratum components is not sufficient, but that the generation of additional intracellular signals is required. Studies by Doherty and co-workers have shown that neurite outgrowth mediated by L1 depends on Ca^{2+} influx (Williams et al., 1992), as well as phosphorylation by an erbstatin analogue-sensitive tyrosine kinase (Williams et al., 1994*a*). Recent evidence also implicates an activation of the FGF receptor in the signaling pathway underlying L1-mediated neurite outgrowth (Williams et al., 1994*b*; Doherty et al., 1995). Maness and co-workers have presented results from studies on src^-/src^- mice implicating the nonreceptor tyrosine kinase src in the transduction of L1-dependent neurite outgrowth (Ignelzi et al., 1994). Moreover, Lemmon and co-workers have demonstrated that L1 signaling may be accompanied by a phosphorylation of the intracellular domain of L1 by a casein kinase II (Wong et al., 1996). In the experimental system used here, the homophilic interaction of receptor NgCAM with substratum NgCAM is not sufficient to elicit neurite outgrowth. The results presented suggest that axonin-1 may be one element required in addition to NgCAM for neurite outgrowth of DRG neurons on an NgCAM substratum.

The generation of a neurite outgrowth signal upon establishment of NgCAM/axonin-1 complexes may be achieved via different mechanisms (Fig. 10). NgCAM may exist in two conformations, one signaling and one nonsignaling. NgCAM without a *cis*-interaction with axonin-1 is in a nonsignaling state (Fig. 10 *a*). Transition to the signaling state occurs by a combination of a *trans*-binding to the substratum and a *cis*-binding to axonin-1 (dual activation model). In this model, axonin-1 would not transduce a signal on its own but rather modulate the signal transduction of NgCAM (Fig. 10 *b*). Alternatively, it is conceivable that axonin-1 contributes an independent intracellular signal. As a GPI-anchored glycoprotein, axonin-1 might be capable of transmembrane signaling by direct or lipid-mediated interaction with a putative transmembrane linker protein. Such linkers have been suggested to relay the signal derived from GPI-anchored molecules to intracellular nonreceptor tyrosine kinases (for a recent review see Brown, 1993). Recent evidence indicates that F11 (contactin) (Ranscht, 1988; Brümmendorf et al., 1989), the closest relative of axonin-1, is linked to fyn, a nonreceptor tyrosine kinase of the src family (Zisch et al., 1995). It is conceivable that the *cis*-interaction of axonin-1 and NgCAM is required to bring the signaling complexes of NgCAM and axonin-1 into proximity so that they can interact to generate a novel signal distinct from the signals evoked by monomeric NgCAM and axonin-1 (signal cooperation model; Fig. 10 *c*).

Conclusion

Evidence from experiments described in this paper suggests that the NgCAM-mediated neurite outgrowth of DRG neurons depends crucially on a cooperation of NgCAM with axonin-1 that is implemented by the formation of NgCAM/axonin-1 heterodimers in the plane of the growth cone membrane. Hence, an activation of neurite outgrowth on an NgCAM substratum depends on two interactions of growth cone NgCAM that are both indispensable for a neurite outgrowth signal: one interaction with substratum NgCAM (*trans*-interaction) and one interaction with axonin-1 residing in the growth cone membrane (*cis*-interaction).

The fact that axonin-1 is spatially more restricted than NgCAM during neural development speaks against the general applicability of the dual activation model where NgCAM receptor function requires axonin-1 as a *cis*-bound activator in addition to substratum contact. However, it is conceivable that some isoforms of NgCAM are capable of stimulating neurite outgrowth independent of *cis*-ligand activation. Alternatively, other regulators could replace axonin-1 in areas where it is not expressed. For instance, the FGF receptor has been implicated as a proximal component of the L1-signaling pathway (Williams et al., 1994*b*). F11(contactin) (Ranscht, 1988; Brümmendorf et al., 1989), the closest relative of axonin-1, which has been demonstrated to bind to NgCAM (Brümmendorf et al., 1993) and exhibits an expression pattern clearly distinct from that of axonin-1, might be a possible candidate molecule for another *cis*-activator of NgCAM.

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