Neurite Outgrowth on Immobilized Axonin-1 Is Mediated by a Heterophilic Interaction with L1(G4)

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Abstract. Axonin-1 is an axon-associated cell adhesion molecule with dualistic expression, one form being glycophosphatidylinositol-anchored to the axonal membrane, the other secreted from axons in a soluble form. When presented as a substratum for neuronal cultures it strongly promotes neurite outgrowth from chicken embryonic dorsal root ganglia neurons. In this study, the axon-associated cell adhesion molecule G4, which is identical with Ng-CAM and 8D9, and homologous or closely related to L1 of the mouse and NILE of the rat, was investigated with respect to a receptor function for axonin-1. Using fluorescent microspheres with covalently coupled axonin-1 or L1(G4) at their surface we showed that these proteins bind to each other. Within the sensitivity of this microsphere assay, no interaction of axonin-1 with itself could be detected. Axonin-1-coated microspheres also bound to the neurites of cultured dorsal root ganglia neurons. This interaction was exclusively mediated by L1(G4), as indicated by complete binding suppression by monovalent anti-L1(G4) antibodies. The interaction between neuritic L1(G4) and immobilized axonin-1 was found to mediate the promotion of neurite growth on axonin-1, as evidenced by the virtually complete arrest of neurite outgrowth in the presence of anti-L1(G4) antibodies. Convincing evidence has recently been presented that neurite growth on L1(8D9) is mediated by the homophilic binding of neuritic L1(G4) (1989. *Neuron.* 2: 1597–1603). Thus, both L1(G4)- and axonin-1-expressing axons may serve as "substrate pathways" for the guidance of following axons expressing L1(G4) into their target area. Conceivably, differences in the concentration of axonin-1 and L1(G4), and/or modulatory influences on their specific binding parameters in leading pathways and following axons could represent elements in the control of axonal pathway selection.

THE formation of nerve fiber tracts during neurogenesis is based on the tendency of growing axons to elongate along other axons. This developmental phenomenon has been proposed to be due to the capability of the leading axons to serve as "substrate pathways" (Katz and Lasek, 1980) or "labeled pathways" (Ghysen and Janson, 1980; Raper et al., 1983) for trailing growth cones by means of guidance cues expressed on their surface. Over the past decade, a number of cell surface glycoproteins concentrated primarily on axons and implicated in neurite extension have been described in the vertebrate nervous system. To this group of axonal glycoproteins belong mouse L1 (Rathjen and Schachner, 1984) and several molecules related thereto (rat NILE, chick Ng-CAM, chick G4, chick 8D9, human 5G3), chick neurofascin (Rathjen et al., 1987*b*), chick F11 (Rathjen et al., 1987*a*) and its mouse homologue F3 (Gennarini et al., 1989), chick axonin-1 (Ruegg et al., 1989*a*; Stoeckli et al., 1989), chick Bravo (De la Rosa et al., 1990), and rat TAG-1 (Dodd et al., 1988). cDNA cloning of some of these molecules has shown that they are structurally related to each other and to N-CAM in that they contain both multiple immunoglobulin- and fibronectin type III–like domains (Cunningham et al., 1987; Moos et al., 1988, Brümmendorf et al., 1989; Gennarini et al., 1989; Furley et al., 1990; Burgoon et al., 1991). Furthermore, among these glycoproteins, L1(8D9), TAG-1, axonin-1, and F11(F3) have been reported to be potent substrata for neurite outgrowth (Lagenaur and Lemmon, 1987; Furley et al., 1990; Gennarini et al., 1991; Stoeckli et al., 1991). Among the described axon-associated glycoproteins, chick axonin-1 is of particular interest in that it is expressed in two forms, one being integrally associated with the axonal membrane (Ruegg et al., 1989*b*) by a glycophosphatidylinositol anchor (Osterwalder, T., and P. Sonderegger, manuscript in preparation), the other secreted from axons (Stoeckli et al., 1989). Secreted axonin-1 diffuses through the extracellular space of the central nervous system and accumulates in the cerebrospinal fluid and the vitreous humor of the eye to relatively high concentrations (Ruegg et al., 1989*b*; Stoeckli et al., 1991). It is released from an intracellular pool (Ruegg et al., 1989*b*) in a functionally competent form, as demonstrated by its strong neurite growth-promoting activity when presented as a substratum to cultured neurons (Stoeckli et al., 1991). In view of the high degree of structural similarity between membrane-bound and secreted axonin-1, we have postulated a competitive binding of the two forms to the same binding site(s), implicating secreted

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axonin-1 as a regulatory element of growth cone–neurite interaction in the control of axonal elongation, pathway selection, and possibly target recognition (Stoeckli et al., 1991). The cDNA encoding axonin-1 has recently been cloned (Zuellig, R. A., C. Rader, A. Schroeder, M. Kalousek, F. von Bohlen, A. Fritz, E. Hafen, H. U. Affolter, and P. Sonderegger. 1991. *Soc. Neurosci. Abstr.* In press.). From an open reading frame of 3,108 nucleotides, a polypeptide of 1,036 amino acids has been deduced, which exhibits an amino acid sequence identity of 75% to TAG-1 of the rat, 53% to F11 of the chicken, and 29% to Ng-CAM. The predicted sequence contains six immunoglobulin-like repeats in its amino-terminal portion and four fibronectin type III–like repeats in its carboxy-terminal portion.

Neither the molecular nature nor the cellular location of the binding sites for axonin-1 has been elucidated thus far. The strong expression of axonin-1 on fasciculated axons of developing nerve fiber tracts found in immunohistochemical studies, the fact that anti-axonin-1 Fab fragments perturb fasciculation of neurites expressing axonin-1, and the neurite growth-promoting activity of axonin-1 when presented as a substratum to cultured neurons (Stoeckli et al., 1991) represent circumstantial evidence for at least one axonin-1 receptor residing in the axonal membrane. Hence, axonin-1 and other axonal molecules coexpressed with axonin-1 in time and space during neural development may be receptor candidates.

In this study, we have investigated another axon-associated cell adhesion molecule as a potential adhesive receptor of axonin-1, namely, G4, which is identical with Ng-CAM (Rathjen et al., 1987*a*; Burgoon et al., 1991; Rathjen, F., unpublished data) and 8D9 (Lemmon et al., 1989), and thus is either homologous or closely related to L1 of the mouse (Moos et al., 1988) and NILE of the rat (Prince et al., 1989). L1(G4) is one of the most abundant AxCAMs in the developing chicken nervous system, where it shows remarkable coexpression with axonin-1 in several nerve fiber tracts, as revealed by immunohistochemical localization in tissue sections (Ruegg et al., 1989*b*). L1(G4) was found to be coexpressed with axonin-1 also at the cellular level, as revealed by double stained patches on cell somas and on neurites of cultured dorsal root ganglia (DRG)[1] neurons. DRG neurites were also found to express receptors for both axonin-1 and L1(G4). As a means to demonstrate weak macromolecular interactions, we coupled soluble axonin-1 to fluorescent microspheres and tested for aggregation with L1(G4)-conjugated microspheres linked to another fluorochrome. By the fact that multiple macromolecular interactions occur upon contact of such protein-conjugated spheres, relatively weak affinities can be visualized (Grumet and Edelman, 1988; Kadmon et al., 1990). Both the aggregation analysis of fluorescent microspheres coated with axonin-1 or L1(G4) and the observation of coated microspheres' binding to cultured neurons demonstrate the specific interaction of axonin-1 with L1(G4). L1(G4)-conjugated beads showed self-aggregation, a finding consistent with previous reports on the homophilic binding of L1(8D9) (Lemmon et al., 1989). Axonin-1 was found to exhibit only heterophilic binding activities in the microsphere assay. As a functional correlate of the observed molecular binding, neurite outgrowth with axonin-1 as a substratum was found to depend on the interaction with L1(G4) of the neuritic membrane.

1. *Abbreviations used in this paper*: DRG, dorsal root ganglia; NL_{50}, neurite length developed by 50% of the neurite-bearing neurons.

Materials and Methods

Materials

Soluble axonin-1 was purified from the ocular vitreous fluid of 14-d-old chicken embryos (Ruegg et al., 1989*a*). L1(G4) was isolated from embryonic chicken brain membranes by immunoaffinity chromatography, using a monoclonal anti-L1(G4) antibody (Rathjen et al., 1987*a*). The functional integrity of purified axonin-1 and L1(G4) was tested in a neurite growth assay (Stoeckli et al., 1991). Fibronectin was a gift from Dr. Annemarie Honegger. Laminin was purchased from GIBCO-BRL Laboratories (Gaithersburg, MD). BSA was from Miles Inc. (Kankekee, IL), ovalbumin from Fluka Chemie AG (Buchs, Switzerland), and transferrin from Sigma Chemical Co. (St. Louis, MO). Antibodies against axonin-1 were raised in goat (Ruegg et al., 1989*a*); anti-L1(G4) antibodies were from rabbits (Rathjen et al., 1987*a*) Fab fragments were prepared by proteolytic digestion followed by chromatographic purification as previously specified (Stoeckli et al., 1991).

Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out as described by Laemmli (1970). For silver staining of proteins the procedure described by Switzer et al. (1979) as modified by Oakley et al. (1980) was used. Immunoblotting was carried out according to Towbin et al. (1979). For immunodetection, the procedure described by Hawkes et al. (1982) was adopted. As primary antibodies, anti-axonin-1 Fab fragments (from goat) and anti-L1(G4) Fab fragments (from rabbit) were used at a concentration of 25 μg/ml. Secondary peroxidase-conjugated antibodies goat and rabbit IgG were used at a dilution of 1:1,000.

Covalent Coupling of Proteins to Fluorescent Microspheres

Fluorescent polystyrene microspheres (Covaspheres) with a nominal diameter of 0.5 μm were purchased ready for the covalent coupling of proteins (Duke Scientific Corp., Palo Alto, CA). Both green fluorescent microspheres containing FITC and red fluorescent microspheres containing TRITC were used. The coupling of proteins to Covaspheres was carried out in PBS. Immediately before coupling, the suspension of microspheres was sonicated in a bath sonicator (Branson Ultrasonics Corp., Danbury, CT) for 2 min and 100-μl aliquots were incubated for 1 h at 37°C with 50 μg of axonin-1, L1(G4), fibronectin, laminin, transferrin, IgG, and BSA, respectively. The spheres were then sedimented by centrifugation and resuspended in PBS containing 5 mg/ml BSA and 10 mM sodium azide, followed by sonication for 2 min, and incubated for 30 min at room temperature. After a second centrifugation, they were resuspended in 100 μl of the same buffer and stored at 4°C. This stock solution contained an estimated concentration of Covaspheres of 10^{11}/ml. To determine the coupling yield serial dilutions of the initial protein solution and the unbound protein from the supernatant of the coupling reaction were subjected to SDS-PAGE on an automated system (Phast System; Pharmacia Fine Chemicals, Uppsala, Sweden). The protein bands were visualized by silver staining. Protein bands with identical intensity were identified, and from their dilution factor the ratio between coupled and uncoupled protein was calculated. In all coupling reactions a yield >80% was obtained, indicating that ~16,000 molecules of axonin-1 or L1(G4) were coupled per Covasphere.

Flow Cytometric Analysis of the Aggregation of Protein-conjugated Covaspheres

The aggregation behavior of protein-conjugated Covaspheres was investigated by incubation at various concentrations for 1 h at room temperature. The stock solutions of protein-conjugated Covaspheres were sonicated for 2 min in a bath sonicator. Immediately after, the desired test mixture was composed in an Eppendorf tube to a final volume of 20 μl PBS containing 5 mg/ml BSA and 10 mM sodium azide. The Covasphere concentrations assayed were varied between 10^9/ml and 5×10^{10}/ml, and the concentration ratios between pairs of Covasphere species were from 1:8 to 8:1.

Antibody perturbation of Covasphere aggregation was carried out by preincubation of one of the two samples to be tested with Fab fragments

of polyclonal IgG at a concentration of 500 μg/ml in PBS. Incubations were for 2 h at room temperature. Subsequently, the unbound antibodies were removed by three consecutive washes with PBS and possible aggregates of Covaspheres were dissolved by ultrasonication of each sample in a bat sonicator for 2 min at room temperature. Thus, no unbound antibodies were present during coincubations of Covaspheres.

Flow cytometric analysis of the interactions of protein-coated Covaspheres was performed on an Epics Profile equipped with standard Powerpak filter configuration (Coulter Corp., Hialeah, FL). The fluorescence excitation was effected by an air-cooled 15-mW argon laser using 488 nm as the exciting wavelength. The 90° fluorescence emission leaving the flow chamber was separated from scattered light by two filters reflecting the 488-nm laser wavelength. Green fluorescence (FITC) was reflected by a 550-nm long pass filter and directed through a 525-nm band pass filter for measurement. Red fluorescence (TRITC) was reflected by a 600-nm short pass filter and passed through a 625 nm band pass filter. The spectral overlap of FITC and TRITC emission was electronically compensated for. Fluorescence parameters were collected using a four decade logarithmic amplifier over a range of 1–1,024 channels, analyzing a minimum of 100,000 events for each test.

In agreement with the supplier's specifications, the Covaspheres were found to be highly homogenous with respect to the fluorescence intensities they emit, as revealed by analytical runs with 100,000 uncoated beads. The FITC Covaspheres exhibited an average relative fluorescence intensity of 0.64 with an SD of 0.13; the TRITC Covaspheres had an average relative fluorescence intensity of 0.96 and an SD of 0.12.

Cell Cultures

The DRGs used for cultivation were dissected from 10-d-old chicken embryos. Dissociation was carried out after enzymatic digestion as detailed previously (Sonderegger et al., 1985).

For the immunocytochemical localization of axonin-1 and L1(G4), dissociated cultures of DRG neurons were plated on poly-D-lysine/laminin-coated tissue culture dishes. For coating, 35-mm cell culture dishes were incubated with 20 μg/ml poly-D-lysine in 150 mM sodium borate, pH 8.4 at 37°C for 16 h. The coating solution was then removed and the plates were washed at least four times with distilled water and dried. For coating with laminin, dry poly-D-lysine-coated dishes were incubated with 10 μg/ml laminin in PBS for 1 h at 37°C and washed twice with PBS immediately before plating the dissociated neurons.

The binding of protein-conjugated Covaspheres was studied with dissociated DRG cultures. The cells were cultivated on collagen-coated culture dishes for 5 d under conditions described previously (Sonderegger et al., 1985). To reduce the medium requirements to 300 μl the cell culture surface was reduced to a 1.5-cm-diam circular area by a donut-shaped teflon ring that was fixed onto the dry surface with silicon grease (Bayer AG, Leverkusen, Germany). Into each of these wells, 25,000 dissociated cells were plated. To minimize proliferation of nonneuronal cells, 0.12 mM fluorodeoxyuridine and 0.3 mM uridine (both from Sigma Chemical Co.) were present in the medium over the entire period of cultivation. In such cultures, only a few nonneuronal cells survived; they were never found to cover or ensheathe the neurites in scanning EM inspections.

Neurite outgrowth was assessed on axonin-1, L1(G4), and laminin, which were absorbed directly to tissue culture plastic as specified previously (Stoeckli et al., 1991) using coating concentrations of 75 μg/ml. After incubation for 2 h the plates were washed twice with PBS and blocked with 10 mg/ml ovalbumin in PBS for 45 min. Dissociated DRG cells were cultivated from the time of plating in serum-free medium, as given in detail by Stoeckli et al. (1991); however, ovalbumin was used instead of BSA. Anti-axonin-1 Fab fragments of goat IgG or anti-L1(G4) Fab fragments of rabbit IgG were added at concentrations of 500 μg/ml.

Immunocytochemical Stainings

Immunocytochemical stainings were carried out on paraformaldehyde-fixed cells. For fixation, the cells were washed with PBS and incubated with 2% paraformaldehyde in 0.1 M sodium phosphate, pH 7.2 for 1 h at 37°C. After removal of the fixing solution, the cells were washed three times with PBS. For indirect double immunofluorescent staining, the protocol previously given in detail was followed (Sonderegger et al., 1985). Anti-axonin-1 antiserum (from goat) and anti-L(G4) antiserum (from rabbit) were incubated simultaneously at a dilution of 1:300. After washing off the first antibodies, the fluorescent second antibodies were added and incubated simultaneously. FITC-conjugated rabbit anti-goat IgG was from Bio-Science Products AG (Emmenbrücke, Switzerland) and used at a dilution of 1:10. TRITC-conjugated goat anti-rabbit IgG was from Zymed Laboratories Inc. (South San Francisco, CA) and used at 1:10.

Binding of Protein-conjugated Covaspheres to Axons in Culture

Cultures of dissociated DRGs were washed twice with PBS and twice with serum-free medium (Stoeckli et al., 1991). Protein-conjugated Covaspheres were diluted 1:1,000 in the same serum-free medium. The diluted Covasphere suspension was sonicated 2 min in a bath sonicator and immediately added to the cultures in substitution of the medium. Incubation was for 1 h in a cell culture incubator at 37°C, 10% CO_2. To remove unbound Covaspheres the cultures were subjected to three consecutive washings with PBS at 37°C. Inspection on an inverted microscope (Nikon) equipped with fluorescence optics followed without fixation.

Antibody perturbation experiments were carried out by preincubation of either the Covaspheres or the cells with Fab fragments of polyclonal IgG at a concentration of 500 μg/ml. Incubations of the Covaspheres were for 2 h at 20°C; incubations of the cultures were for 1 h at 37°C in a CO_2 incubator. At the end of the incubation period the Fab fragments were removed and the spheres or the cells were washed twice with PBS or culture medium, respectively.

Measurements of Neurite Length

Neurite lengths were measured as described previously in detail (Stoeckli et al., 1991). In brief, cultures of dissociated DRG cells on different substrata were grown for 20 h in defined, serum-free medium. A plating area of ~20 mm^2 was inspected with an inverted microscope using phase contrast optics. Following the suggestion of Lagenaur and Lemmon (1987), a neurite was defined as a process extending from the neuronal cell body by more than a cell diameter. Only neurites that emerged from an isolated neuron (not a clump of cells) and did not contact other neurites or cells were included for length determination. The total length of all neurite branches elaborated by a neuron was measured. The percentage of neurons with neurites longer than a given length was plotted versus neurite length (Chang et al., 1987). As a characteristic for neurite growth under a given condition, the neurite length developed by 50% of the neurite-bearing neurons (NL_{50}) was determined (Stoeckli et al., 1991).

Results

Immunocytochemical Localization of Axonin-1 and L1(G4) on and Binding of Fluorescent Microspheres Coated with Axonin-1 and L1(G4), Respectively, to Neurites of Cultured DRG Neurons

Immunohistochemical localization of axonin-1 and L1(G4) by double immunofluorescence staining under native conditions revealed that both glycoproteins are proteins at the surface of somas and neurites of DRG neurons in dissociated culture (Fig. 1, *a–c*). Among the neurites, the staining was not uniformly distributed, but occurred as irregularly spaced bright spots along the neurites. Most spots emitted both green and red fluorescence. No staining was found in control experiments in which the primary antibody against axonin-1 (from goat) and L1(G4) (from rabbit) were followed by the inappropriate secondary antibody, namely, anti-rabbit IgG and anti-goat IgG, respectively. Hence, insufficient specificity of the second antibodies leading to crossreactive staining was excluded (data not shown). Crossreactivity of the antibodies against axonin-1 and L1(G4) employed was excluded by using antigen in different forms. No crossreactions were observed either when the antigens were run on SDS-PAGE after reduction and subsequently electrotransferred to nitrocellulose paper (Fig. 2), or when native, nonreduced axonin-1 and L1(G4) were dotted to paper (not shown). Anti-axonin-1 antibodies and anti-L1(G4) antibodies were also specific when confronted with native axonin-1 and L1(G4)

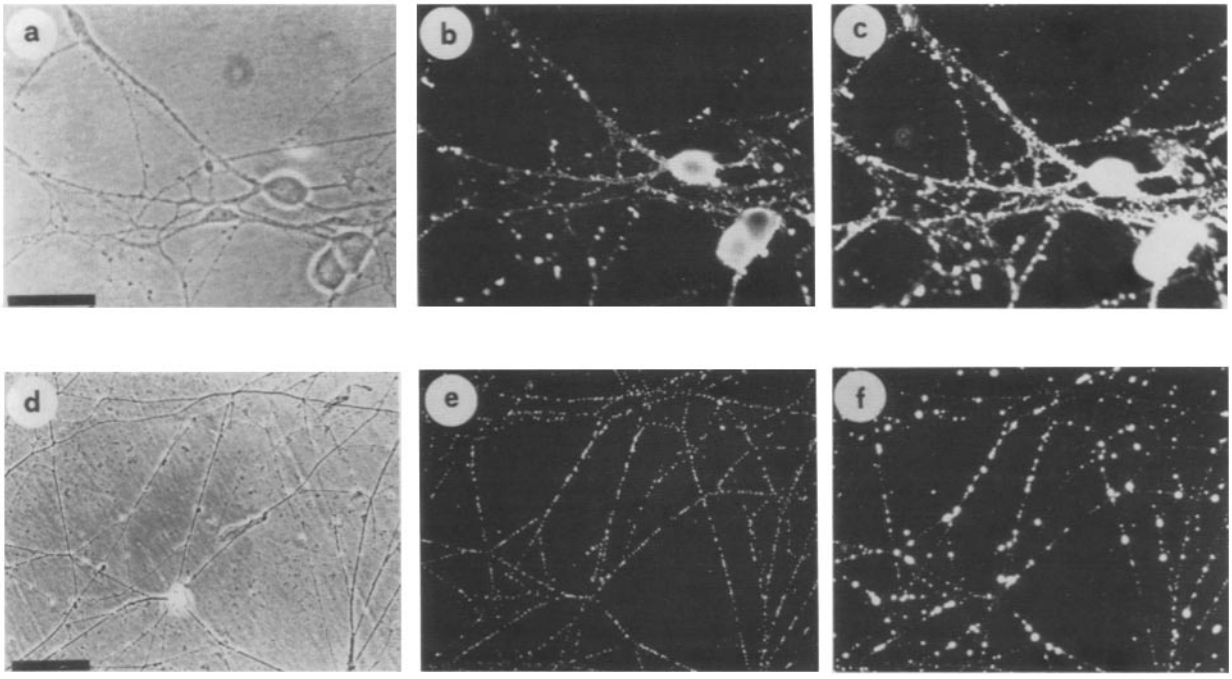

Figure 1. Localization of axonin-1 and L1(G4), and their receptors, on cultured DRG neurons. (*a–c*) Immunocytochemical localization of axonin-1 and L1(G4) on paraformaldehyde-fixed cells by double indirect immunofluorescence. (*a*) Phase-contrast optics; (*b*) fluorescent staining of axonin-1 by goat anti-axonin-1 antiserum followed by FITC-conjugated anti-goat IgG; (*c*) fluorescent staining of L1(G4) by rabbit anti-L1(G4) antiserum followed by TRITC-conjugated anti-rabbit IgG. (*d–f*) Binding of fluorescent microspheres conjugated with axonin-1 and L1(G4) to unfixed cells. (*d*) Phase-contrast optics; (*e*) red fluorescent Covaspheres conjugated with axonin-1; (*f*) green fluorescent Covaspheres conjugated with L1(G4). *Bar*: 50 μm (*a–c*); 100 μm (*d–f*).

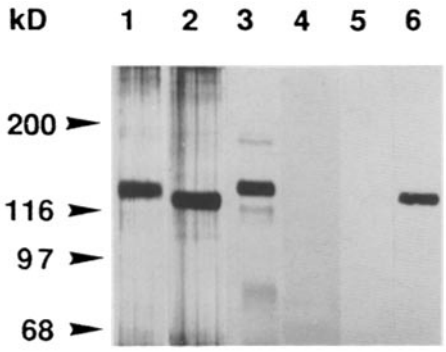

Figure 2. Control for cross-contamination in the preparations of axonin-1 and L1(G4), and for crossreactivity of anti-axonin-1 and anti-L1(G4) antibodies. (Lanes *1* and *2*) Test for cross-contaminations in the preparations of axonin-1 and L1(G4), respectively. Aliquots taken from the preparations of axonin-1 and L1(G4) used in this study were subjected to SDS-PAGE and analyzed for cross-contamination by silver staining. (Lane *1*) 0.25 μg L1(G4); (lane *2*) 0.25 μg axonin-1. (Lanes *3–6*) Direct and crossed immunoblots on preparations of axonin-1 and L1(G4) with Fab fragments against axonin-1 and L1(G4). The Fab fragments against axonin-1 and L1(G4) used in this test were from the same preparation as those used in all antibody perturbation experiments presented in this paper. (Lanes *3* and *4*) 0.25 μg L1(G4) and 0.25 μg axonin-1, respectively, stained for L1(G4) with rabbit anti-L1(G4) Fab fragments; (lanes *5* and *6*) 0.25 μg L1(G4) and 0.25 μg axonin-1, respectively, stained for axonin-1 with goat anti-L1(G4) Fab fragments.

that were covalently coupled to the surface of fluorescent microspheres (Covaspheres), as revealed by indirect immunofluorescence using a second antibody with a fluorochrome distinct from that contained in the Covaspheres.

When Covaspheres coated with either axonin-1 or L1(G4) were incubated with viable DRG neurons in culture, they aligned along all the detectable neurites or neurite bundles (Fig. 1, *d–f*). Spots of fluorescence emission with irregular spacing were found along the neurites. Signals emitted from L1(G4) Covaspheres were larger and brighter than those of axonin-1 Covaspheres, regardless of the color of the spheres chosen. In view of the reported homophilic binding of L1(G4), this observation probably reflects the fact that L1(G4) Covaspheres form aggregates during incubation. If both axonin-1 and L1(G4) Covaspheres were incubated simultaneously, double green and red light emission was observed at virtually identical locations, as well as red fluorescence at locations where there was no green fluorescence, and vice versa. These data may be interpreted as evidence for the presence of a putative receptor on neuritic membranes for both axonin-1 and L1(G4).

Flow Cytometric Studies of Aggregate Formation of Protein-coated Fluorescent Microspheres

The affinity between axonin-1, L1(G4), and a variety of other proteins was tested in a bead aggregation assay previously found suitable for the detection of weak macromolecular in-

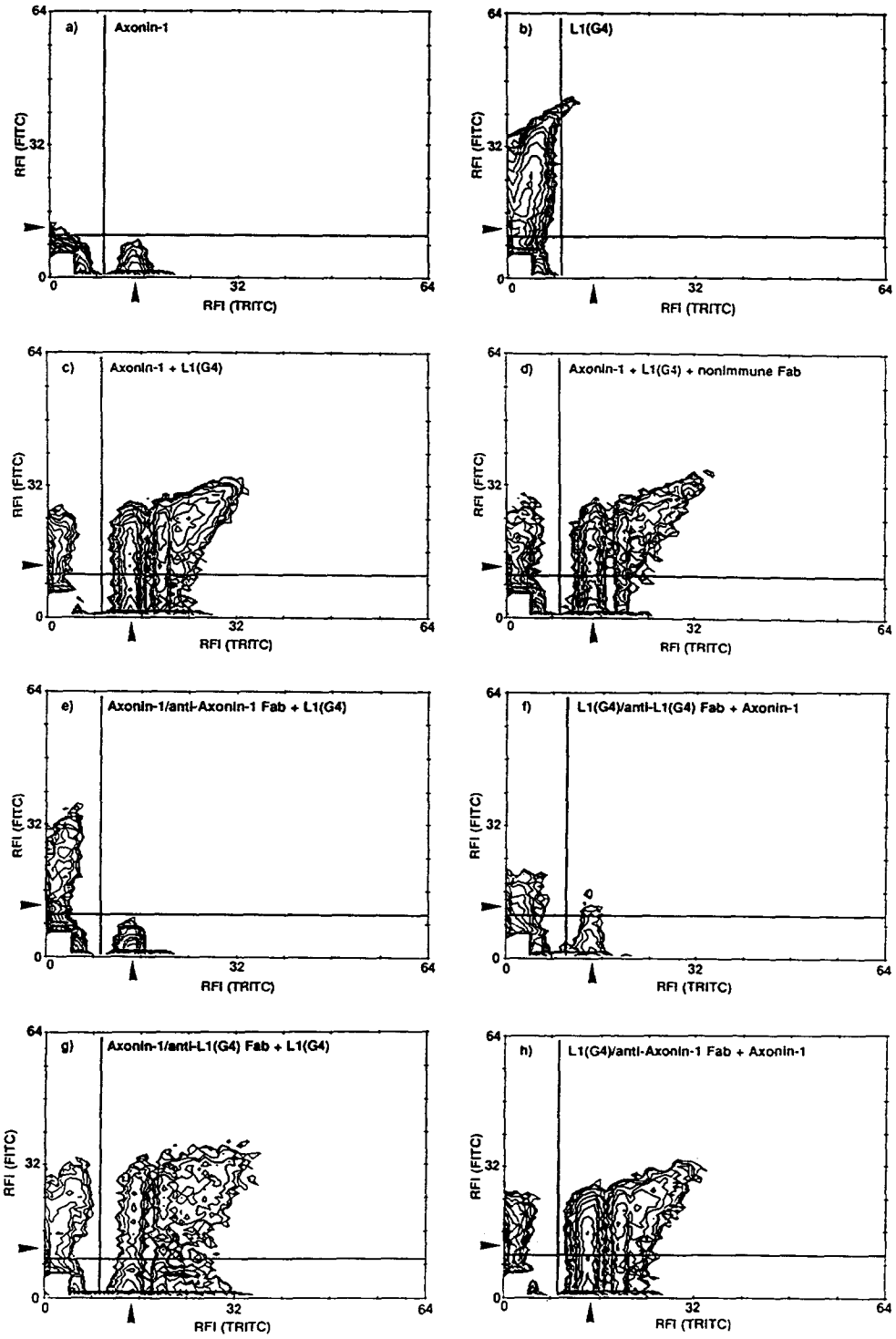

Figure 3. Aggregation of Covaspheres coated with axonin-1 and L1(G4). Protein-coated Covaspheres were allowed to aggregate by incubation for 1 h. For flow cytometric analysis, small aliquots were diluted 10,000-fold and injected. The state of aggregation was visualized by contour plots in a two-dimensional representation of the relative fluorescence intensity. The relative fluorescence intensity of Covaspheres passing as single units is indicated by arrows. In all the experimental conditions presented in this figure, axonin-1 was bound to TRITC Covaspheres and L1(G4) to FITC Covaspheres. Mixtures of axonin-1 and L1(G4) Covaspheres were assayed with an initial ratio of 1:1. For antibody perturbation tests, Fab fragments were used in preincubations with one species of Covasphere only; before mixing the Covaspheres for aggregation testing, unbound antibodies were removed. (*a*) Axonin-1-conjugated TRITC Covaspheres; (*b*) L1(G4)-conjugated FITC Covaspheres; (*c*) coincubation of FITC Covaspheres coated with L1(G4) and TRITC Covaspheres coated with axonin-1; (*d*) coincubation of Covaspheres coated with L1(G4) and axonin-1 in the presence of 1 mg/ml nonimmune Fab fragments; (*e*) coincubation of L1(G4) Covaspheres with axonin-1 Covaspheres preincubated with anti-axonin-1 Fab fragments; (*f*) coincubation of axonin-1 Covaspheres with L1(G4) Covaspheres preincubated with anti-L1(G4) Fab fragments; (*g*) coincubation of L1(G4) Covaspheres with axonin-1 Covaspheres preincubated with anti-L1(G4) Fab fragments; (*h*) coincubation of axonin-1-Covaspheres with L1(G4) Covaspheres preincubated with anti-axonin-1 Fab fragments.

teractions (Hoffman and Edelman, 1987). Pure axonin-1, L1(G4), BSA, laminin, fibronectin, IgG, and transferrin were covalently attached to red or green fluorescent spheres (TRITC and FITC Covaspheres, respectively) and their affinity was measured as a function of their capability to cause the formation of mixed aggregates. Aggregate formation and the relative content of red or green spheres were analyzed with a fluorescence-activated flow cytometer.

Covaspheres coated with covalently attached axonin-1 did not aggregate, as indicated by the fact that their fluorescent light emission occurred at the intensity of single beads only (Fig. 3 *a*). In agreement with a previous report by Grumet

and Edelman (1988) on Ng-CAM, L1(G4)-conjugated Covaspheres formed aggregates (Fig. 3 *b*). As calculated from the relative fluorescence intensity of the aggregates in both colors, aggregate sizes of up to 12 beads were found. When axonin-1- and L1(G4)-conjugated Covasphere were incubated together, they formed mixed aggregates, as indicated by the concomitant emission of both red and green fluorescent light (Fig. 3 *c*). Approximately 70% of the TRITC fluorescence of the axonin-1 Covaspheres was detected in mixed aggregates, ~25% of the TRITC fluorescence exhibited a relative fluorescence intensity corresponding to single beads, and ~5% of the fluorescence was associated with particles having higher than unity TRITC fluorescence together with lower than unity FITC fluorescence. Multiple axonin-1 Covaspheres apparently occurring without the participation of L1(G4) Covaspheres was an unexpected observation, since self-aggregation of axonin-1 Covaspheres was never observed. The most straightforward explanation of this phenomenon may be that in these particles the FITC fluorescence of an L1(G4) sphere, for geometrical reasons, cannot be excited or its emission is quenched as a consequence of its particular situation with respect to other spheres in the aggregate during the passage through the detection chamber. The occurrence of such a geometrical quenching phenomenon concerning the FITC-labeled L1(G4) Covaspheres would have remained undetected, because of its overlap with real homoaggregates of L1(G4) Covaspheres.

In an initial series of experiments, aggregate formation between axonin-1- and L1(G4)-coated Covaspheres was tested at various concentrations and ratios of each Covasphere species (Fig. 4). At each of four concentrations of axonin-1 Covaspheres, a series of five different concentrations of L1(G4) Covaspheres was assayed. As demonstrated in Fig. 4 *a*, at each given concentration of axonin-1 Covaspheres, an increase in the initial concentration of L1(G4) Covaspheres resulted in an increase in the proportion of L1(G4) included in mixed aggregates; with a higher initial concentration of axonin-1 Covaspheres, a higher concentration of L1(G4) Covaspheres was necessary to obtain a given L1(G4)/axonin-1 ratio. However, at the Covasphere concentrations assayed, the ratio of L1(G4) Covaspheres to axonin-1 Covaspheres in mixed aggregates was determined by the input ratio, rather than by the absolute concentration of Covaspheres, as indicated by the fact that the ratio of L1(G4) Covaspheres to axonin-1 Covaspheres in mixed aggregates was virtually identical at all initial ratios of L1(G4) and axonin-1 Covaspheres, regardless of the absolute input concentration of Covaspheres (Fig. 4 *b*). Based on the fluorescence intensity of single beads, the composition of mixed aggregates was determined: formations with up to 12 spheres were observed, the median size aggregate containing eight beads. With equal initial concentrations of axonin-1 and L1(G4) Covaspheres, 60–80% of the Covaspheres were found in mixed aggregates. To obtain an optimum signal to noise ratio for both Covasphere species, we chose an input ratio of 1:1 at a concentration of 10^{10} Covaspheres/ml for all subsequent antibody perturbation experiments of the axonin-1/L1(G4) binding. The same parameters were used in all additional experiments performed to test for binding of axonin-1 or L1(G4) to other proteins bound to Covaspheres (Fig. 5).

Several observations indicate that the formation of mixed aggregates of axonin-1 and L1(G4) Covaspheres was mediated by a specific molecular mechanism (Fig. 5). Neither axonin-1-conjugated Covaspheres nor L1(G4)-conjugated Covaspheres aggregated with Covaspheres coated with other proteins, including BSA, laminin, fibronectin, IgG, and transferrin (Fig. 5, *a–e* and *g–k*). Axonin-1 Covaspheres of one color did not form mixed aggregates with axonin-1 Covaspheres of the other color (Fig. 5 *f*), regardless of the presence of absence of Ca^{2+} and Mg^{2+} ions, whereas L1(G4) Covaspheres, as expected (Grumet and Edelman, 1988), were found in aggregates (Fig. 5 *l*). The formation of mixed

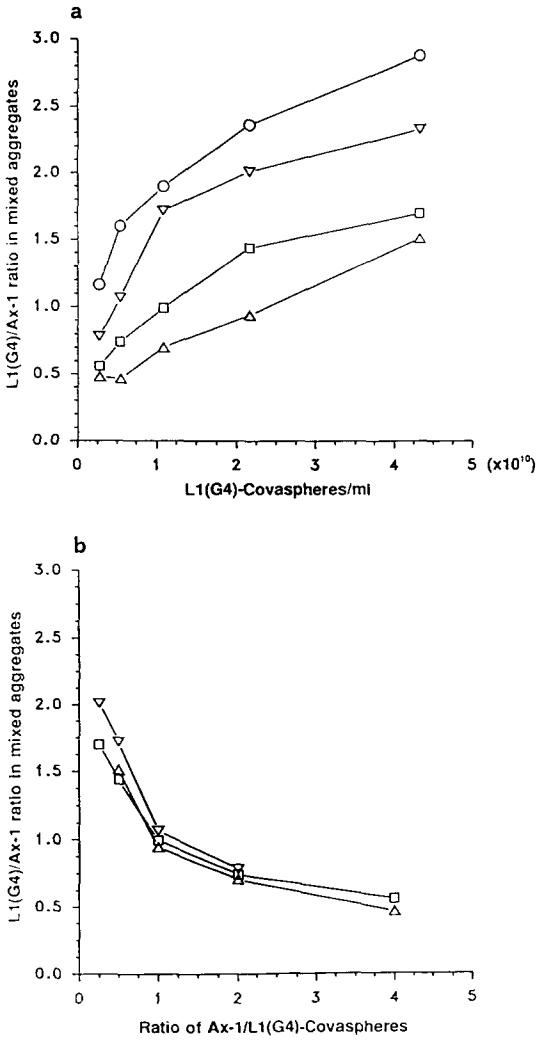

Figure 4. Aggregation of axonin-1 and L1(G4) Covaspheres at different initial concentrations and ratios of Covaspheres. At each of four concentrations of axonin-1 Covaspheres, a series of five different concentrations of L1(G4) Covaspheres were assayed for aggregation and the ratio of the sum of the L1(G4) Covaspheres and the sum of the axonin-1 Covaspheres that were detectable in mixed aggregates was determined. (*a*) The ratios of L1(G4) and axonin-1 Covaspheres were plotted versus the initial concentration of L1(G4) Covaspheres. (△) 2×10^{10} axonin-1 Covaspheres/ml; (□) 1×10^{10} axonin-1 Covaspheres/ml; (▽) 0.5×10^{10} axonin-1 Covaspheres/ml; (○) 0.25×10^{10} axonin-1 Covaspheres/ml. (*b*) The ratios of L1(G4) and axonin-1 Covaspheres in mixed aggregates were plotted versus the initial ratios between the two Covasphere species. (△) 2×10^{10} axonin-1 Covaspheres/ml; (□) 1×10^{10} axonin-1 Covaspheres/ml; (▽) 0.5×10^{10} axonin-1 Covaspheres/ml.

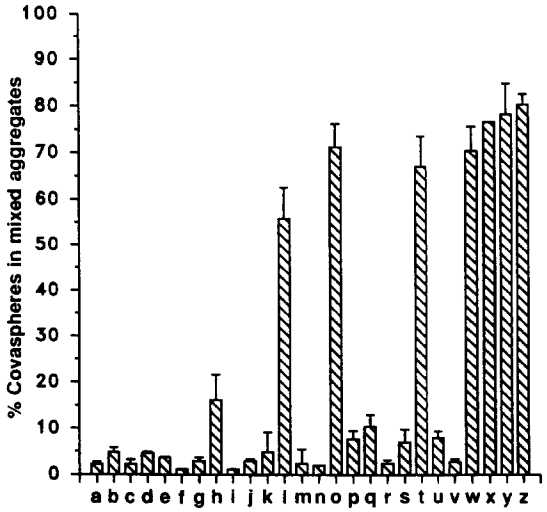

Figure 5. Incorporation of Covaspheres into mixed aggregates. The aggregation of L1(G4)- and axonin-1-coated Covaspheres with each other and either one of them with other proteins coupled to Covaspheres was assayed by pairwise coincubation of equal concentrations of Covaspheres, and the percentage of Covaspheres (of both species) found in mixed aggregates, as opposed to single spheres, was determined with the flow cytometer. In all conditions where Fab fragments of antibodies were used, they were preincubated with one species of Covasphere and unbound antibodies were removed before mixing the spheres for aggregation testing. Each experimental condition was measured in triplicate; the average value and the SD are given. (*a*) Axonin-1 versus BSA; (*b*) axonin-1 versus laminin; (*c*) axonin-1 versus fibronectin; (*d*) axonin-1 versus rabbit nonimmune IgG; (*e*) axonin-1 versus transferrin; (*f*) axonin-1 versus axonin-1; (*g*) L1(G4) versus BSA; (*h*) L1(G4) versus laminin; (*i*) L1(G4) versus fibronectin; (*j*) L1(G4) versus rabbit nonimmune LgG; (*k*) L1(G4) versus transferrin; (*l*) L1(G4) versus L1(G4); (*m*) L1(G4) versus trypsin-treated L1(G4); (*n*) L1(G4) versus heat-treated L1(G4); (*o*) axonin-1 versus L1(G4); (*p*) trypsin-treated axonin-1 versus L1(G4); (*q*) axonin-1 versus trypsin-treated L1(G4); (*r*) heat-treated axonin-1 versus L1(G4); (*s*) axonin-1 versus heat-treated L1(G4); (*t*) axonin-1 preincubated with nonimmune Fab fragments versus L1(G4); (*u*) axonin-1 preincubated with anti-axonin-1 Fab fragments versus L1(G4); (*v*) axonin-1 versus L1(G4) preincubated with anti-L1(G4) Fab fragments; (*w*) axonin-1 preincubated with anti-L1(G4) Fab fragments versus L1(G4); (*x*) axonin-1 versus L1(G4) preincubated with anti-axonin-1 Fab fragments; (*y*) axonin-1 preincubated with anti-fibronectin Fab fragments versus L1(G4); (*z*) axonin-1 preincubated with anti-laminin Fab fragments versus L1(G4).

aggregates of axonin-1 Covaspheres of the one color with L1(G4) Covaspheres of the other was successfully prevented by incubated with 0.5% trypsin for 14 h or by heating the Covaspheres in a boiling water bath for 10 min (Fig. 5, *p–s*).

Preincubation of the axonin-1-conjugated Covaspheres with anti-axonin-1 Fab fragments, and preincubation of L1(G4)-conjugated Covaspheres withanti-L1(G4) Fab fragments, prevented the formation of mixed aggregates (Fig. 3, *e* and *f*; Fig. 5, *u* and *v*). No interference with the formation of mixed aggregates was observed when nonimmune Fab fragments were used instead of anti-axonin-1 or anti-L1(G4) Fab fragments (Fig. 3 *d*; Fig. 5 *t*). Similarly, no perturbation of the formation of mixed aggregates occurred after preincubation of either species of Covaspheres with anti-fibronectin or anti-laminin Fab fragments (Fig. 5, *y* and *z*).

Preincubation of axonin-1-conjugated Covaspheres with anti-L1(G4) Fab fragments did not interfere with the formation of mixed aggregates with L1(G4)-conjugated Covaspheres (Fig. 3 *g*; Fig. 5 *w*). Similarly, after preincubation with anti-axonin-1 Fab fragments, L1 (G4)-conjugated Covaspheres were still capable of the formation of both L1(G4) homopolymers and mixed aggregates with axonin-1-conjugated Covaspheres (Fig. 3 *h*; Fig. 5 *x*). In quantitative terms the proportion of Covaspheres included in mixed aggregates was identical to that found without antibodies or in the presence of nonimmune serum. In conjunction, the presented data show that a contamination of axonin-1 with L1(G4) and of L1(G4) with axonin-1, as an explanation of the mixed aggregates, may be excluded.

Interaction of Protein-coated Fluorescent Microspheres with Axons of Cultured Neurons

To locate cellular binding sites for axonin-1, neural cultures were exposed to axonin-1 Covaspheres (Figs. 6 and 7). As illustrated in Fig. 6 *a*, axonin-1-conjugated spheres exposed to dissociated dorsal root ganglion cells bound preferentially to neurites. On both single neurites and neurite fascicles axonin-1-conjugated fluorescent spheres were aligned as pearls on a string.

The binding of axonin-1-conjugated spheres to neurites was specifically mediated by axonin-1, as verified by a series of controls. No binding was observed when BSA was substituted for axonin-1 on the spheres (Fig. 6 *b*), and when axonin-1-conjugated Covaspheres were preincubated with 500 μg/ml of anti-axonin-1 Fab fragments (Fig. 6 *c*). Preincubation of the axonin-1 Covaspheres with 500 μg/ml anti-L1(G4) Fab fragments did not prevent their binding to neurites (Fig. 6 *d*). This indicates that the binding molecule on the axonin-1 Covaspheres was not L1(G4), derived from contamination of the axonin-1 preparation used for coating of the Covaspheres.

The ligand for axonin-1 Covaspheres on the DRG neurites was identified by preincubations of the cultures with Fab fragments of polyclonal IgG. Preincubation of the cells with anti-L1(G4) Fab fragments resulted in a complete absence of binding of axonin-1-conjugated spheres to the neurite membranes (Fig. 6 *e*). The binding of axonin-1-coated Covaspheres to neurites was not prevented by preincubation of the cells with anti-axonin-1 Fab fragments (Fig. 6 *f*). These data indicate that the binding of axonin-1-conjugated spheres to DRG neurites is mediated by axonal L1(G4). Furthermore, the complete prevention of Covasphere binding after preincubation of the cultures with anti-L1(G4) Fab fragments identified L1(G4) as the only axonin-1-binding ligand detectable on the axonal membranes of embryonic dorsal root ganglia neurons of the chicken, within the sensitivity of the used Covasphere assay.

When fluorescent spheres with covalently attached L1(G4) were incubated with cultured DRG neurons, they also aligned with neurites (Fig. 7 *a*). Preincubation of the spheres with anti-L1(G4) Fab fragments completely prevented their binding to neurites (Fig. 7 *b*), whereas preincubation with anti-axonin-1 Fab fragments was without effect (Fig. 7 *c*). These data indicate that binding of the L1(G4)-conjugated Covaspheres is indeed mediated by L1(G4) and presents further evidence for the absence of axonin-1 in the preparation of L1(G4).

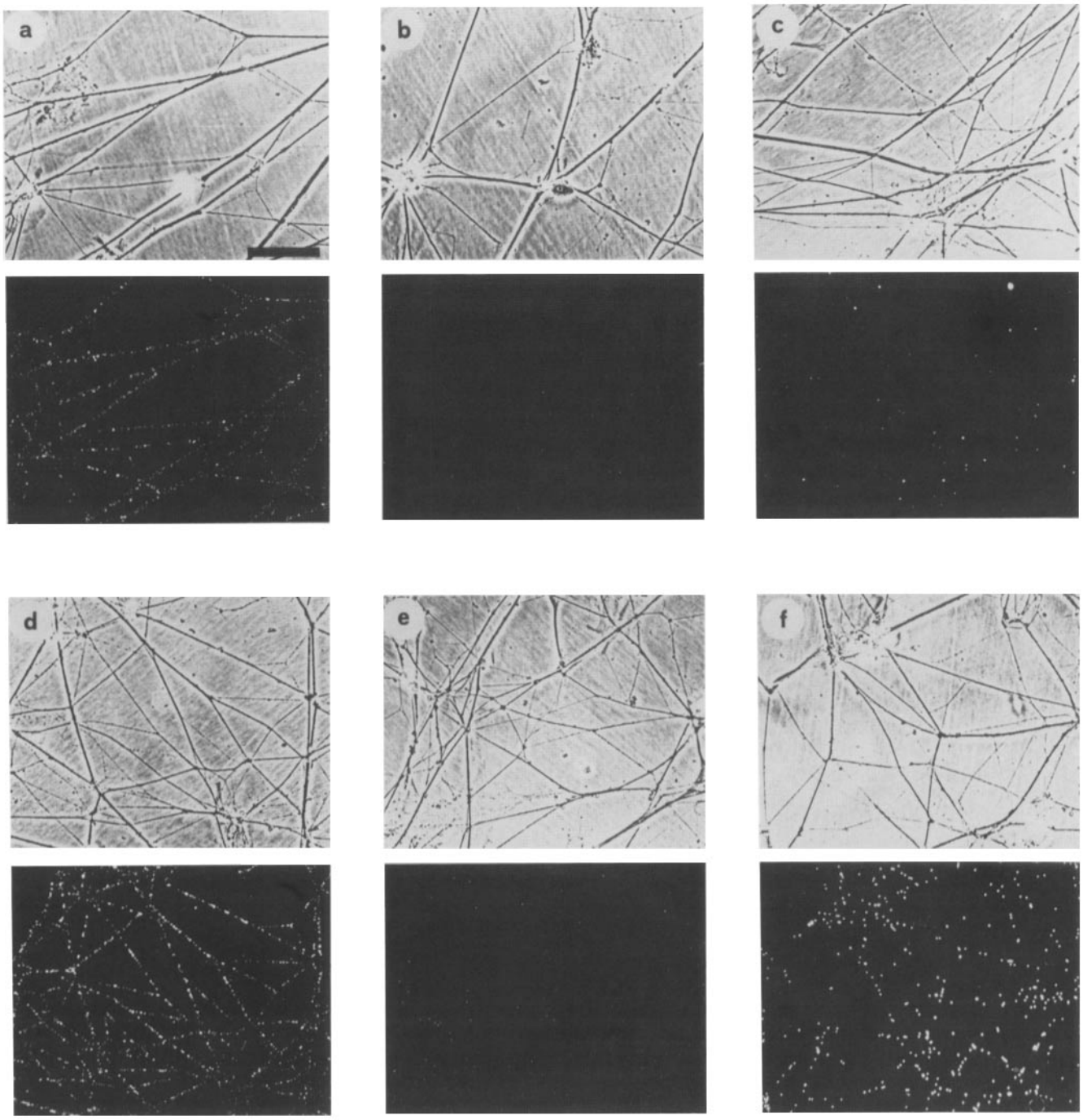

Figure 6. Binding of axonin-1-coated Covaspheres to cultured DRG neurons and their axons. Covaspheres coated with axonin-1 (or BSA for a control) were added to the medium of cultured DRG neurons without prior fixation and incubated for 1 h at 37°C. At the end of the incubation, the unbound Covaspheres were removed and the cell-bound Covaspheres were visualized on an inverted microscope equipped with phase (*upper panels*) and fluorescence (*lower panels*) optics. For antibody perturbation of Covasphere binding, either the Covaspheres or the cells were preincubated with 0.5 mg/ml Fab fragments. Before the addition of the Covaspheres to the cells, unbound antibodies were removed. (*a*) Axonin-1-coated Covaspheres on DRG cultures; (*b*) BSA-coated Covaspheres on DRG cultures; (*c*) preincubation of axonin-1-coated Covaspheres with anti-axonin-1 Fab fragments before addition to the culture; (*d*) preincubation of axonin-1-coated Covaspheres with anti-L1(G4) Fab fragments before addition to the culture; (*e*) preincubation of cells with anti-L1(G4) Fab fragments before addition of axonin-1-coated Covaspheres; (*f*) preincubation of cells with anti-axonin-1 Fab fragments before addition of axonin-1-coated Covaspheres. *Bar*: 50 μm (*a–f*).

After preincubation of DRG cultures with anti-L1(G4) Fab fragments, L1(G4) Covaspheres were still able to bind (Fig. 7 *d*). However, the number of particles bound to neurites was reduced (not quantified). Similarly, a reduction of the number of bound L1(G4) Covaspheres also resulted from preincubation of the neurons with anti-axonin-1 Fab fragments (Fig. 7 *e*). Thus, on neurites of DRG neurons both L1(G4) and axonin-1 appear to be binding partners for L1(G4) bound to Covaspheres. The blockage of both axonin-1 and L1(G4) on neurites by simultaneous incubation of the cultures with

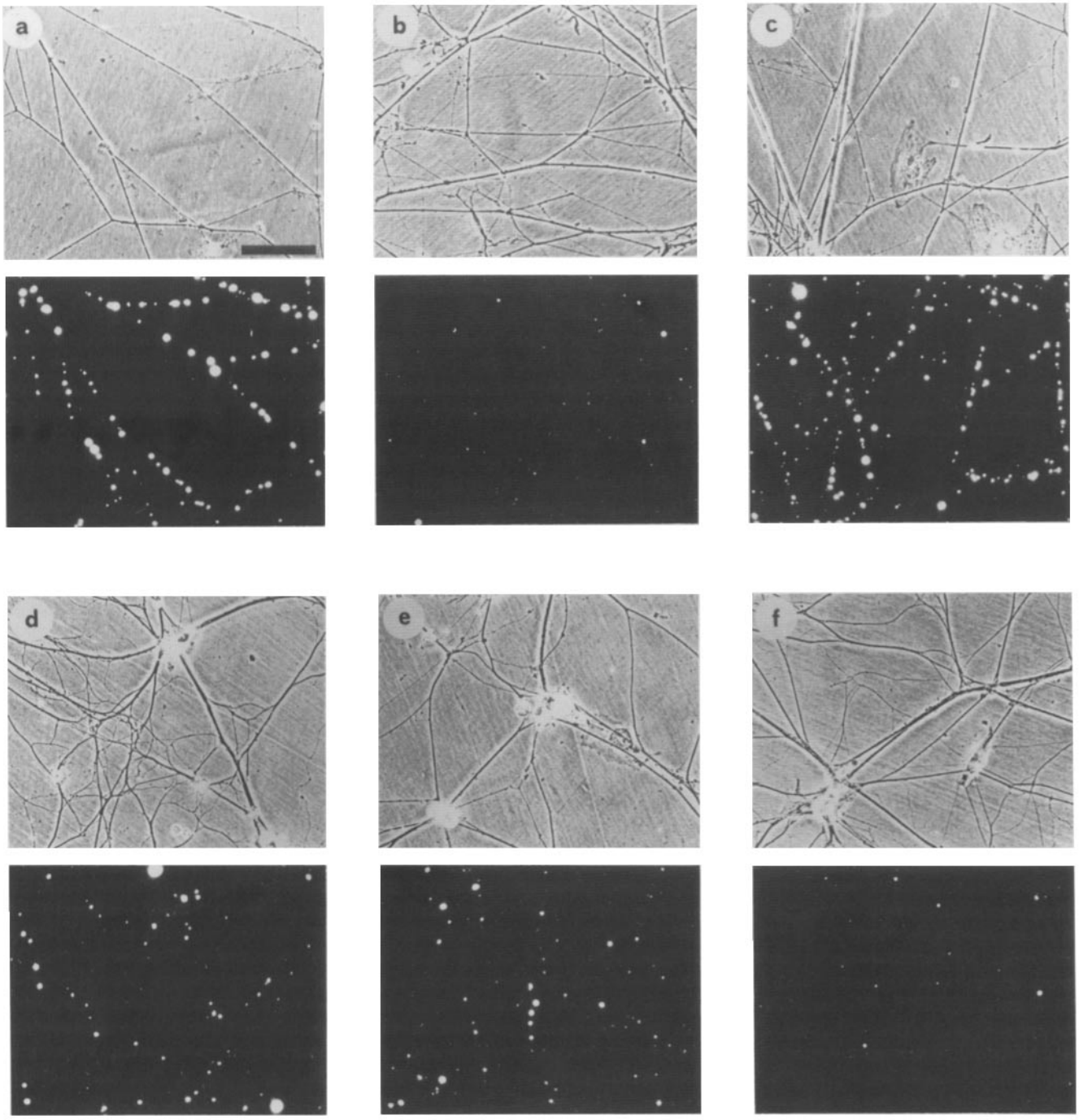

Figure 7. Binding of L1(G4)-coated Covaspheres to cultured DRG neurons and their axons. Covaspheres coated with L1(G4) were incubated together with DRG neurons and their binding visualized as specified in Fig. 6. (*a*) L1(G4)-coated Covaspheres on DRG cultures; (*b*) preincubation of L1(G4)-coated Covaspheres with anti-L1(G4) Fab fragments before addition to the culture; (*c*) preincubation of L1(G4)-coated Covaspheres with anti-axonin-1 Fab fragments before addition to the culture; (*d*) preincubation of cells with anti-L1(G4) Fab fragments before addition of L1(G4)-coated Covaspheres to the culture; (*e*) preincubation of cells with anti-axonin-1 Fab fragments before addition of L1(G4)-coated Covaspheres; (*f*) simultaneous preincubation of cells with anti-axonin-1 and anti-L1(G4) Fab fragments before addition of L1(G4)-coated Covaspheres. *Bar*: 50 μm (*a–f*).

both antibodies prevented the binding of L1(G4) Covaspheres completely (Fig. 7*f*), indicating the absence of further major binding partners for L1(G4) on DRG neurites.

Identification of the Axonal Receptor Involved in Neurite Outgrowth on Immobilized Axonin-1

The finding that the only observable interaction of axonin-1-conjugated Covaspheres with neurites of cultured DRG neurons was mediated by L1(G4) implicated L1(G4) of the neuritic membrane as the receptor mediating the recently reported neurite growth-promoting effect of immobilized axonin-1 observed with cultured DRG neurons (Stoeckli et al., 1991). To test this, neurite growth from dissociated DRG neurons on immobilized axonin-1, L1(G4), and laminin, respectively, was studied in the presence of 500 μg/ml poly-

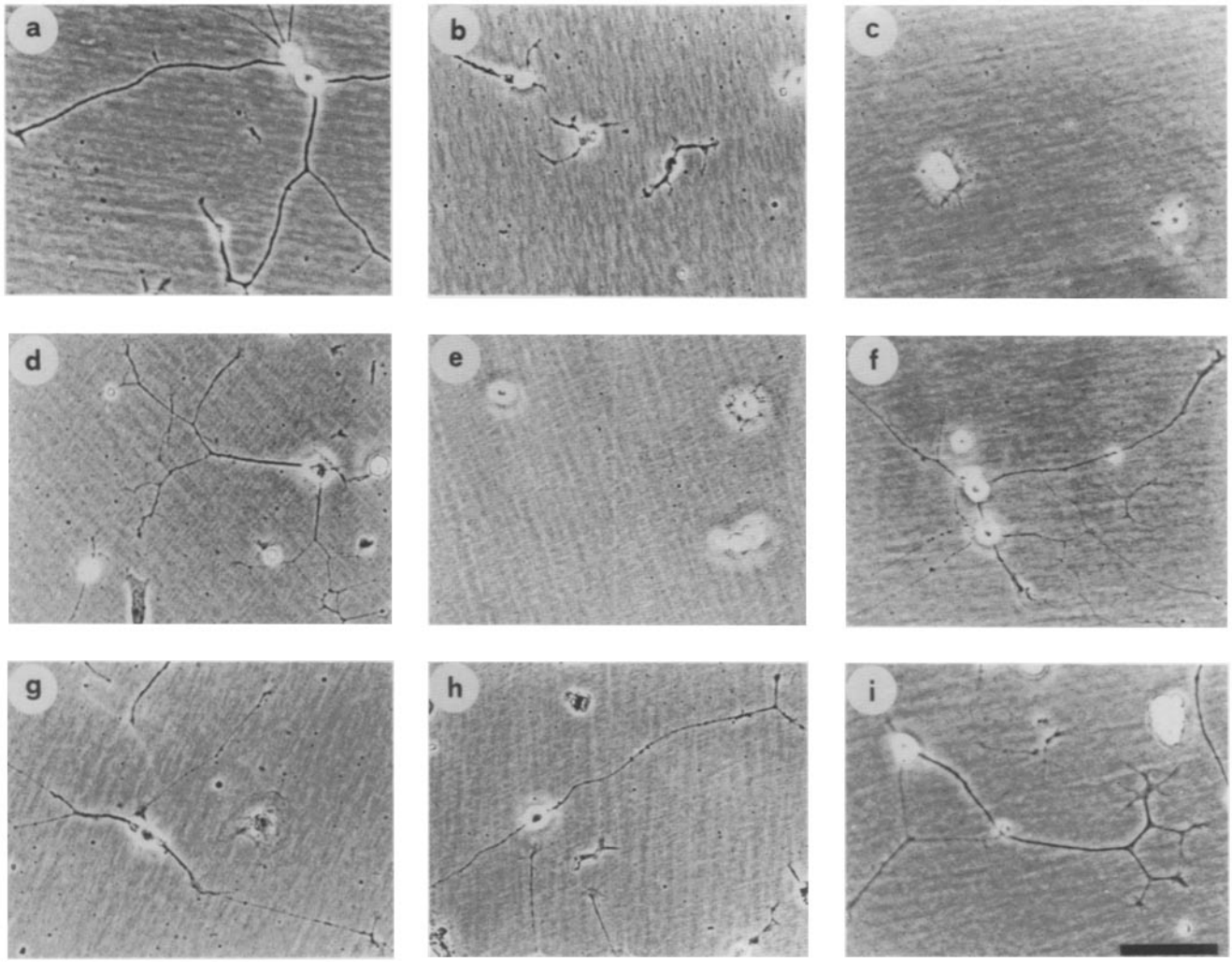

Figure 8. Perturbation of neurite growth on immobilized axonin-1, L1(G4), and laminin by Fab fragments of anti-L1(G4) or anti-axonin-1 IgG. Dissociated DRG neurons were plated on immobilized axonin-1, L1(G4), and laminin in a serum-free, defined medium and incubated for 20 h. To test for antibody perturbation of neurite outgrowth, Fab fragments of anti-axonin-1 or anti-L1(G4) antibodies were added at the time of plating to a final concentration of 500 μg/ml. Phase-contrast optics. *Bar*: 100 μm (*a–i*). (*a–c*) Cultures on immobilized axonin-1; (*d–f*) cultures on immobilized L1(G4); (*g–i*) cultures on immobilized laminin; (*b*, *e*, and *h*) Anti-L1(G4) Fab fragments; (*c*, *f*, and *i*) anti-axonin-1 Fab fragments.

clonal Fab fragments against axonin-1 or L1(G4). The results of this study are illustrated in Fig. 8. Both anti-L1(G4) and anti-axonin-1 Fab fragments strongly inhibited neurite outgrowth on axonin-1 (Fig. 8, *b* and *c*). Using L1(G4) as a substratum (Fig. 8, *d–f*), the presence of anti-L1(G4) Fab fragments also prevented neurite outgrowth (Fig. 8 *e*); in the presence of anti-axonin-1 Fab fragments, however, good neurite growth was found (Fig. 8 *f*). With laminin as a substratum (Fig. 8, *g–i*), neither anti-axonin-1 nor L1(G4) Fab fragments evoked any detectable perturbation of neurite growth (Fig. 8, *h* and *i*, respectively). These controls exclude a general or toxic inhibition of neurite growth by the Fab fragment preparations used.

In Fig. 9, a quantitative assessment of neurite outgrowth in the presence or absence of Fab fragments against axonin-1 and L1(G4), respectively, is given. The total length of all neurite branches extending from a neuron was determined for at least 150 neurons per experiment, except when the neurites were so short that most of them did not extend over the length of one cell diameter, and hence, did not fulfill the requirements to be considered as a neurite. In these cases, namely, anti-axonin-1 Fab fragments on axonin-1 substratum and anti-L1(G4) Fab fragments on L1(G4) substratum, the number of measured neurite-bearing neurons was between 20 and 30. The data obtained are presented using the graphic representation introduced by Chang et al. (1987). In this representation, the percentage of neurons with neurites longer than a given length is plotted versus neurite length. The resulting curve then represents the distribution of neurite lengths in a given neuronal population, and the neurite length reached by 50% of the neurite-bearing neurons (NL_{50}) was given as a characteristic for neurite growth (Stoeckli et al., 1991).

The quantitative data confirm that on axonin-1 (Fig. 9 *a*) neurites are almost completely inhibited by anti-axonin-1 Fab fragments: antibody inhibited neurites had an NL_{50} of

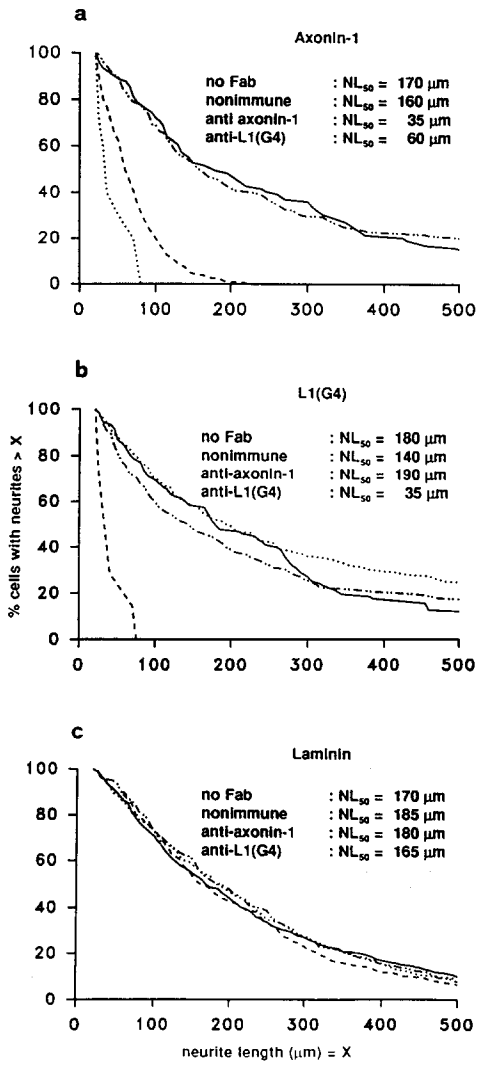

Figure 9. Quantification of neurite growth-perturbing effects of anti-axonin-1 and anti-L1(G4) Fab fragments. Dissociated DRG neurons were grown on immobilized axonin-1, L1(G4), and laminin, respectively, in the absence of antibodies or in the presence of either 500 µg/ml anti-axonin-1 Fab fragments, 500 µg/ml anti-L1(G4) Fab fragments, or 500 µg/ml nonimmune Fab fragments. The total length of all neurite branches was measured for at least 150 neurons per experimental condition. In two cases, namely, anti-axonin-1 on axonin-1 substratum and anti-L1(G4) on L1(G4), respectively, when the neurites were so short that most of them did not reach one cell diameter and hence did not qualify to be measured, neurite lengths of 20–30 neurons were determined. The neurite lengths were plotted as percentage of neurons with neurites longer than a given length (*y* axis) versus the neurite length (*x* axis), as introduced by Chang et al. (1987). (*a*) Dissociated DRG neurons cultivated on tissue culture plastic coated with axonin-1. (*b*) DRG neurons cultured on plastic-adsorbed L1(G4). (*c*) DRG neurons cultured on plastic-adsorbed laminin. *Solid line*, neurites grown in the absence of antibodies; *dashed line*, neurites grown in the presence of anti-L1(G4) Fab fragments; *dotted line*, neurites grown in the presence of anti-axonin-1 Fab fragments; *dash-dotted line*, neurites grown in the presence of nonimmune Fab fragments.

35 µm, as opposed to 170 µm without antibodies and 160 µm with nonimmune Fab fragments. The neurites grown on axonin-1 in the presence of anti-L1(G4) Fab fragments were only slightly longer than those with anti-axonin-1 Fab fragments (NL_{50} = 60 µm). In light of the fact that the Fab fragments used did not reduce neurite length on laminin (Fig. 9 *c*), these effects must be attributed to specific antibody binding, and antibody toxicity is excluded. Overall, these data identify L1(G4) as the neuritic receptor involved in the process of neurite growth on axonin-1 substratum. In addition, the large extent of inhibition of neurite growth in the presence of anti-L1(G4) Fab fragments suggests that L1(G4) may be a major molecule of the neurite membrane involved in the promotion of neurite growth from these neurons on immobilized axonin-1.

Neurites grown on L1(G4) (Fig. 9 *b*) had an NL_{50} of 180 µm in the absence of antibodies and 140 µm in the presence of nonimmune Fab fragments. When anti-L1(G4) Fab fragments were present, only a small proportion of the neurons had processes that could be considered as neurites (at least one cell diameter long) and those qualifying as neurites were very short (NL_{50} = 35 µm). In the presence of anti-axonin-1 Fab fragments, NL_{50} was 190 µm, hence neurite outgrowth was not perturbed.

Discussion

The results reported here demonstrate that the AxCAMs axonin-1 and L1(G4) bind to each other. In DRG neurons cultured on immobilized axonin-1, L1(G4) was found to be the neuritic receptor involved in the promotion of neurite growth. Thus, surface-exposed L1(G4) of neuritic membranes mediates neurite growth not only on a L1(G4) substratum by means of the well-established homophilic L1/L1 interaction (Grumet and Edelman, 1988; Lemmon et al., 1989; Kadmon et al., 1990), but also on axonin-1 by means of a heterophilic L1/axonin-1 interaction.

The binding behavior of axonin-1 was highly selective. Axonin-1-conjugated Covaspheres did not interact with Covaspheres coated with other proteins, such as laminin, fibronectin, IgG, and transferrin. In particular, under binding conditions and ligand concentrations in which both the homophilic L1/L1 and the heterophilic L1/axonin-1 interactions readily occurred, no evidence for a homophilic interaction of axonin-1 molecules was found, irrespective of the presence or absence of Ca^{2+} or Mg^{2+} ions in the medium. Furthermore, its heterophilic binding to L1(G4) was found to be the only adhesive interaction of axonin-1 Covaspheres with cultured DRG neurites, as revealed by the fact that both binding of axonin-1 Covaspheres and neurite growth on axonin-1 substratum were completely suppressed when its interaction with neuronal L1(G4) was perturbed by anti-L1(G4) antibodies. This represents indirect evidence that, at the sensitivity of detection of the microsphere assay, axonin-1 does not interact with the following adhesive and neurite growth-promoting proteins reportedly expressed on the surface of cultured chicken DRG neurons: N-CAM (Rutishauser et al., 1978; Doherty et al., 1990), *N*-cadherin (Matsunaga et al., 1988; Letourneau et al., 1990), and the receptors for the extracellular matrix glycoproteins fibronectin (Rogers et al., 1983; Bozyczko and Horwitz, 1986; Humphries et

al., 1988), laminin (Manthorpe et al., 1983; Bozyczko and Horwitz, 1986), and tenascin (Wehrle and Chiquet, 1990).

In view of the fact that L1(G4) molecules bind to other L1(G4) molecules in a so-called homophilic binding (Grumet and Edelman, 1988; Kadmon et al., 1990) and that this interaction has been reported to be involved in neurite growth promotion on immobilized L1(G4) (Lemmon et al., 1989), contamination of the axonin-1 preparation with L1(G4) could also explain the observations made in this study. Hence, it was essential for the interpretation of both the binding and the functional data, that the presence of L1(G4) in the axonin-1 preparations could be excluded with certainty. Although the chromatographic purification of axonin-1 from the vitreous humor of the chicken embryo has been shown to lead to electrophoretically pure axonin-1 that is free from detectable contaminations of chicken AxCAMs, such as F11, neurofascin, and, especially relevant for the present study, L1(G4) (Ruegg et al., 1989*a*), further control experiments have been carried out with the preparations used in the studies presented here. Both the absence of L1(G4) contamination in purified axonin-1 and the absence of contamination of axonin-1 in the L1(G4) preparations were verified by direct comparison of the protein patterns after gel electrophoresis and by immunological detection in gels. The possibility of cross-contamination of the axonin-1 and L1(G4) preparations was further addressed by including the appropriate controls in all binding experiments. In particular, we have shown that preincubations of axonin-1-conjugated Covaspheres with anti-L1(G4) Fab fragments are without effect on their binding to L1(G4) Covaspheres, whereas preincubation of L1(G4) Covaspheres with the same anti-L1(G4) Fab fragments prevented their binding to both L1(G4)-conjugated spheres and axonin-1 Covaspheres completely (Fig. 3). Similarly, the binding of L1(G4)-conjugated Covaspheres to the neurites of cultured DRG neurons was successfully prevented by preincubation of the spheres with anti-L1(G4) Fab fragments, whereas preincubation of the spheres with anti-axonin-1 Fab fragments had no effect. Altogether, these data clearly exclude a contamination of the L1(G4) preparation used for coating the spheres as an explanation of the observed results.

Previous studies on the neuronal receptor of L1-related molecules have failed to reveal any evidence for their binding to the neuronal surface other than that mediated by the homophilic interaction. Grumet and Edelman (1988) showed that Ng-CAM interact with neurons by a homophilic binding and uses another, heterophilic ligand for interacting with nonneuronal cells. However, the proposed heterophilic ligand has not yet been identified. Lemmon et al. (1989) demonstrated that neurite outgrowth on 8D9 substratum depends on the homophilic interaction with its counterpart of the neurite membrane. In both studies, blockage of the membrane-associated component with monovalent antibodies completely prevented the interaction with the solid phase component presented on Covaspheres or as a culture substratum. The most likely explanation for the absence of evidence for an additional, heterophilic, neuronal receptor in these studies may lie in the cellular object used. In Grumet and Edelman's work (1988), embryonic chicken brain neurons in suspension were used as the cellular binding partner for Ng-CAM-conjugated Covaspheres; Lemmon and co-workers (1989) studied neurite growth on 8D9 as a substratum with embryonic tectal neurons. As illustrated by immunohistochemical localization studies (Ruegg et al., 1989*b*), the expression of axonin-1 in DRG neurons and especially their axons is very high, whereas in the central nervous system axonin-1 expression is restricted to a relatively small fraction of the neurons. Hence, in a mixture of cells derived from dissociated central nervous system tissue, relatively few axonin-1-expressing neurons may be present. Alternatively, the heterophilic interactions with other axon-associated molecules, like axonin-1, may have been overlooked because of their relative weakness due to the absence of molecules with affinity-enhancing capability or the presence of molecules exerting an affinity-reducing effect. Interestingly, it has recently been reported that another member of the immunoglobulin superfamily, N-CAM, when it occurs in the same membrane, undergoes a so-called *cis*-binding interaction with L1, resulting in an enhanced homophilic interaction of L1 with L1 in the membrane of other cells (Kadmon et al., 1990).

The antibodies to L1(G4) have been shown to be specific (Chang et al., 1987; this paper); especially, it has been demonstrated that they do not inhibit or modulate N-CAM- or *N*-cadherin-dependent neurite outgrowth (Doherty et al., 1991). Thus, the present data on the blockage of neurite outgrowth on axonin-1 with anti-L1(G4) antibodies clearly demonstrate the function of axonin-1 as a substrate pathway component capable of promoting neurite outgrowth by interacting with L1(G4) of the neuritic membrane. Axonin-1 then acts as an emitter of a neurite outgrowth-promoting signal, whereas L1(G4) may represent the receptive element of the responding neurites. In the reverse situation, when L1(G4) is presented as a substratum for growing neurites, both L1(G4) and axonin-1 may act as receptive neuritic elements. In either case, both homophilic interaction of L1(G4) molecules and heterophilic L1(G4)/axonin-1 interaction may also occur by *cis*-binding in the neuritic membrane. This suggests that a complex pattern of interactions between axonin-1 and L1(G4) may be effective in the transmission of the neurite growth-promoting signals of both axonin-1 and L1(G4) substratum. Present data do not allow a detailed account of the actually occurring interactions and their functional consequences, yet they suggest that a major signaling pathway may be mediated by the homophilic L1(G4) interactions: in the presence of anti-axonin-1 Fab fragments in cultures on L1(G4) substratum, where both the *trans*- and *cis*-binding interactions of axonin-1 are expected to be blocked, neurite outgrowth on L1(G4) substratum was not detectably altered, indicating that neuritic L1(G4) does not need a contribution from a *cis*-binding interaction with axonin-1 to promote neurite outgrowth upon binding to L1(G4) substratum. Via its transmembrane domain (Moos et al., 1988; Burgoon et al., 1991), L1(G4) could also be capable of the signal transfer across the membrane. Conclusions as to the role of axonin-1 as a receptive or modulatory neuritic element for processes elongating on L1(G4) substratum cannot be drawn, since its substrate-binding and intramembrane interactions with L1(G4) could not be studied in isolation with the presently available antibodies. In consideration of the fact that axonin-1 is anchored to the neuritic membrane by a glycophosphoinositol-lipid anchor rather than by a transmembrane domain, a direct transmembrane signaling function appears less likely than a modulatory activity by *cis*-

binding to L1(G4) or another transmembrane protein. It will be crucial for a more detailed understanding of the role of axonin-1 and its macromolecular interactions to be able to selectively block *trans*- and *cis*-binding contacts with L1(G4). This may become feasible by a cross-species experimental design in which functionally crossreacting substrata from mouse and chicken are used in combination with immunologically non-crossreacting antibodies (Lemmon et al., 1989). Current work is aimed at establishing such an experimental system.

A pathway selection mechanism may be postulated in view of the observation presented here that neurites expressing L1(G4) may elongate on substrate pathways labeled with either L1(G4) or axonin-1, in conjunction with the previously reported finding that the binding capabilities of L1(G4) may be modulated by *cis*-interactions with other molecules of the same membrane, such as N-CAM (Kadmon et al., 1990). At the bifurcation of a nerve fiber tract, a growing axon is thought to choose the branch more favorable to its elongation. In molecular terms, preference for one branch or the other may depend on the number of L1/L1 and L1/axonin-1 interactions and their relative potency in promoting neurite elongation. Speculating that *cis*-binding contacts in both leading substrate pathways and following neurites were able to modify the efficiency of *trans*-binding neurite growth-promoting L1/L1 or L1/axonin-1 pairs, a small number of modulatory molecules would suffice to generate combinatorial patterns for encoding a large number of neurite group-specific guidance pathways. Besides L1(G4), axonin-1, and N-CAM, axonal cell surface molecules such as F11 and neurofascin, which are found colocalized with L1(G4) and axonin-1 in some parts of the developing chick nervous system, might be involved in the generation of such neural recognition patterns; their availability will allow us to subject this hypothesis to detailed experimental testing.

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