The Fourth Immunoglobulin-like Domain of NCAM Contains a Carbohydrate Recognition Domain for Oligomannosidic Glycans Implicated in Association with L1 and Neurite Outgrowth

Rüdiger Horstkorte, Melitta Schachner, Josef Peter Magyar, Thomas Vorherr,‡ and Brigitte Schmitz

Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, 8093 Zürich, Switzerland; and ‡Hoffmann La-Roche, Grenzacherstr., 4002 Basel, Switzerland

Abstract. We have previously shown that the neural adhesion molecules L1 and NCAM interact with each other to form a complex which binds more avidly to L1 than L1 to L1 alone (Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990*a*. *J. Cell Biol.* 110:193–208). This *cis*-association between L1 and NCAM is carbohydrate-dependent (Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990*b*. *J. Cell Biol.* 110:209–218). In the present study, we report that L1 and NCAM bind to each other via oligomannosidic carbohydrates expressed by L1, but not by NCAM, as shown in several experiments: (*a*) complex formation between L1 and NCAM is inhibited by a mAb to oligomannosidic carbohydrates and by the oligosaccharides themselves; (*b*) NCAM binds to oligomannosidic carbohydrates; (*c*) within the L1/NCAM complex, the oligomannosidic carbohydrates are hidden from accessibility to a mAb against oligomannosidic carbohydrates; (*d*) the recombinant protein fragment of NCAM containing the immunoglobulin-like domains and not the fragment containing the fibronectin type III homologous repeats binds to oligomannosidic glycans. Furthermore, the fourth immunoglobulin-like domain of NCAM shows sequence homology with carbohydrate recognition domains of animal C-type lectins and, surprisingly, also with plant lectins. A peptide comprising part of the C-type lectin consensus sequence in the fourth immunoglobulin-like domain of NCAM interferes with the association between L1 and NCAM.

The functional importance of oligomannosidic glycans at the cell surface was shown for neurite outgrowth in vitro. When neurons from early postnatal mouse cerebellum were maintained on laminin or poly-L-lysine, neurite outgrowth was inhibited by oligomannosidic glycans, by glycopeptides, glycoproteins, or neoglycolipids containing oligomannosidic glycans, but not by nonrelated oligosaccharides or oligosaccharide derivates. Neurite outgrowth was also inhibited by the peptide comprising part of the C-type lectin consensus sequence in the fourth immunoglobulin-like domain of NCAM. The combined results suggest that carbohydrate-mediated *cis*-associations between adhesion molecules at the cell surface modulate their functional properties.

THE specificity and efficacy of interactions between cells depend on the cooperativity between molecules at the cell surface. In the nervous system, several cell surface molecules have been found to be expressed by distinct cell types, with one cell capable of expressing more than one molecule that participate in recognition and adhesion at a particular developmental stage and at a particular topographic localization (Edelman and Crossin, 1991; Schachner, 1991). The implication is that the developmentally and topographically changing associations of cell surface molecules are important for the regulation of cell behavior by triggering intracellular signals depending on the partner molecules with which they are associated.

Although the mechanisms of such interactions have remained largely unknown, one pair capable of forming a functional complex through *cis*-interaction within the surface membrane of one cell has recently been recognized: the neural adhesion molecules L1 and neural cell adhesion molecule (NCAM)[1] can associate with each other thus leading

Please address all correspondence to Dr. B. Schmitz, Institute of Anatomy, Physiology and Hygiene of Domestic Animals, University of Bonn, Katzenburgweg 7-9, 5300 Bonn, Germany.

1. *Abbreviations used in this paper*: AMOG, adhesion molecule on glia; GlcNAc, N-acetylglucosamine; MAG, myelin-associated glycoprotein; Man, mannose; NCAM, neural cell adhesion molecule; NCAM-Ig, NCAM fragment containing the five immunoglobulin-like domains; NCAM-FN, NCAM fragment containing the two fibronectin-type III homologous repeats; TS101, trisaccharide containing galactose-β1-4-N-acetyl-glucosamine-β1-2-mannose.

to a strengthened adhesion to L1 on another cell. In this adhesion mechanism, termed "assisted homophilic binding" (Kadmon et al., 1990*a*), the *cis*-interaction between L1 and NCAM modifies the efficacy of L1 to associate with another L1 molecule in a *trans*-interaction. The *cis*-, but not the *trans*-interaction was dependent on the structure of the oligosaccharides synthesized by the L1 and NCAM expressing cells: when the L1 and NCAM dependent aggregation of cultured neuroblastoma cells was determined after growing the cells in the presence of oligosaccharide processing inhibitors, a dependence on high mannose- or hybrid-type oligosaccharides was observed (Kadmon et al., 1990*b*).

After this lead, we have probed the possibility that oligomannose-type oligosaccharides recognized by the monoclonal L3 antibody on L1, but not on NCAM[2] (Kücherer et al., 1987) could be involved in the association of the two molecules. We report here that the oligomannose-type oligosaccharides carried by L1 mediate the binding of L1 to NCAM. Analysis of sequence homologies between NCAM and carbohydrate binding molecules revealed motifs characteristic for the carbohydrate recognition domains of C-type lectins and for plant lectins in the fourth immunoglobulin-like domain of NCAM. A peptide comprising part of the C-type lectin consensus sequence in the fourth immunoglobulin-like domain not only interfered with the interaction between L1 and NCAM, but also inhibited neurite outgrowth. Similarly, oligomannosidic glycans and their derivates inhibited neurite outgrowth. The results implicate carbohydrate-mediated *cis*-interactions between adhesion molecules in important functional roles.

Materials and Methods

Glycoproteins

The neural cell adhesion molecules L1 (Rathjen and Schachner, 1984), NCAM (Hoffman et al., 1982), myelin-associated glycoprotein (MAG; Poltorak et al., 1987), adhesion molecule on glia (AMOG; Antonicek et al., 1987), and "rest-L2" (L2/HNK-1 positive glycoproteins from detergent extracts of adult mouse brain after removal of NCAM, L1, and MAG; Kruse et al., 1984) comprising mostly J1-160/180 (Pesheva et al., 1989) were immunoaffinity purified as described. Proteins were checked for purity by silver staining of 8% SDS-polyacrylamide gels according to Merril et al. (1982). No additional bands other than the expected appeared. Ovalbumin was obtained from Serva (Heidelberg, FRG) and ribonuclease B and asialofetuin from Sigma Chem. Co., St. Louis, MO.

Antibodies

The following antibodies were used: monoclonal L1 antibodies 324 (Rathjen and Schachner, 1984) and 555 (Conscience, J.-F., F. Appel, J. Holm, F. von Bohlen und Halbach, A. Faissner, and M. Schachner, manuscript submitted for publication), monoclonal NCAM antibody H28 (Hirn et al., 1981), monoclonal L2 antibody 412 (Kruse et al., 1984), monoclonal L3 antibody 492 (Kücherer et al., 1987), monoclonal L5 antibody 487 (Streit et al., 1990), and monoclonal 473 antibody (Faissner, 1988. *Soc. Neurosci. Abstr.* 920). mAbs 324, 412, 555, and H28 are of the IgG subclass and mAbs 473, 492, and 487 of the IgM subclass. The monoclonal L1, L2, and NCAM antibodies were used as protein G (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) purified fractions. The monoclonal L3 antibody was used as ascites or as immunopurified IgM using a monoclonal Mark-1 antibody column reacting with rat Ig-kappa chain (Bazin, 1982). Immunopurified preparations were also partially digested with trypsin to obtain monovalent fragments of IgM antibody according to Matthew and Reichardt (1982). Monoclonal 473 and monoclonal L5 antibodies were used as ammonium sulfate precipitated preparation of hybridoma supernatant. Polyclonal antibodies were prepared in rabbits against immunoaffinity purified L1 (Rathjen and Schachner, 1984), MAG (Poltorak et al., 1987), AMOG (Antonicek et al., 1987), and NCAM (Faissner et al., 1984) from adult mouse brain. Polyclonal L1 and NCAM antibodies were immunoaffinity purified on L1 and NCAM coupled to Sepharose 4B (Martini and Schachner, 1986) and reacted exclusively with their respective antigens as determined by ELISA (not shown). Polyclonal AMOG and MAG antibodies were used as ammonium sulfate precipitated preparations of antisera. Secondary HRP- and alkaline phosphatase-conjugated antibodies were obtained from Dianova (Hamburg, FRG).

Fragments of NCAM

Protein fragments of NCAM were produced in bacteria in the pET expression system (Frei et al., 1992). One fragment contained the five immunoglobulin-like domains from amino acid number 18 to 492 (NCAM-Ig) and the other one the two fibronectin type III homologous repeats from amino acid number 492 to 692 (NCAM-FN).

Glycopeptides

Glycopeptides were prepared from ribonuclease B, ovalbumin, asiolofetuin (all from Sigma Chem. Co.), and AMOG by digestion of the glycoproteins with pronase (Sigma Chem. Co., catalog no. P-5147; Tai et al., 1977). Enzyme, salts, and digested peptides were removed from glycopeptides by a P2 gel filtration column (Biorad Labs., Hercules, CA), using 0.1% acetic acid in water as elution solvent. Orcinol positive fractions were collected.

Neoglycolipids

Neoglycolipids were synthesized from oligosaccharides released by treatment with endoglycosidase H (Boehringer Mannheim Corp., Mannheim, Germany) of AMOG and ribonuclease B, which expresses only oligomannosidic glycans (Liang et al., 1980). Neoglycolipids were also derived from the oligosaccharide $Man_5GlcNAc_2$, the trisaccharide containing galactose-β1-4-*N*-acetylglucosamine-β1-2-mannose (TS101), and from lactose (Sigma Chem. Co.). The method used and the purification of neoglycolipids is described by Stoll et al. (1988)[2]. All neoglycolipids were checked by TLC for purity using chloroform/methanol/water (105/100/28, vol/vol) as solvent and found to contain no starting material of the synthetic reaction.

Peptide Synthesis

The following acetylated peptide carboxamides have been synthesized: peptide MS1 (Ac-Q-V-A-G-D-A-K-D-K-D-I-S-W-F-S-P-N-G-E-NH_2) and peptide MS2 (Ac-E-A-S-G-D-P-I-P-S-I-T-W-R-T-S-T-R-N-I-NH_2). Peptide synthesis was performed on a Milligen-9050 continuous-flow synthesizer using an adapted software package. The Fmoc group was used for *N*-alpha protection throughout, the side-chain protection was the following: N^{ω}-(2,2,5,7,8-pentamethylchroman-6-sulfonyl) for arginine, trityl for cysteine, glutamine and asparagine, (tert-butoxy)carbonyl for lysine, and tertbutyl for serine, threonine, glutamic acid, and aspartic acid. Synthesis started on the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy polydimethylacrylamide resin and activation was performed by 1,1,3,3-tetramethyl-2-(2-oxo-1(2H)-pyridyl)-uronium tetrafluoro-borate:diisopropylethyl-amine (1:2, vol/vol) using a fivefold excess of amino acid derivate. Final acetylation was carried out manually using acetanhydride in *N*,*N*-dimethylformamide in the presence of pyridine. The cleavage reaction was performed for 1 h 45 min in a mixture of 1.6 ml H_2O, 1.6 ml thioanisole, 0.8 ml ethanedithiol, 2 g phenol, and 32 ml TFA at room temperature. Peptides were purified by Delta-Prep-3000 HPLC (Waters Chromatography Division, Milford, MA). From 64 mg of the crude peptide MS1, 10 mg of purified peptide were obtained after preparative HPLC. 14.9 mg of purified peptide MS2 were obtained from 75 mg crude material. Ion spray MS on an API III (Sciex, Thornhill, Ontario, Canada) showed the expected ion series.

Enzyme-linked Immunosorbent Assays

Binding of L1 to NCAM, the NCAM fragment containing all five immunoglobulin-like domains (NCAM-Ig), MAG, or AMOG. Ninety-six-well microtiter plates (Falcon 3912) were incubated overnight at 4°C with 5 μg/ml monoclonal L1 antibody 324 or 555 in PBS (pH 7.4) for substrate-

2. Schmitz, B., J. Peter-Katalinic, H. Egge, and M. Schachner, manuscript submitted for publication.

coating, washed three times with PBS and blocked for 1 h at room temperature with 1% fatty acid-free BSA (Sigma Chem. Co., catalog no. A-7030) in PBS. This and all following incubation steps were carried out at room temperature. L1 (5 μg/ml) was preincubated with NCAM, MAG, or AMOG in PBS containing 0.1% BSA and 0.025% deoxycholate at a concentration of 5 μg/ml and with NCAM-Ig at a concentration of 3 μg/ml. The mixtures of molecules were allowed to react with each other for 2 h, and then incubated with substrate-coated monoclonal L1 antibody 324 or 555 for 1.5 h. After washing the plates three times with PBS, wells were incubated for 1.5 h with polyclonal antibodies to L1, NCAM, MAG, or AMOG in PBS containing 1% BSA. Plates were again washed three times with PBS and incubated for 1 h with HRP-conjugated anti-rabbit antibodies in PBS containing 1% BSA. Bound secondary antibodies were visualized by incubation with H_2O_2 and ABTS (2,2 azino-di-3-ethylbenzthiazoline sulfonate 6; Boehringer Mannheim Corp.) as HRP-substrates. The color reaction was terminated by addition of 0.6% aqueous SDS and the OD of the reaction product was determined at 405 nm in a Microtec ELISA reader (Flow Labs., Inc., McLean, VA).

Determination of the binding of L1 to NCAM in the presence of additives. L1 was preincubated with mAbs against carbohydrate epitopes (L2, L3, or L5), monovalent fragments of the monoclonal L3 antibody or synthetic peptides (MS1 or MS2) before the incubation with NCAM. Since both L1 and NCAM express the L2 carbohydrate epitope, not only L1, but also NCAM was preincubated with this antibody before allowing them to react with each other. NCAM and NCAM-Ig were also preincubated with carbohydrate carrying molecules (AMOG, "rest-L2," glycopeptides prepared from AMOG or asialofetuin, and neoglycolipids prepared from oligomannosidic carbohydrates of ribonuclease B or TS10l) before the addition to L1. These incubation steps were carried out for 1 h at room temperature in PBS at concentrations indicated in the figure legends, before the mixtures of molecules were added to substrate-coated monoclonal L1 antibody. The binding of L1 and NCAM in the presence of the additives to substrate-coated monoclonal L1 antibody was determined as described in the previous paragraph.

Determination of the accessibility of the oligomannosidic carbohydrate epitope expressed by L1 in the L1/NCAM complex. To determine the binding of the monoclonal L3 antibody to L1 after complex formation between L1 and NCAM, L1 and NCAM were coated overnight at 4°C in wells of microtiter plates either individually or together after a preincubation period of 1 h at room temperature at a concentration of 2 μg/ml in PBS each. In some experiments, NCAM was preincubated for 1 h at room temperature with a glycopeptide fraction from ovalbumin (500 μg/ml), which is enriched in oligomannosidic glycans (Tai et al., 1977) before the addition of L1 and substrate-coating of the mixture onto microtiter plates. After washing and blocking as described above, monoclonal L1, L3, and NCAM antibodies in PBS containing 1% BSA were incubated for 2 h at room temperature to probe for the accessibility of their respective epitopes. After washing with PBS, bound mAbs were detected with HRP-conjugated anti-rat antibodies as described above.

The accessibility of the oligomannosidic carbohydrate epitope in the L1/NCAM complex was also determined in another experimental approach. Monoclonal L3 antibody was adsorbed overnight at 4°C at 5 μg/ml in PBS to microtiter plates and after washing and blocking as above, the mixture of preincubated L1 and NCAM was added to the substrate-coated monoclonal L3 antibody. Binding of L1 and NCAM to monoclonal L3 antibody was determined as described above.

Binding of L1, NCAM, or NCAM fragments to glycolipids. Glycolipids dissolved in ethanol (4 μg/ml) were added to the wells of microtiter plates and adsorbed to the plastic by evaporating the ethanol at room temperature. Plates were blocked with PBS containing 1% fatty-acid free BSA for 1 h at room temperature. L1, NCAM, or NCAM fragments were added to the wells at concentrations of 3 μg/ml (NCAM fragments) or 5 μg/ml (L1 and NCAM) in PBS containing 0.1% BSA and 0.025% deoxycholate, and incubated for 2 h at room temperature. In some experiments, substrate-coated glycolipids were preincubated with monoclonal L3 antibody for 1 h at room temperature before the addition of NCAM. Bound antigens were detected with their respective polyclonal antibodies and HRP-coupled secondary antibodies as described above.

Binding of polyclonal L1 and NCAM antibodies to covalently linked antigens. L1 and NCAM (0.5 μg/ml) were covalently linked to covaLink microtiter plates (Nunc, Roskilde, Denmark) according to the manufacturers' instructions. After washing and blocking the plates with PBS containing 1% BSA for 1 h at room temperature, polyclonal L1 and NCAM antibodies were incubated in a 1:2 dilution series on their respective antigens for 2 h at room temperature. Bound polyclonal antibodies were detected using HRP-coupled secondary antibodies as described above. The coating efficiencies were over 90% as determined by recoating the nonbound L1 and NCAM again to covaLink microtiter plates and repeating the ELISA. After the second coating, OD values were 10 times lower than after the first coating indicating that most of the molecules were linked to the covaLink microtiter plates after the first coating.

Determination of the Percentage of L1 Molecules Containing the L3 Epitope by Sequential Immunoprecipitation

Immunoaffinity purified L1 antigen (1 μg/ml) in a final volume of 500 μl PBS was incubated overnight at 4°C with monoclonal L3 antibody (20 μg/ml) and, for control, with monoclonal 473 antibody which does not recognize L1. The mixtures were then incubated with polyclonal anti-rat IgM antibodies conjugated with biotin (30 μg/ml) for 2 h at 4°C. For precipitation, 50 μl streptavidin-agarose beads (Sigma Chem. Co., catalog no. S-1638) were added and after another hour of incubation at 4°C, samples were centrifuged for 1 min at 13,000 rpm. Supernatants were removed, incubated with 50 μl streptavidin-agarose, and after another hour of incubation again pelleted by centrifugation. This procedure was repeated six times, and after every centrifugation step an aliquot of the supernatant was removed for SDS-PAGE (8% slab gels) and immunoblotting using immunoaffinity purified polyclonal L1 antibodies and alkaline phosphatase-conjugated anti-rabbit antibodies according to Faissner et al. (1985). The detection limit for L1 was at least 20 ng as determined by parallel assessment of defined amounts of L1 in a standard curve. After the sixth round of clearance with streptavidin-agarose beads, very low amounts of L1 were detected by immunoblotting in the supernatants of solutions of L1 treated with monoclonal L3 antibody, whereas the full immunoreactivity was detected in the solutions of L1 treated with monoclonal 473 antibody.

Determination of Sequence Homologies

The Swissprot data base (release 21.0, 3/92) was used for the search of sequence homologies with the sequence Cxxxxxx(V,I,L)x(S,T)(V,I)xxxx(E,S), which is present in all eight carbohydrate recognition domains of the human mannose receptor (Taylor et al., 1990) using the PILEUP program (GCG; Devereux et al., 1984). Alignment of plant lectins, animal C-type lectins, and NCAM as well as determination of sequence homologies were also performed using this program. Alignment parameters are indicated in the legend to Fig. 7.

Cell Culture

Freshly dissociated cell populations enriched in small cerebellar neurons (~99% pure) were prepared from 5- to 7-day-old ICR mice (Schnitzer and Schachner, 1981; Keilhauer et al., 1985). Cells were plated at a concentration of 2×10^6 cells/ml onto glass coverslips in serum-free, hormone-supplemented medium (Fischer et al., 1986) containing 25 mM Hepes (50 μl per 16-mm diameter coverslip). Coverslips were coated either with poly-*L*-lysine (10 μg/ml H_2O) or laminin from Engelbreth-Holm-Swarm sarcoma (Boehringer Mannheim Corp.; 20 μg/ml in basal medium Eagle's). Three coverslips were then placed in 35-mm diameter tissue culture plastic petri dishes (Nunc). The medium was removed by gentle suction 3–4 h after plating of cells on laminin or 20 h after plating of cells on poly-*L*-lysine. Neoglycolipids, glycopeptides, oligosaccharides, glycoproteins, or synthetic peptides were then added in 50 μl medium per coverslip. Cultures were examined 24 and 48 h later by phase contrast microscopy.

Statistical Analysis

Statistical analysis was performed on nonnormalized data by one way ANOVA and Scheffé comparison among means. Differences between values were reported as significant (*) when p was at least <0.05.

Results

Determination of the Complex Formation between L1 and NCAM

To analyze the interaction between L1 and NCAM, a solid phase assay was developed on the basis of the ELISA. Im-

munoaffinity purified molecules from adult mouse brain were allowed to react with each other in Ca^{++}-free solution for 2 h at room temperature. The mixture was then added to the monoclonal L1 antibody, which had been preadsorbed to plastic microtiter plates. As solely L1 could bind to the substrate-coated mAb, detection of NCAM with its polyclonal antibodies was only possible when NCAM had formed a stable molecular complex with L1 (Fig. 1). Complex formation between L1 and NCAM (i.e., binding of NCAM to L1) was concentration-dependent and in saturation when the molecules were incubated at a concentration of 5 μg/ml each (not shown). Incubation of L1 with MAG or AMOG did not result in complex formation between L1 and MAG, or AMOG as determined by ELISA with polyclonal antibodies to MAG or AMOG, since these antibodies did not detect their antigens on substrate-coated monoclonal L1 antibody after preincubation with L1 (Fig. 1). The amount of L1 bound to the substrate-coated monoclonal L1 antibody was controlled using polyclonal L1 antibodies and found to be similar in all experiments (Fig. 1). To estimate the quantities of L1 and NCAM bound in the complexes, OD values measured by ELISA were calibrated for each antigen/antibody pair (see Materials and Methods). It was found that approximately the same OD values corresponded to the same quantities of bound protein for both antibodies. The molar ratio of L1:NCAM in the measured complexes was therefore ~1:1. However, more precise estimates regarding the stoichiometry of this complex cannot be made, since the contribution of L1–L1 and NCAM–NCAM interactions are unknown.

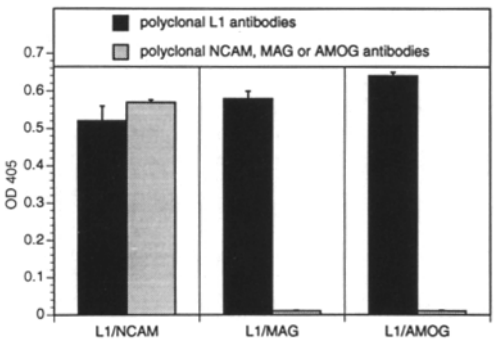

Figure 1. Determination of the binding of NCAM, MAG, and AMOG to L1. L1 was preincubated with NCAM, MAG, or AMOG before the addition of the mixtures to substrate-coated monoclonal L1 antibody. Bound antigens were detected by ELISA using polyclonal antibodies to L1, NCAM, MAG, or AMOG. The OD of the reaction product was measured at 405 nm (*OD 405*). Mean values ± SDs from one representative experiment out of three independent experiments carried in triplicates are shown.

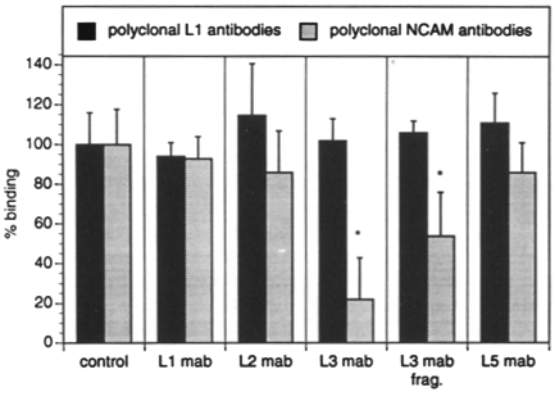

Figure 2. Determination of the association between L1 and NCAM in the absence or presence of different antibodies or antibody fragments. L1 was preincubated at saturating concentrations with monoclonal L1 (555) (*L1 mab*, 50 μg/ml), L2 (*L2 mab*, 50 μg/ml), L3 (*L3 mab*, 50 μg/ml), L5 (*L5 mab*, 40 μg/ml) antibodies or monoclonal L3 antibody fragments (*L3 mab frag.*, 50 μg/ml), before addition of NCAM. Also, NCAM was preincubated at saturating concentration with monoclonal L2 antibody before addition to L1. After further incubation, the mixtures of molecules were added to substrate-coated monoclonal L1 antibody (324). Bound antigens were detected by ELISA using their respective polyclonal antibodies. Binding of L1 or NCAM to monoclonal L1 antibody in the absence of additives was set to 100% (*control*) and binding of L1 and NCAM in the presence of additives was expressed in % of the control. Mean values ± SDs from at least five independent experiments carried out in duplicates are shown.

Oligomannosidic Carbohydrates Mediate Binding between L1 and NCAM

To investigate whether the interaction between L1 and NCAM depends on oligomannosidic glycans, L1 was preincubated with the oligomannosidic glycan–specific monoclonal L3 antibody and with monovalent fragments of this antibody before allowing complex formation with NCAM (Fig. 2). As shown by sequential immunoprecipitation (see Materials and Methods) most if not all L1 molecules carry oligomannosidic glycans. A strong inhibition (~80%) of the interaction of L1 with NCAM was observed in the case of the monoclonal L3 antibody and a slightly lower inhibition (~50%) in the case of the monovalent antibody fragments, as indicated by the reduced binding of polyclonal NCAM antibodies. This reduction was not due to a diminished binding of L1 to substrate-coated monoclonal L1 antibody, since L1 was bound in similar amounts in the absence or presence of the IgM antibody or monovalent fragments. As controls, monoclonal L1 antibody (555; recognizing the protein backbone), monoclonal L5 antibody (reacting with a carbohydrate epitope on L1, but not on NCAM), and monoclonal L2 antibody (reacting with the L2/HNK-1 carbohydrate on L1 and NCAM) were used. To prevent cross-linking between L1 and NCAM in the presence of the monoclonal L2 antibody, both molecules were incubated separately with the antibody at saturating concentrations before the mixtures of molecules were allowed to interact with each other. None of the control antibodies significantly inhibited the association of L1 with NCAM (Fig. 2).

The interaction between L1 and NCAM could also be inhibited by the oligomannosidic carbohydrate itself as shown by preincubation of NCAM with either neoglycolipids prepared from ribonuclease B, which carries only oligomannosidic glycans (Liang et al., 1980). Furthermore, inhibition was also observed in the presence of glycopeptides prepared from AMOG of adult mouse brain, which carries ~30% of its molecular weight in form of N-linked oligosaccharides, of which 75–80% are of the oligomannosidic type[2] or AMOG (Fig. 3). At 2 μg/ml, AMOG hardly interfered with the interaction between L1 and NCAM whereas at 4 μg/ml an inhibition of ~50% was observed (not shown). At 20

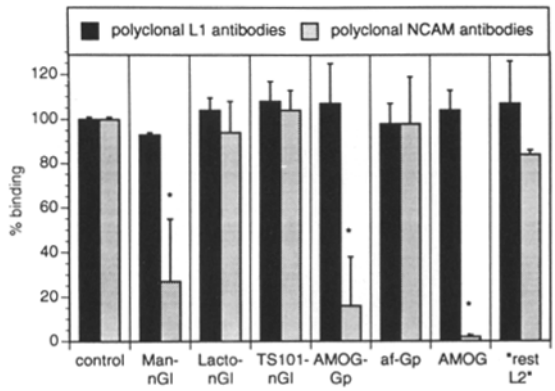

Figure 3. Determination of the association between L1 and NCAM in the absence or presence of neoglycolipids, glycopeptides, or glycoproteins. NCAM was preincubated with oligomannosidic neoglycolipids prepared from ribonuclease B (*Man-nGl*); 20 μg/ml), oligomannose containing glycopeptides prepared from AMOG (*AMOG-Gp*; 500 μg/ml) and AMOG (*AMOG*) 20 μg/ml) or, for control, with lactosylneoglycolipid (*Lacto-nGl*; 20 μg/ml), TS101-neoglycolipid (*TS101-nGl*; 200 μg/ml), a glycopeptide prepared from asialofetuin (*af-Gp*; 500 μg/ml), and with "rest L2," the L2/HNK-1 carbohydrate containing glycoprotein fraction from adult mouse brain (*"rest L2*; 20 μg/ml) before the addition of L1. After further incubation, the mixtures of molecules were added to substrate-coated monoclonal L1 antibody. Bound antigens were detected by ELISA using their respective polyclonal antibodies. Binding of L1 and NCAM to monoclonal L1 antibody in the absence of additives was set to 100% (*control*) and binding of L1 and NCAM in the presence of additives was expressed in % of control. Mean values ± SDs from at least two independent experiments carried out in duplicates are shown.

μg/ml a complete inhibition of the association between L1 and NCAM was observed (Fig. 3). No significant inhibition of the L1/NCAM association was observed in the presence of neoglycolipids derived from the neutral disaccharide lactose and the trisaccharide TS101, which are not recognized by the monoclonal L3 antibody, neutral glycopeptides prepared from asialofetuin (Hatton et al., 1979) and the L2/HNK-1 carbohydrate containing glycoprotein fraction from adult mouse brain, which is also not recognized by the monoclonal L3 antibody (Fig. 3).

Interaction of L1 and NCAM Leads to a Reduced Accessibility of the Oligomannosidic Carbohydrate Epitope to the Monoclonal L3 Antibody

To investigate the accessibility of the oligomannosidic carbohydrate in the L1/NCAM complex, the ability of the monoclonal L3 antibody to react with its epitope in the complex was determined. Substrate-coated L1 gave a strong reactivity with the monoclonal L3 antibody, which was reduced to 50% when L1 was preincubated with NCAM before substrate-coating the mixtures of molecules onto microtiter plates. The reduced binding of monoclonal L3 antibody to the L1/NCAM complex could be reversed by preincubating NCAM with glycopeptides from ovalbumin, which are strongly enriched in oligomannosidic glycans (Tai et al., 1977), before complex formation with L1 (Fig. 4). The accessibility of monoclonal L1 or NCAM antibodies to their respective antigens was not changed by complex formation

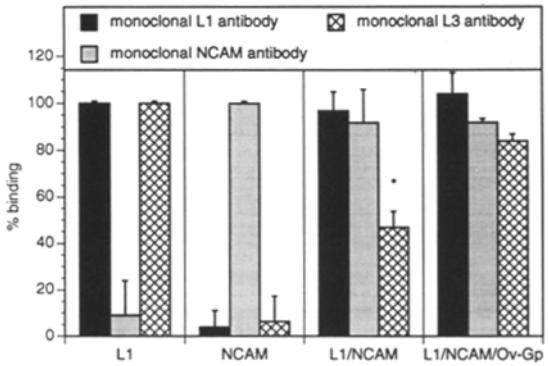

Figure 4. Determination of the accessibility of the L3 carbohydrate epitope in the L1/NCAM complex in absence or presence of oligomannosidic glycopeptides derived from ovalbumin. L1 (*L1*) and NCAM (*NCAM*) were coated as substrates either individually or as mixture (*L1/NCAM*). The mixture of L1 and NCAM was also coated after preincubation of NCAM with glycopeptides of ovalbumin (*L1/NCAM/Ov-GP*). Glycopeptides of ovalbumin did not bind to the plastic of the microtiter plates (not shown). Binding of monoclonal L1 and L3 antibodies to L1 and binding of monoclonal NCAM antibody to NCAM was measured by ELISA and set to 100% for individually coated antigens. Binding of these antibodies to the mixtures was expressed in % of mAb binding to the individually coated antigens. Mean values ± SDs from three independent experiments carried out in duplicates are shown.

(Fig. 4). Further evidence that the oligomannosidic carbohydrate epitope carried by L1 is hidden from accessibility to the monoclonal L3 antibody in the L1/NCAM complex came from experiments, in which monoclonal L3 antibody was coated as substrate and the L1/NCAM complex was then allowed to interact with the antibody. No binding of the complex could be detected, whereas in the control experiment with substrate-coated monoclonal L1 antibody the L1/NCAM complex was readily bound (not shown).

NCAM Binds to Substrate-bound Oligomannosidic Glycans

As oligomannosidic glycans carried by L1 are required for the L1/NCAM complex formation, NCAM must be a receptor or lectin for these carbohydrates. The ability of NCAM to serve as lectin for oligomannosidic glycans could be shown by the binding of NCAM to substrate-coated oligomannosidic glycan containing neoglycolipids (Fig. 5). NCAM did not bind to the neoglycolipid containing the neutral trisaccharide TS101 or to BSA. The binding of NCAM to the substrate-coated oligomannosidic neoglycolipids was reduced after preincubation of the oligomannosidic neoglycolipid substrate with monoclonal L3 antibody (Fig. 5). As control, L1 was shown not to bind to the substrate-coated oligomannosidic neoglycolipids (Fig. 5).

The Immunoglobulin-like Domains and Not the Fibronectin Type III Homologous Repeats of NCAM Bind to Oligomannosidic Glycans and Are Involved in L1/NCAM Complex Formation

The bacterially expressed protein fragment of NCAM containing the five immunoglobulin-like domains also bound to substrate-coated oligomannosidic neoglycolipids, but not to

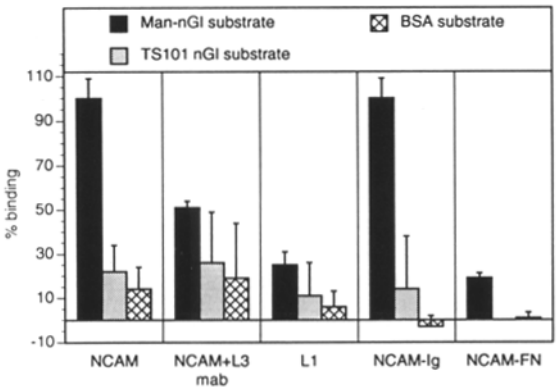

Figure 5. Determination of the binding of NCAM or NCAM fragments to oligomannosidic neoglycolipids. NCAM, NCAM fragments comprising the immunoglobulin-like domains (*NCAM-Ig*) or fibronectin type III homologous repeats (*NCAM-FN*) and L1 were added to substrate-coated oligomannosidic neoglycolipids prepared from ribonuclease B (*Man-nGl substrate*), to TS101-neoglycolipid (*TS101-nGl substrate*), or to BSA (*BSA substrate*). All three substrates were also preincubated with monoclonal L3 antibody before addition of NCAM (*NCAM+L3 mab*). Bound antigens were detected by ELISA using their respective polyclonal antibodies. Binding of NCAM to substrate-coated Man-nGl was set to 100% and binding of NCAM to TS101-nGl and BSA or of L1 to all substrates was expressed in % of binding of NCAM to Man-nGl. Binding of NCAM-Ig to substrate-coated Man-nGl was also set to 100%, and binding of NCAM-Ig to TS101-nGl or BSA and binding of NCAM-FN to substrate-coated Man-nGl or BSA was expressed in % of binding of NCAM-Ig to Man-nGl. Mean values ± SDs from three different experiments carried out in duplicates are shown.

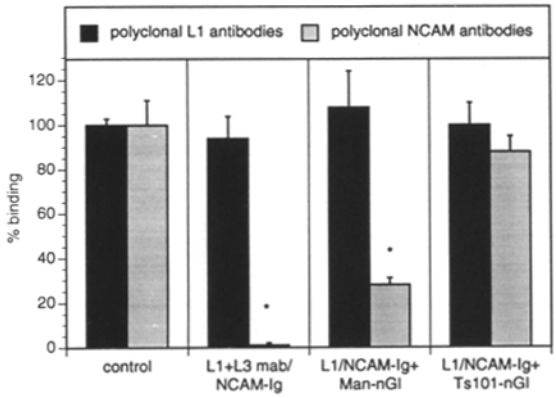

Figure 6. Determination of the binding of L1 to the bacterially expressed NCAM fragment containing the immunoglobulin-like domains (*NCAM-Ig*) in the absence or presence of monoclonal L3 antibody or oligomannosidic neoglycolipids. L1 was preincubated with monoclonal L3 antibody (*L3 mab*) and NCAM-Ig was preincubated with an oligomannosidic neoglycolipid prepared from ribonuclease B (*Man-nGl*, 20 μg/ml) or, for control, with TS101-neoglycolipid (TS101-nGl, 200 μg/ml) before addition of NCAM-Ig or L1, respectively. After further incubation, the mixtures were added to substrate-coated monoclonal L1 antibody. Bound antigens were detected by ELISA using their respective polyclonal antibodies. Binding of L1 and NCAM-Ig in the absence of additives were set to 100% (*control*) and binding of L1 and NCAM-Ig in the presence of additives expressed in % of the control. Mean values ± SDs from one representative out of three independent experiments carried out in triplicates are shown.

the control neoglycolipid or to BSA (Fig. 5). The bacterially expressed protein fragment of NCAM containing the two fibronectin type III repeats hardly bound to the oligomannosidic neoglycolipid substrate (Fig. 5).

The five immunoglobulin-like domains of NCAM also interacted with L1 (Fig. 6). Preincubation of L1 with monoclonal L3 antibody reduced the binding of L1 to the protein fragment containing the immunoglobulin-like domains of NCAM (Fig. 6). Furthermore, preincubation of this NCAM fragment with oligomannosidic neoglycolipids also prevented the binding of the NCAM fragment to L1. The control neoglycolipid TS101 was ineffective in preventing the binding of the NCAM fragment to L1 (Fig. 6).

The Fourth Immunoglobulin-like Domain of NCAM Shows Sequence Similarity to Carbohydrate Recognition Domains

Since the carbohydrate recognition domains of several types of mannose-specific carbohydrate binding proteins have been determined in their primary structure, it seemed worthwhile to determine whether NCAM shows sequence homologies with any of these domains. The primary sequences of all eight carbohydrate recognition domains of the human mannose receptor (Taylor et al., 1990) could be aligned with the amino acid sequence Cxxxxxx(V,I,L)x(S,T)(VI)xxxx (E,S). This sequence was identified within the fourth immunoglobulin-like domain of mouse NCAM starting at amino acid number 330 and also in 37 other proteins. Among the other proteins expressing this sequence, several are known to be lectins, as for example the Kupffer cell receptor (Hoyle and Hill, 1991), the low affinity Fc receptor of IgE (Bettler et al., 1989), the cartilage-specific proteoglycan core protein (Doege et al., 1987), the hepatic lectin homolog (Tomley et al., 1988), or versican (Krusius et al., 1987).

We further compared the amino acid sequence of the fourth immunoglobulin-like domain of NCAM without the alternatively spliced π exon (Santoni et al., 1989) with the sequence of the carbohydrate recognition domain four of the human mannose receptor, shown to be functionally most active in carbohydrate binding (Taylor et al., 1992), with the consensus sequence for C-type lectins according to Weis et al. (1991), and to the alpha chain of the mannose/glucose specific lectin from *Lathyrus ochrus* (Richardson et al., 1984). We found a similarity of 37% at the protein level between the fourth and the beginning of the fifth immunoglobulin-like domain of mouse NCAM (from amino acid 323 to 439) and the carbohydrate recognition domain four of the human mannose receptor (from amino acid 673 to 781). In the amino-terminal half of the fourth immunoglobulin-like domain of NCAM we could align 6 out of 10 amino acids of the consensus sequence of C-type lectins according to Weis et al. (1991) (Fig. 7). Surprisingly, the carboxy-terminal half of the fourth and the beginning of the fifth immunoglobulin-like domains (from amino acid 368 to 421 of mouse NCAM) showed sequence homology to plant lectins from leguminoses. The amino acid sequence of the alpha chain of mannose/glucose specific lectin from *Lathyrus ochrus*, which is nearly identical with *Lathyrus sativus* (Sletten et al., 1983)

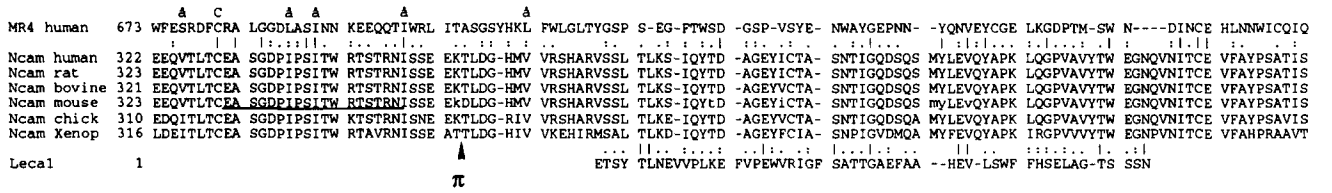

Figure 7. Comparison of the amino acid sequence of the fourth immunoglobulin-like domain of NCAMs from different species with C-type and plant lectins. Alignment of MR4 (carbohydrate recognition domain four of the human mannose receptor; Taylor et al., 1990) with the fourth immunoglobulin-like domain of NCAM from different species (human NCAM: Barton et al., 1988; rat NCAM: Small et al., 1987; bovine NCAM: Lipkin et al., 1989; mouse NCAM: Barthels et al., 1987; Montag, D., and F. Lahrtz, personal communication; chicken NCAM: Cunningham et al., 1987; *Xenopus laevis* NCAM: Krieg et al., 1989) was performed using the following parameters: gap wt = 2.0, gap length wt = 0.1. Alignment of the entire sequence of Lecal (alpha 1 chain of the mannose/glucose-specific lectin from *Lathyrus ochrus*; Richardson et al., 1984) with the fourth immunoglobulin-like domain of NCAM was performed using the following parameters: gap wt = 1.0, gap length wt = 1.0. Vertical bars between the alignments indicate identical amino acids in all sequences. Double points indicate a minimal comparison value of 0.5 and single points indicate a minimal comparison value of 0.1, when compared to the amino acid with the lowest homology, in the NCAM sequences using the standard Dayhoff's amino acid comparison table. Numbers indicate the amino acids at the start sites of the protein segments used for the alignment. Characters above the alignment indicate the relevant sites in the mouse NCAM sequence which are specific for C-type lectins (Weis et al., 1991; *C*, cysteine, *â*, aliphatic amino acid). The π below the alignment indicates the site where the alternatively spliced π-exon is located in the NCAM sequence (not included). The sequence of peptide MS2 of mouse NCAM is underlined.

and *Vicia cracca* (Bauman et al., 1982), was used for this comparison. The fourth immunoglobulin-like domain showed a similarity of 43% at the protein level to the alpha chain of *Lathyrus ochrus* lectin (total length 53 amino acids) (Fig. 7).

A Peptide Comprising Part of the C-type Lectin Consensus Sequence in the Fourth Immunoglobulin-like Domain of NCAM Interferes with L1/NCAM Interaction

As it is possible to prevent the binding of oligosaccharide structures to their corresponding receptors by using synthetic peptides containing the amino acid sequence of the receptor domain (see for example, Geng et al., 1991), we used the peptide MS2 representing 19 amino acids starting at glutamic acid-331 at the amino-terminal end of the fourth immunoglobulin-like domain of mouse NCAM as competitor for the L1/NCAM interaction. This peptide comprises part of the C-type lectin consensus sequence. Preincubation of L1 with this peptide prevented the association of L1 with NCAM in a concentration-dependent manner (Fig. 8). The control peptide MS1 starting at glutamine-42, which represents the first 19 amino-terminal amino acids of the first immunoglobulin-like domain of NCAM did not interfere with the interaction between L1 and NCAM (Fig. 8).

Oligomannosidic Glycans Are Implicated in Neurite Outgrowth from Early Postnatal Cerebellar Neurons

The oligomannosidic L3 epitope has been detected by indirect immunofluorescence studies at the cell surface of cerebellar neurons (Kücherer et al., 1987). To study their function at the neuronal cell surface, neurons from early postnatal mouse cerebellum were maintained on substrates of poly-*L*-lysine or laminin in the presence or absence of oligomannosidic glycans, neoglycolipids, glycopeptides, or glycoproteins. For control, other neutral glycans or glycoconjugates were used (Table I). Laminin and poly-*L*-lysine do not carry oligomannosidic glycans (not shown). Outgrowth of neurites could be observed after ~4 h on poly-*L*-lysine, whereas on laminin neurites were formed already shortly after cells had adhered. The addition of mannosidic glycoconjugates after the first neurites had extended resulted in a dramatic decrease of neurite outgrowth on both substrates. One day after addition of glycoconjugates, neurites were shorter on laminin in the presence of 1 and 5 μM oligomannosidic neoglycolipids than in their absence,

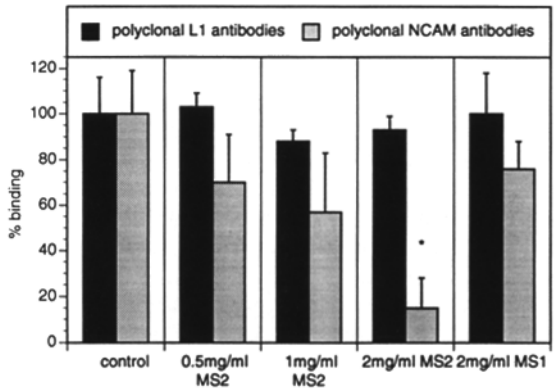

Figure 8. Determination of the association between L1 and NCAM in the absence or presence of peptides MS1 and MS2 containing the first 19 amino acids of the first and fourth immunoglobulin-like domain of NCAM, respectively. L1 was preincubated with increasing amounts of peptide MS2 and, for control, with peptide MS1 (2 mg/ml) before the addition of NCAM. After further incubation, the mixtures were added to substrate-coated monoclonal L1 antibody. Bound antigens were detected by ELISA using their respective polyclonal antibodies. Binding of L1 and NCAM to monoclonal L1 antibody in the absence of peptide MS2 was set to 100% (*control*) and binding of L1 and NCAM in the presence of peptide MS2 expressed in % of control. In the presence of peptide MS1, binding of L1 to substrate-coated monoclonal L1 antibody was inhibited when added either alone or together with NCAM. Therefore, binding of NCAM was expressed relative to the binding of L1, which was set to 100%. Mean values ± SDs from three independent experiments carried out in duplicates are shown.

Table I. List of Oligosaccharides, Neoglycolipids, and Glycoproteins Used for the Study of Inhibition of Neurite Outgrowth from Early Postnatal Cerebellar Neurons

Type of oligosaccharide	Type of glycoconjugate	Concentration
		(μM)
Oligomannosidic (from AMOG, ovalbumin or ribonuclease B)	Oligosaccharide	50–200 μM*
	Neoglycolipid	1–10 μM*
	Glycopeptide	50–200 μM*
	Glycoprotein (not AMOG)	100 μM
$Man_5GlcNAc_2$	Neoglycolipid	10 μM
Lactose	Oligosaccharide	200 μM
	Neoglycolipid	10 μM
Gal-GlcNAc-Man	Oligosaccharide	200 μM
	Neoglycolipid	10 μM
Lacto-*N*-tetraose	Oligosaccharide	200 μM
Non-sialylated complex type (from asialofetuin)	Glycopeptide	200 μM
	Glycoprotein	100 μM

* Complete inhibition was observed at the highest concentration indicated.

whereas in the presence of 10 μM oligomannosidic neoglycolipids or 200 μM oligomannosidic glycopeptides, virtually no neurites could be seen (Fig. 9, *C* and *D*). Neurites which had been formed before the addition of oligomannosidic compounds appeared to become degraded. One day after addition of 10 μM oligomannosidic neoglycolipids, a reduced neurite outgrowth was seen on poly-*L*-lysine compared to the control in the absence of added oligomannosidic compounds, with many neurons (~20–30%) not developing any neurites. After two days, more than 90% of all cells were devoid of neurites on both substrates (not shown). No significant difference in viability of cells was observed between cultures maintained in the presence or absence of additives as judged by the exclusion of trypan blue. Removal of the oligomannosidic compounds by change of the culture medium one day after their addition led to a partial regrowth of neurites from ~30–40% of all neurons (not shown). The inhibitory effects of oligomannosidic oligosaccharides or glycopeptides were the same as of neoglycolipids, although much higher concentrations were necessary (Table I; Fig. 9, *C* and *D*). There was also no difference to observe whether mannosidic oligosaccharides, glycopeptides, or neoglycolipids were derived from either AMOG, ribonuclease B, or ovalbumin (not shown). Neurite outgrowth was not affected in the presence of several other neoglycolipids, oligosaccharides, glycopeptides containing neutral oligosaccharides, or in the presence of asialofetuin (Table I, Fig. 9 *B*). No binding of oligomannosidic neoglycolipids to laminin or poly-*L*-lysine substrates was detectable by ELISA (not shown).

Neurite Outgrowth Is Inhibited in the Presence of the Synthetic Peptide Comprising Part of the C-type Lectin Consensus Sequence in the Fourth Immunoglobulin-like Domain of NCAM

Since oligomannosidic glycans may interfere with the L1/NCAM interaction at the cell surface, the peptide comprising part of the C-type lectin consensus sequence in the fourth immunoglobulin-like domain of NCAM should have similar effects on neurite outgrowth as the oligomannosidic glycans. When the peptide was added to the culture medium of cerebellar neurons, a strong inhibition of neurite outgrowth was observed after 1 d (Fig. 10 *C*). After 2 d, more than 90% of all cells were devoid of neurites (Fig. 10 *D*). When cells were maintained in the presence of the control peptide derived from the first immunoglobulin-like domain of NCAM, neurite outgrowth was unaffected (Fig. 10, *A* and *B*).

The combined observations from these cell culture experiments indicate that an interaction between cell surface expressed oligomannosidic glycans with the carbohydrate recognition domain of the fourth immunoglobulin-like domain of NCAM is implicated in neurite outgrowth.

Discussion

In our study we present evidence that the ability of L1 and NCAM to interact with each other is based on oligomannosidic glycans carried by L1, but not by NCAM. This was shown in experiments in which the association between L1 and NCAM could be inhibited by different glycoconjugates carrying oligomannosidic glycans. The same results were obtained independent of the source of oligomannosidic glycans, i.e., whether they were derived from AMOG, ovalbumin, or ribonuclease B. The association between L1 and NCAM was also strongly inhibited by the oligomannosidic glycan recognizing monoclonal L3 antibody. Although this antibody is of the IgM subtype, the inhibition observed appears to be specific and not due to sterical hindrance for the following reasons: First, monovalent fragments of this antibody also gave significant inhibition. Second, the monoclonal L5 antibody, which is also an IgM and binds to a carbohydrate epitope on L1 (Streit et al., 1991) did not interfere with the interaction between L1 and NCAM. Third, inhibition was not observed in the presence of the mAb recognizing the sulfated L2/HNK-1 carbohydrate which is carried by L1 and NCAM (Kruse et al., 1984). Fourth, other glycoproteins expressing the L2-HNK-1 epitope, which has been shown in several studies to be functionally important in cell

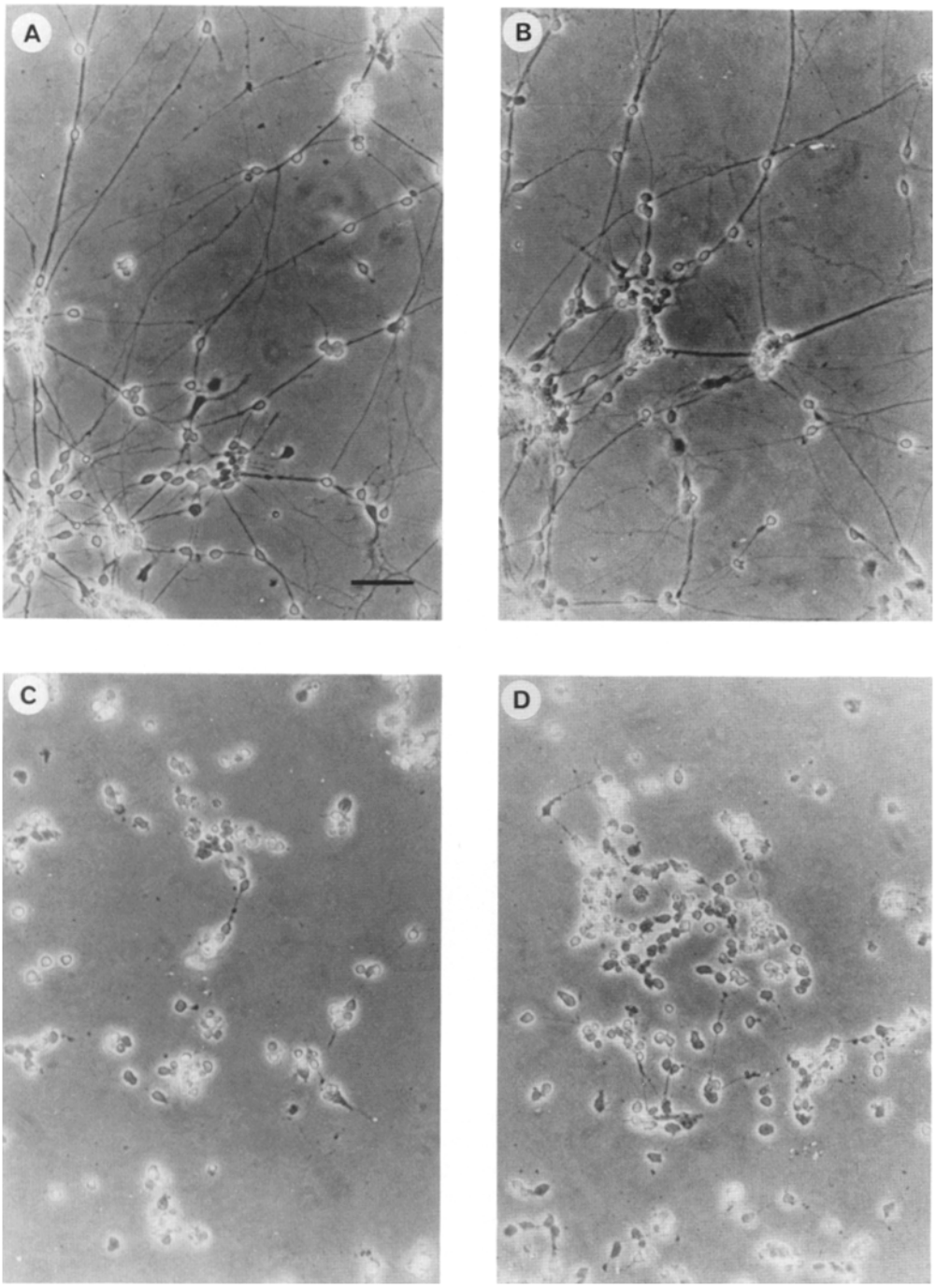

Figure 9. Phase contrast micrographs of small cerebellar neurons cultured in the absence or presence of different glycoconjugates. Early postnatal small cerebellar neurons were plated on laminin-coated glass coverslips. After 3 h, the culture medium was exchanged with culture medium containing no additives (*A*), or containing 10 μM lactosyl-neoglycolipid (*B*), 10 μM oligomannosidic neoglycolipids derived from AMOG (*C*), or 200 μM oligomannosidic glycopeptides derived from ribonuclease (*D*). Phase contrast micrographs were taken 24 h after the change of culture medium. Bar in *A* represents 20 μm for *A–D*.

adhesion and neurite outgrowth (Bronner-Fraser, 1987; Dow et al., 1988; Künemund et al., 1988; Martini et al., 1992; Hall et al., 1993), also did not interfere with L1/NCAM complex formation. Fifth, a mAb recognizing the protein backbone of L1 did not inhibit the L1/NCAM interaction. The direct binding of NCAM to oligomannosidic glycans could also be shown to be specific. The combined observations support the contention that in the L1/NCAM interaction, NCAM functions as a carbohydrate binding protein. Thus NCAM is, in the strict sense of the definition, a lectin in that it is a receptor for particular carbohydrates, but not for others.

That NCAM is a receptor for oligomannosidic glycans is underscored by the finding that sequences characteristic of two different types of lectins can be localized within the fourth and the beginning of the fifth immunoglobulin-like domain of NCAM. Comparison with the C-type carbohydrate recognition domain four of the human mannose receptor (Taylor et al., 1990) yielded a similarity of 37%, with the highest homology being found at the amino-terminal end of the fourth immunoglobulin-like domain of NCAM. Inhibition studies with a synthetic peptide comprising the first 19 amino acids of the amino-terminal end of the domain confirmed that this part is functionally active. A few amino

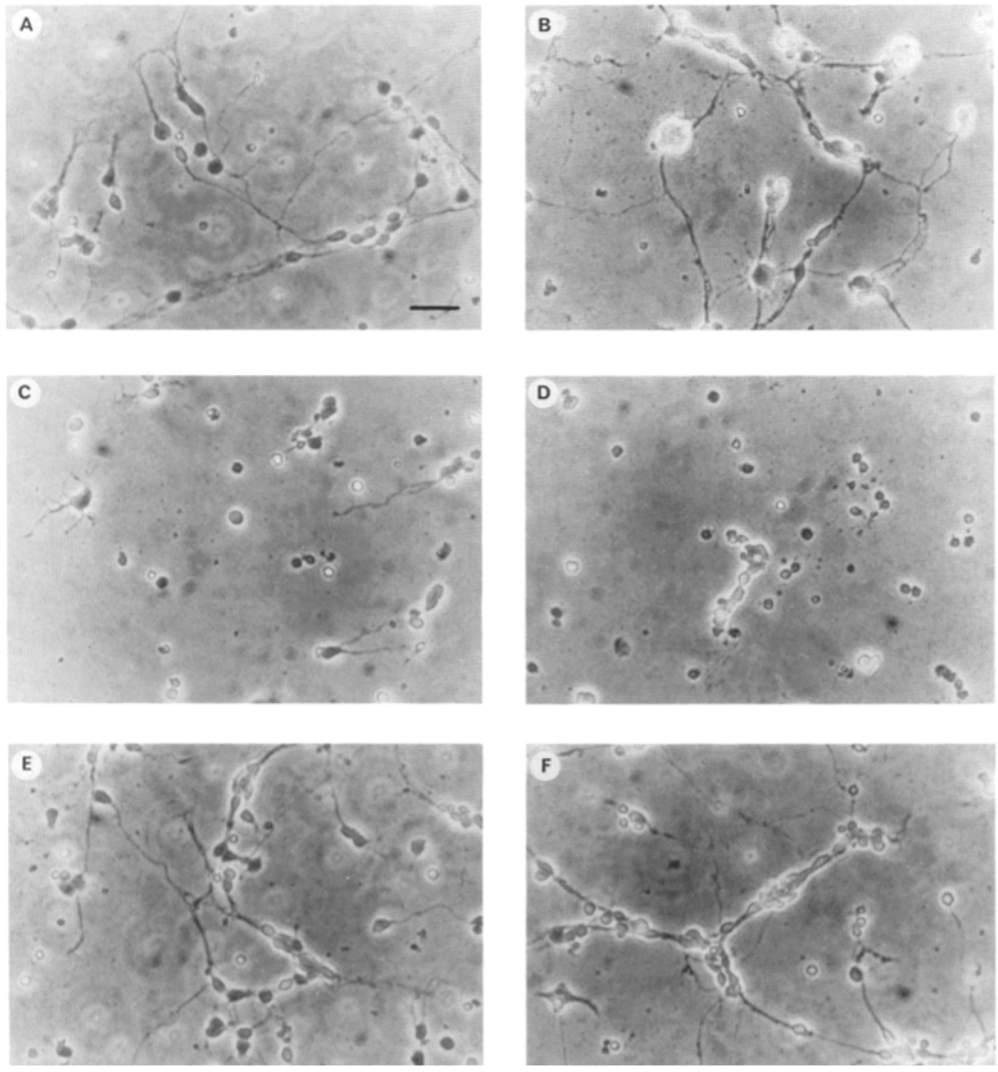

Figure 10. Phase contrast micrographs of small cerebellar neurons cultured in the absence or presence of NCAM-derived peptides. Early postnatal small cerebellar neurons were plated on poly-L-lysine-coated glass coverslips. After 20 h, the culture medium was exchanged with culture medium containing no additives (*E* and *F*) or containing a peptide (1 mM) comprising 19 amino acids of the first (*A* and *B*) or a peptide (1 mM) comprising 19 amino acids of the fourth immunoglobulin-like domain of NCAM (*C* and *D*). Phase contrast micrographs were taken 24 (*A*, *C*, and *E*) or 48 h (*B*, *D*, and *F*) after the change of culture medium. Bar in *A* represents 10 μm for *A–F*.

acids of the consensus sequence for carbohydrate recognition domains of C-type lectins (Weis et al., 1991) could also be aligned at corresponding positions within this peptide. Most surprisingly, the carboxy-terminal half of the fourth and the beginning of the fifth immunoglobulin-like domains of NCAM showed a similarity of 43% to the alpha chains of a group of mannose/glucose specific plant lectins from leguminoses (Richardson et al., 1984). Thus, as to our knowledge, we describe here for the first time a sequence homology between animal and plant lectins. A C-type carbohydrate recognition domain has recently been detected in pertussis toxin, a prokaryotic protein (Saukkonen et al., 1992). This finding and our observations indicate that the sequence for carbohydrate recognition domains of plants, bacteria, and animals are phylogenetically conserved. Another interesting aspect is that within NCAM, structurally different, but functionally similar domains from animal and plant lectins are combined. We do not know whether the presence of two different types of lectin-homologous sequences implies that there are multiple binding sites for oligomannosidic glycans. Nevertheless, at least part of the binding capacity resides in the amino-terminal half of the fourth immunoglobulin-like domain, as the corresponding synthetic peptide inhibited the association between L1 and NCAM.

Some characteristic properties of the lectin-like domains of NCAM are worth being considered in more detail. C-type and plant lectins interact with their ligands in a Ca^{++}-dependent manner. However, L1 and NCAM bind to each other in the absence of Ca^{++}. Interestingly, amino acids which are thought to be important for Ca^{++}-binding (amino acids D, N, E, or Q with side chains carrying carbonyl oxygen atoms at numbers 161 and 165 of the C-type lectin sequence according to Weis et al., 1991) are not contained in the amino acid sequence of NCAM. Ca^{++} is believed to be important for providing the carbohydrate recognition domain with a specific conformation for high affinity binding of ligands at physiological pH and for low affinity binding in the low pH environment of endosomes (Weis et al., 1991). The absence of the amino acids in the NCAM sequence important for Ca^{++} binding suggests that this change in affinity might not be relevant for the binding of oligomannosidic ligands to NCAM at the cell surface.

Oligomannosidic glycans are thought to mainly occur in the ER as precursors of complex-type oligosaccharides

(Kornfeld and Kornfeld, 1985). They also occur on cell surfaces as they could be detected with the monoclonal L3 antibody on cell surfaces of neurons, subpopulations of astrocytes, and oligodendrocytes (Kücherer et al., 1987; Fahrig et al., 1990). These carbohydrates are indeed involved in important cellular functions: Neurite outgrowth was strongly inhibited when oligomannosidic glycoconjugates were added as putative competitors of cell-bound glycans to the culture medium. Oligomannosidic neoglycolipids derived from oligosaccharides of AMOG or ribonuclease B and the $Man_5GlcNAc_2$ glycan were most potent in inhibiting neurite outgrowth. Inhibition of neurite outgrowth was also seen in the presence of free mannosidic oligosaccharides or glycopeptides, although they were about 20 times less effective on a molar basis than the glycolipids. This difference is most likely due to the fact that neoglycolipids exist in aqueous solution as micelles that carry multiple carbohydrate epitopes in contrast to the monovalent oligosaccharides or glycopeptides, which have been shown to display lower affinity to their binding sites than multimers (see for example, Künemund et al., 1988). The inhibition of neurite outgrowth in the presence of the neoglycolipids was probably not the result of their incorporation into cell membranes and subsequently intracellular compartments, because such an uptake is not known to exist for free oligosaccharides or glycopeptides, in the presence of which exactly the same effects as for glycolipids were observed. Furthermore, no influence on neurite growth could be detected in the presence of other neutral neoglycolipids, oligosaccharides, or glycopeptides including those from asialofetuin which were all prepared exactly the same way and mostly even in parallel so that the possibility can be excluded that the effects observed were due to any cytotoxic contaminations not detectable by either thin layer chromatography or mass spectrometry[2].

The effects on neurite growth caused by the oligomannosidic glycans raised the question as to the molecular mechanism(s). Since L1 appears to be the only glycoprotein on cerebellar neurons to carry oligomannosidic glycans (Kücherer et al., 1987) it can be assumed that L1 is the main cell surface glycoprotein which is involved in the oligomannosidic carbohydrate-dependent effect on neurite growth. Also, since L1 and NCAM are coexpressed on cerebellar neurons and associate with each other within the cell surface as described for neuroblastoma cells (Kadmon et al., 1990*a*,*b*), this *cis*-interaction is most likely competitively inhibited in the presence of oligomannosidic carbohydrates. Inhibition of neurite outgrowth in the presence of a synthetic peptide comprising part of the C-type lectin consensus sequence in the fourth immunoglobulin-like domain of NCAM implicates also NCAM in this interaction. The perturbation of the L1/NCAM complex at the cell surface thus may affect intracellular reactions, which in turn could influence neurite outgrowth. Since poly-*L*-lysine and laminin were used as substrates to assay oligomannosidic glycan inhibitable neurite outgrowth, the question remains to be addressed, why a disturbance of oligomannosidic carbohydrate-receptor interaction at the cell surface may influence cell–substrate interactions which are not suspected or known to depend on the L1/NCAM complex. While the cell surface receptors mediating neurite outgrowth on poly-*L*-lysine are not known, laminin is thought to promote neurite outgrowth via integrin cell surface receptors. Since we could exclude that oligomannosidic glycans mediate *trans*-interactions between cells and laminin or poly-*L*-lysine, and since it is unlikely that these cell-substrate interactions are inhibitable by the synthetic peptide comprising part of the C-type lectin consensus sequence of the fourth immunoglobulin-like domain of NCAM, the inhibitory effects of these substances can be attributed to the *cis*-interaction between L1 and NCAM. The ways by which L1 and NCAM may modulate integrin mediated neurite outgrowth are presently not known. It is conceivable that the signal transduction mechanisms involved in neurite outgrowth (Bixby, 1989; Bixby and Jhabvala, 1990; Doherty et al., 1991, 1992) are interconnected, such that perturbance of one may influence another signaling cascade. Preliminary evidence suggests that signal transduction is indeed involved in L1/NCAM interaction, as oligomannosidic neoglycolipids increased intracellular levels of inositol phosphates (von Bohlen und Halbach and Schmitz, unpublished results). It remains to be seen by which intracellular mechanisms the oligomannosidic oligosaccharides or the synthetic peptide evoke changes in neurite outgrowth. The immunoglobulin-like domain four contains a variable alternatively spliced exon (π- or VASE-exon) consisting of 10 amino acids (Santoni et al., 1989; Small et al., 1990). The presence of this exon may lead to an altered three-dimensional structure of the immunoglobulin-like domain and thus to a change in the binding of oligomannosidic glycans. This is of interest in view of the observation that cerebellar and hippocampal cells downregulate their neurite outgrowth at developmental stages where the expression of the π-exon is enhanced and, vice versa, neurite outgrowth is increased when the π-exon is expressed at low levels (Walsh et al., 1992). It remains to be seen whether the π-exon-dependent reduction of neurite outgrowth is related to the oligomannosidic glycan-dependent interaction between L1 and NCAM.

Immunoglobulin-like domain five has been proposed to carry the unusual polysialic acid characteristic of the so-called embryonic form of NCAM (Santoni et al., 1988). Yang et al. (1992) and Zhang et al. (1992) suggested that L1 is modulated in its functions by this polysialic acid. Their data support the view that homophilic interaction between cell surfaces (*trans*-interaction) of L1 is modulated by polysialic acid rather than the heterophilic interaction within the same cell surface (*cis*-interaction). Since the hydrated volume of NCAM containing polysialic acid is approximately three times as large as that of the polypeptide without it (Rutishauser and Landmesser, 1991), it is possible, however, that polysialic acid would also influence the *cis*-binding of L1 to NCAM for steric reasons. It is therefore conceivable that by modulating the interaction between L1 and NCAM, polysialic acid may modify neurite outgrowth (Doherty et al., 1990).

Mannosidic glycans have been implicated in several recognition and adhesion processes. They play a role in the binding of type I fimbriated bacteria to enterocytes of the intestine, a prerequisite for infections caused by bacteria (Lis and Sharon, 1991). The binding and uptake of these bacteria by granulocytes occurs by a process called lectinophagocytosis and is initiated by the attachment of bacteria to mannosidic glycans carried by the leucocyte adhesion molecule CD11/CD18, an integrin (Gbarah et al., 1991). These oligomannosidic glycan-dependent ligand-receptor associations

must be based on *trans*-interactions. It is, however, possible that these glycans may also be involved in *cis*-interactions between the α- and β-integrin subunits as in the interaction between L1 and NCAM. The association of the subunits of human chorionic gonadotropin is mediated by oligomannosidic glycans (Blithe, 1990; Ji and Ji, 1990). Another candidate for a heterodimeric *cis*-interaction is thyroglobulin, which carries oligomannosidic glycans (Arima et al., 1972). The adhesion molecule on glia AMOG, the β2 subunit of Na^+/K^+ ATPase (Gloor et al., 1990) which carries 30% of its molecular weight predominantly as oligomannosidic glycans (Fahrig et al., 1990), can associate with the α-subunit to form a catalytically active enzyme (Schmalzing et al., 1992). It will be interesting to determine whether this association is also mediated by oligomannosidic glycans.

A complex network of interacting molecules can be created through the binding of carbohydrate ligands carried by different glycolipids or glycoproteins to different carbohydrate receptors (Feizi and Childs, 1987). Since adhesion molecules expressing oligomannosidic glycans, such as L1, MAG (Fahrig et al., 1990), AMOG (Antonicek et al., 1987; Kücherer et al., 1987), P0 (Bollensen and Schachner, 1987), and the integrins (Gbarah et al., 1991; Pesheva et al., 1987) are endowed with different functional properties, their interchangeable interactions with NCAM may result in spatially and temporally varying effects on cell–cell and cell–extracellular matrix interactions.

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