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. 1990a. 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. 1990b. 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) can associate with each other thus leading...
to a strengthened adhesion to L1 on another cell. In this
adhesion mechanism, termed "assisted homophilic binding"
(Kadmon et al., 1990a), 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 oligo-
saccharides synthesized by the L1 and NCAM expressing
cells: when the L1 and NCAM dependent aggregation of cul-
tured neuroblastoma cells was determined after growing the
cells in the presence of oligosaccharide processing inhibi-
tors, a dependence on high mannose- or hybrid-type
oligosaccharides was observed (Kadmon et al., 1990b).

After this lead, we have probed the possibility that oligo-
mannose-type oligosaccharides recognized by the mono-
clonal L3 antibody on L1, but not on NCAM (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 charac-
teristic 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 immuno-
globulin-like domain not only interfered with the interaction
between L1 and NCAM, but also inhibited neurite outgrowth.
Similarly, oligomannosidic glycans and their derivatives in-
hibited neurite outgrowth. The results implicate carbohy-
drate-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; Pol-
torak et al., 1987), adhesion molecule on glia (AMOG; Antonicek et
al., 1987), and "rest-L2" (L2/HNK1) positive glycoproteins from detergent ex-
tracts 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 im-
munoaffinity purified as described. Antibodies were prepared in rabbits against immunoaflinity purified 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 Di-
avo (Hamburg, FRG). Polyvalent fragments of IgM antibody according to Matthew and Reichardt
(1982). Monoclonal 473 and monoclonal L5 antibodies were used as amno-
nium sulfate precipitated preparation of hybridoma supernatant. Polyclonal antibodies were prepared in rabbits against immunoprodu ct purified L1 (Rathjen and Schachner, 1984), MAG (Poltorak et al., 1987), AMOG (An-
tonicek 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).

Fragments of NCAM

Protein fragments of NCAM were produced in bacteria in the pET expres-
sion system (Frei et al., 1992). One fragment contained the five immuno-
globulin-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, asialofetuin
(all from Sigma Chem. Co.), and AMOG by digestion of the glycopeptides with pronase (Sigma Chem. Co., catalog no. P-5147; Tai et al., 1977). En-
zyme, 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. Ovomucin positive fractions were collected.

Neoglycolipids

Neoglycolipids were synthesized from oligosaccharides released by treat-
ment with endoglycosidase H (Boehringer Mannheim Corp., Mannheim,
Germany) of AMOG and ribonuclease B, which expresses only oligomann-
osidic glycans (Liang et al., 1980). Neoglycolipids were also derived from
the oligosaccharide Man5GlcNAc2, the triasaccharide containing galactose-
β1-4-N-acetylgalcosamin-β1-2-mannose (TSI01), and from lactose (Sigma
Chem. Co.). The method used and the purification of neoglycolipids is de-
scribed 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: pep-
tide MS1 (Ac-Q-V-A-G-D-A-K-D-K-D-I-S-W-F-S-P-N-G-E-NH2) and pep-
tide MS2 (Ac-A-E-A-S-G-D-P-I-P-S-T-W-R-T-S-T-R-N-H2). Peptide syn-
thesis was performed on a Milligen-9050 continuous-flow synthesizer using an
adapted software package. The Fmoc group was used for N-alpha protec-
tion throughout, the side-chain protection was the following: N(CH2)5-
(tert-butyl)carbonyl for serine, threonine, glutamic acid, and aspartic acid.
Synthesis started on the 4-(2'-diethoxymethylphosphinyl-Fmoc-aminomethyl)-
phenoxy polydimethylacrylamide resin and activation was performed by 2.5,3,3-tetramethyl-2-(2-oxo-2H-
pyrydil)-uracil tetrafluoroborate:disopropylationlamine (1,2, vol/vol)
using a fivefold excess of amino acid derivative. Final acetylation was car-
ried out manually using acetic anhydride 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 H2O, 1.6 ml thioanisole, 0.8 ml ethanediol, 2 g phe-
nol, 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 immuno-
globulin-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 355 in PBS (pH 7.4) for substrate-
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 with 1% fatty acid-free BSA (Sigma Chem. Co., catalog no. A-7030). Bound secondary antibodies were visualized by incubation with H₂O₂ 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), monoclonal 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. L1 and NCAM-Ig were also preincubated with carbohydrates carrying molecules (AMOG, "rest-L2"); glycopolymers prepared from oligomannosidic carbohydrates of ribonuclease B or TSI(01) 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 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 antigens were detected with HRP-conjugated anti-rabbit antibodies in PBS containing 1% BSA. Bound secondary antibodies were visualized by incubation with H₂O₂ and ABTS (2,2 azino-di-3-ethylbenzthiazoline sulfonate 6; Boehringer Mannheim Corp.) as HRP-substrates. The color reaction was determined as described above.

**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 reincubated 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 with 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 polynuclear 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, polynuclear L1 and NCAM antibodies were incubated in a 1:2 dilution series on their respective antigens for 2 h at room temperature. Bound polynuclear 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.** Immunofinity 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 immunofinity purified polyclonal L1 antibodies and alkaline phosphatase-conjugated anti-rabbit antibodies according to Faisnser 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 210, 3/92) was used for the search of sequence homologies with the sequence Cxxxxxx(V,I,L)x(S,T)(V,1)x(E,S), which is present in all eight carbohydrate recognition domains of the human mannose receptor (Taylor et al., 1990) using the PILEUP program (GGC; 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 × 10⁶ 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 H₂O) 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, glycopolypeptides, 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 LI–L1 and NCAM–NCAM interactions are unknown.

**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), TSI01-neoglycolipid (TSI01-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 LI 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 three 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 LI/NCAM association was observed in the presence of neoglycolipids derived from the neutral disaccharide lactose and the trisaccharide TSI01, 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 LI/NCAM complex, the ability of the monoclonal L3 antibody to react with its epitope in the complex was determined. Substrate-coated LI 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 LI/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 (Fig. 4). Further evidence that the oligomannosidic carbohydrate epitope carried by LI is hidden from accessibility to the monoclonal L3 antibody in the LI/NCAM complex came from experiments, in which monoclonal L3 antibody was coated as substrate and the LI/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 LI/NCAM complex was readily bound (not shown).

NCAM Binds to Substrate-bound Oligomannosidic Glycans
As oligomannosidic glycans carried by L1 are required for the LI/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 TSI01 or to BSA. The binding of NCAM to the substrate-coated oligomannosidic glycoproteins 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 LI/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...
the control neoglycolipid or to BSA (Fig. 5). The bacterially expressed protein fragment of NCAM containing the two 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 (TSI01-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.

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)x xxx (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 Kupfer 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 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 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 = 1.0. Alignment of the entire sequence of Local (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 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, δ, 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 MSI starting at glutamine-42, 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, 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.
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                               | Oligosaccharide               | 50–200 μM*         |
| (from AMOG, ovalbumin or ribonuclease B)      | Neoglycolipid                 | 1–10 μM*           |
|                                               | Glycopeptide                  | 50–200 μM*         |
|                                               | Glycoprotein (not AMOG)       | 100 μM             |
| ManαGlcNAc2                                   | Neoglycolipid                 | 10 μM              |
| Lactose                                       | Oligosaccharide               | 200 μM             |
|                                               | Neoglycolipid                 | 10 μM              |
| Gal-βGlcNAc-Man                               | Oligosaccharide               | 200 μM             |
|                                               | Neoglycolipid                 | 10 μM              |
| Lacto-N-tetraose                              | Oligosaccharide               | 200 μM             |
| Non-sialylated complex type                   | Glycopeptide                  | 200 μM             |
| (from asialofetuin)                           | 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 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
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 LI/NCAM complex formation. Fifth, a mAb recognizing the protein backbone of LI did not inhibit the LI/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 LI/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 acids.
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 specific conformation for high affinity binding of ligands at physiological pHe 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.
laminin is thought to promote neurite outgrowth via integrin

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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), PO (Bollensenn 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-extra-cellular matrix interactions.

The authors thank Dr. T. Frei for providing the bacterially expressed NCAM fragments. The trisaccharide TS101 was kindly provided by Dr. T. Ogawa, RIKEN (Wako, Japan), the oligosaccharide ManGlcNAc2 by Dr. A. Haselbeck (Boehringer Mannheim, FRG), and lacto-N-tetraose by Dr. H. Egge (Bonn, FRG). We also thank A. Marax for preparing antibodies, and A. Schwarze for preparing neoglycolipids and cerebellar neurons.

Received for publication 9 August 1992 and in revised form 15 March 1993.

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