Analysis of Neurocan Structures Interacting with the Neural Cell Adhesion Molecule N-CAM*

(Received for publication, May 6, 1996, and in revised form, August 6, 1996)

Charlotte Retzler, Walter Göhring, and Uwe Rauch‡

From the Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, 82152 Martinsried, Germany

Neurocan is a brain-specific chondroitin sulfate proteoglycan, which has been shown to bind to the neural cell adhesion molecule N-CAM and to inhibit its homophilic interaction. To study in more detail the structures of neurocan responsible for this interaction, various recombinant neurocan fragments were generated. The ability of these fragments to interact with N-CAM was investigated in several different in vitro assay systems, enzyme-linked immunosorbent assay-type binding assays, Covasphere-aggregation assays, and assays based on an optical biosensor (BIAcore™) system. The analysis of the homophilic N-CAM interaction in the BIAcore system revealed a $K_D$ of 64 nM. This homophilic interaction could be reduced by preincubation of soluble N-CAM with neurocan. Direct binding of N-CAM to immobilized neurocan core protein and recombinant neurocan fragments could also be demonstrated, and $K_D$ values between 25 and 100 nM were obtained. In addition, direct binding of N-CAM to chondroitin sulfate could be demonstrated.

Binding of N-CAM to the immobilized neurocan core protein could be inhibited with all recombinant fragments containing chondroitin sulfate or major parts of the mucin-like central region of neurocan. For the inhibition of homophilic N-CAM interactions, however, a combination of globular and extended structures was required.

The development of the nervous system, which is characterized by the migration of individual cells and axonal outgrowth, depends on the ability to modulate cell-cell and cell-matrix interactions. Two molecules that may be particularly involved in these processes are the neural cell adhesion molecule N-CAM (1) and the brain-derived chondroitin sulfate proteoglycan neurocan (2). These molecules have been found to colocalize in brain during certain developmental stages and to interact with each other in binding and aggregation inhibition assays (3, 4).

N-CAM was the first described member of the immunoglobulin superfamily of cell adhesion receptors (5). The extracellular part of the molecule consists of five immunoglobulin-related (Ig) and two fibronectin type III domains. N-CAM appears early in development in tissues of all germ layers. In later histogenesis N-CAM is mainly involved in muscle formation and the development of the nervous system. Three major isoforms of N-CAM are known, a glycosinolat phospholipid-linked form and two transmembrane molecules, which differ in the size of their intracellular domain (1). N-CAM and other members of the immunoglobulin family have been shown to bind homophilically in a divalent cation-independent fashion. The homophilic binding of N-CAM is dependent on the presence of the third of the five immunoglobulin-related domains (6). A physiological modulator of the homophilic N-CAM interaction is polysialic acid, which is covalently attached to N-CAM via an N-linked oligosaccharide, preferentially during early developmental stages (7). In addition to this N-CAM intrinsic regulatory mechanism, the ability of proteoglycans of the brain extracellular matrix to inhibit the homophilic interaction of N-CAM has been demonstrated (3).

Neurocan, one of these proteoglycans, is a member of the aggrecan family of chondroitin sulfate proteoglycans (2, 8). Other proteoglycans of this family are PG-M-versican and brevican. These proteoglycans are homologous by having hyaluronan binding domains at their N terminus and epidermal growth factor-like, C-type lectin-like, and complement regulatory-like domains at their C-terminal end. The central regions of these proteoglycans, which are not homologous to each other or other proteins, can differ considerably, in size and in substitution with oligo- and polysaccharide structures (9). The filamentous, 60–90-nm, central region of neurocan is substituted with 2–3 chondroitin sulfate chains and multiple sialylated oligosaccharides, which give this protein a partially mucin-like character (10). During the first postnatal month, in rat brain neurocan is increasingly proteolytically processed in the central region. The generated C-terminal half of the molecule, neurocan-C, still has the ability to interact with N-CAM and to inhibit the homophilic interaction of this cell adhesion molecule (3). A significant reduction of the N-CAM binding activity of neurocan-C was, however, observed after enzymatic removal of the glycosaminoglycan chains (4).

Several neurocan fragments have been produced in a eucaryotic cell line and were compared to tissue-derived neurocan by rotary shadowing electron microscopy, and by their electrophoretic migration behavior on SDS-PAGE.1 Western blot experiments revealed their substitution with oligosaccharide structures, oligomannosidic glycans and the HNK-1 epitope, which both have been reported to be involved in neuronal cell and protein interactions (10).

These recombinant fragments have now been used in different assay systems to elucidate the molecular basis of the interaction of neurocan with N-CAM. The results indicate that more than one structural component of neurocan is responsible for the interaction with N-CAM and that only certain combinations of these structural components are able to inhibit the homophilic interaction of this neural cell adhesion molecule.

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; RU, resonance unit(s); PVC, polyvinyl chloride.
Materials and Methods

Monoclonal Antibodies—Hybridoma cells secreting the monoclonal antibody 5B8, which was used for purification of N-CAM, were developed as described (10). J. Doolittle obtained the Developmental Studies Hybridoma Bank maintained by the Department of Physiology and Molecular Science, Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biological Science, University of Iowa, Iowa City, IA under Contract NO1-HD-2-3144 from the NICHD, National Institutes of Health. The monoclonal antibody 1D1 directed against neurocan has been described previously (11). The construction of expression vectors, transfections, and cell culture—A detailed description of the constructs employed in this study, except 952P, is given elsewhere (10). Construct 952P was designed to start after four unrelated amino acids (AFLPA) with arginine 356 and to end with proline 952 of the authentic rat neurocan sequence (2). As in all other constructs, the signal peptide of human BM40 terminating in an artificial NheI site was placed in front of the neurocan sequence (11). Positive clones of human embryonic kidney cells (293, American Type Culture Collection) transfected with these constructs in the pRCCMV vector (Invitrogen) were identified by SDS-PAGE1 and maintained as described (10).

Protein Purification—Fragment 952P was purified in two steps by hydrophobic interaction chromatography and ion exchange chromatography. 800 ml of medium were adjusted to 2 mM ammonium sulfate, and after centrifugation the supernatant was loaded on a 15-ml octyl-Sepharose 4B-CL column in 50 mM sodium phosphate, pH 7.2. After washing the column with 1 x ammonium sulfate in the same buffer, fragment 952P was eluted with 50 mM sodium phosphate buffer, pH 7.2, without ammonium sulfate. Fractions containing fragment 952P were detected by immunostaining with a polyclonal rabbit serum raised against 1D1 affinity-purified neurocan from 7-day rat brain (provided by Dr. R. U. Margolis, New York University Medical Center, New York, NY). Positive fractions were dialyzed against water, lyophilized, and incubated with 1 ml of DEAE-Sepharose in 150 mM NaCl, 50 mM Tris, pH 8.0. The DEAE resin was washed with 250 mM NaCl in the same buffer, and the protein was eluted with 500 mM NaCl in 50 mM Tris, pH 8.0. Fragment 952P-containing fractions were dialyzed against water and lyophilized. The recombinant neurocan fragments L639, T925, D950, 773M, and 359H were purified as described (10). Native neurocan was purified from the PBS-soluble supernatant of 7-day postnatal rat brain as described previously (8), and N-CAM was isolated from the corresponding membrane fraction by immunooaffinity chromatography using the 5B8 monoclonal antibody (5).

Analytical Methods—SDS-PAGE was performed on 10% slab gels (12) and stained with Coomassie Blue (Sera, Heidelberg, Germany) according to standard protocols. Protein concentrations were determined with the Micro BCA reagent (Pierce) according to the manufacturer’s protocol. Digestion with protease-free chondroitinase ABC (Seikagaku, Tokyo, Japan) was carried out for 1 h at 37 °C in 100 mM Tris/Cl, pH 8.5, 30 mM sodium acetate using 0.5 milliunits of enzyme. N-CAM was treated with Endo-N in 100 mM Tris/Cl, pH 8, 40 mM MgCl2, 4 mM DTT for 30 min at 37 °C.

Covasphere Aggregation Assay—N-CAM was covalently coupled to 0.5-μm Covaspheres (Duke Scientific Corp., Palo Alto, CA) according to the manufacturers protocol. Aggregation assays were performed as described (3) with the following changes; 5 μl of N-CAM-coupled Covaspheres in 20 μl of PBS, 1% BSA were diluted in a total volume of 50 μl of a 10 μg/ml solution of purified test protein in PBS. After sonication, the samples were kept at 37 °C for 2.5 h, and several 1-μl droplets of each sample were analyzed in the fluorescence microscope.

Enzyme-linked Immunosorbent Assay-type Protein Ligand Binding Assay—1 μg of protein/well (native neurocan or recombinant neurocan fragments) was immobilized overnight on polyvinylchloride (96-well PVC plates; Falcon Plastics, Cockeysville, PA) in 0.1 x NaHCO3, pH 8.0. The wells were blocked for 2 h with 1% BSA in the same buffer and then incubated with 100 μl of purified N-CAM at concentrations of 50–0.5 μg/ml in PBS containing 2 mM MgCl2, 2 mM CaCl2, and 1% BSA overnight. The plates were then washed five times with the same buffer containing 0.02% Tween 20 (TBS-T) and incubated with 5B8 monoclonal antibody (hybridoma supernatant diluted 1:10 in TBS containing 2 x NaHCO3 and 1% BSA) overnight. After extensive washing with TBS-T, the wells were incubated with peroxidase-derivatized secondary antibody for 1 h, washed twice in TBS-T and once in 20 mM Na2HPO4, pH 6.8, and developed with 4 mM 5-amino-2-hydroxybenzoic acid in 20 mM Na2HPO4, pH 6.8. The reaction was stopped after 10 min with 2 mM NaOH and the optical density measured at 490 nm. The binding assay with free chondroitin sulfate was performed in a similar fashion with the following modification: 20 μg/ml protein-free chondroitin sulfate from bovine cartilage (Sigma) were at room temperature. In the inhibition assay, different concentrations of protein-free chondroitin sulfate were preincubated with 50 μg/ml N-CAM in TBS containing 2 mM MgCl2, 2 mM CaCl2 at 37 °C for 30 min and then incubated with native neurocan coated on PVC-microtiter plates (1 μg/well) overnight at 4 °C. The detection and development of the binding assay were performed as described above.

BIAcore System—This system (Pharmacia Biosensor AB, Uppsala, Sweden) has been described in detail (13). In this system, binding of soluble ligands to immobilized ligands is measured in arbitrary units (RU). There is a linear relationship between the mass of the protein bound to the immobilized protein and the RU observed (1000 RU = 1 μg/mm2 bound protein). Purified neurocan or recombinant neurocan fragments could only be immobilized to the carboxymethylated dextran matrix of the sensor chip after cleavage of the glycosaminoglycan chains with chondroitinase ABC. The core proteins were coupled at concentrations of 200 μg/ml in 0.1 mM sodium acetate, pH 5.0, by the aminocoupling procedure according to the manufacturer’s protocol. Each ligand was immobilized at concentrations of about 2 ng/mm2 (2000 RU). Purified N-CAM was diluted in TBS containing 2 mM MgCl2, 2 mM CaCl2, and 0.05% P-20 and added in concentrations ranging from 6.25 to 50 μg/ml. The sample was injected at a flow rate of 5 μl/min for 7 min at 25 °C. The complexes were dissociated by washing with the same buffer containing 0.5 or 1 x NaCl. The association rate constant (kA) and the dissociation rate constant (kD) were calculated according to the BIAl evaluation software version 2.1 provided by the manufacturer. The affinity constant was calculated from the equation Kd = kD / kA.

Expression and Purification of the Recombinant Fragment 952P—Expression and structural features of the recombinant fragments 359H, 773M, L639, T925, and D950 will be described elsewhere (10). Like these fragments, the rat neurocan cDNA fragment 952P coding for 597 amino acids of the central region of neurocan was linked to the BM-40 signal peptide (Fig. 1). The transfected cells secreted this fragment as a chondroitin sulfate proteoglycan with a core protein with an apparent molecular mass of 190 kDa, which was recognized by a polyclonal anti-neurocan antiserum (results not shown). By hydrophobic interaction and ion exchange chromatography, this fragment could be enriched to considerable purity (Fig. 2A).

Analysis of Homophilic N-CAM Interaction in the BIAcore System—The polysialylated N-CAM molecules used in our assays were isolated from the brain of 7-day-old rats with the aid of the monoclonal antibody 5B8, and represent a mixture of the 140- and 180-kDa transmembrane isoforms (Fig. 2B). These molecules were tested for their ability to interact homophilically in the BIAcore system. With this system, kinetic constants for the association and dissociation of isolated molecules can be obtained, and from these parameters the dissociation constant can be calculated. The observed interaction between soluble and immobilized N-CAM indicated a single-phase association and dissociation kinetic, with a Kd of 64 mM (Fig. 3; Table I). In addition to this, binding of tissue-derived neurocan to immobilized N-CAM was analyzed. Although an interaction has previously been demonstrated in radioimmunoassays, no binding could be observed in the BIAcore system. An inhibition of the binding of soluble N-CAM to immobilized N-CAM, however, was apparent after preincubation of soluble N-CAM with the core protein of tissue-derived neurocan. Two large, recombinantly expressed, neurocan fragments, 773M and L639, containing one of the terminal globular domains and a major part of the central region, were potent inhibitors of the N-CAM/N-CAM interaction. The C-terminal globular region alone, fragment T950, displayed considerably reduced inhibitory capacity (results not shown).
Since it was not possible to observe binding of soluble neurocan to covalently immobilized N-CAM, the binding of soluble N-CAM to covalently immobilized neurocan was tested. Thus, all recombinant neurocan fragments had to be immobilized separately and tested for their N-CAM binding ability. Fortunately, in the BIAcore system the dissociation constants are solely calculated from the dissociation and association profiles and do not depend on the absolute amount of binding. Therefore, the affinities of the different fragments were comparable, even when they were immobilized with variable efficiencies. Initial attempts to immobilize chondroitin sulfate containing neurocan and recombinant neurocan fragments to the dextran layer of a sensor chip were unsuccessful, probably due to electrostatic repulsion of the polyanionic glycosaminoglycan structures and the carboxymethylated dextran matrix. However, after chondroitinase ABC treatment tissue-derived neurocan and the recombinant fragments 773M and L639 could be immobilized, whereas even after chondroitinase ABC treatment an immobilization of the core protein of fragment 952P could not be achieved. In this configuration, binding of N-CAM to the immobilized neurocan core protein could clearly be demonstrated (Fig. 4A). The binding was concentration-dependent, and the mean $K_D$ value calculated from the kinetics observed with four different N-CAM concentrations was 28 nM (Table I). The $K_D$ values for the binding of N-CAM to the recombinant neurocan core protein could clearly be demonstrated (Fig. 4A). The binding was concentration-dependent, and the mean $K_D$ value calculated from the kinetics observed with four different N-CAM concentrations was 28 nM (Table I). The $K_D$ values for the binding of N-CAM to the recombinant neurocan core protein were similar in the range of 25–33 nM (Fig. 4B; Table I). Only the $K_D$ value of N-terminal fragment 359H was with 100 nM significantly higher. These results indicate that N-CAM is able to interact with similar affinities with non-overlapping parts of the neurocan core protein. The binding of N-CAM to neurocan was not affected by treatment of N-CAM with Endo-N (results not shown), an enzyme that specifically cleaves $\alpha$-2,8-linked polysialic acid (7).

N-CAM Binding to Chondroitin Sulfate Chains—Although the BIAcore assay revealed a high affinity binding between N-CAM and the core proteins of neurocan and the recombinant fragments, it was not possible to evaluate the contribution of the glycosaminoglycan chains with this system. A considerable contribution of these polysaccharides has been shown previously in radioimmunoassays, where non-covalently adsorbed cell adhesion molecules were incubated with 125I-labeled proteoglycans. Although a similar approach, the detection of neurocan binding to immobilized N-CAM with a polyclonal antiserum and an enzyme-linked secondary antibody, failed to give reasonable signals, N-CAM binding to immobilized neurocan and recombinant neurocan fragments could be observed. Bound N-CAM was detected with the monoclonal antibody 5B8. In these assays, only the proteoglycan forms of neurocan and the recombinant fragments were recognized by N-CAM,
Table I

| Immobilized ligand     | $k_a$  | $k_d$  | $K_D$ |
|------------------------|--------|--------|--------|
| N-CAM                  | 5.85 x 10^{-4} | 9.1 x 10^{3} | 64     |
| Neurocan core protein  | 9.4 x 10^{-4}  | 3.4 x 10^{3}  | 28     |
| Fragment 773M          | 1.8 x 10^{-3}  | 5.5 x 10^{4}  | 33     |
| Fragment L639          | 1.9 x 10^{-3}  | 7.6 x 10^{4}  | 25     |
| Fragment T950          | 1.9 x 10^{-3}  | 7.5 x 10^{4}  | 25     |
| Fragment 359H          | 1.7 x 10^{-3}  | 1.7 x 10^{4}  | 100    |

**FIG. 4.** Analysis of direct binding of N-CAM to native neurocan core protein or recombinant neurocan fragments (core proteins) using the BIAcore system. A, tissue-derived neurocan was covalently immobilized on the carboxymethylated dextran matrix of the sensor chip, and N-CAM was used as the soluble ligand in the indicated concentrations. B, the recombinant fragments indicated in the figure were covalently immobilized on the BIAcore chip, and N-CAM was used as the soluble ligand with a concentration of 500 nM. The arrows indicate the beginning (A) and the end (B) of the application of the soluble ligand.

**FIG. 5.** Binding of N-CAM to chondroitin sulfate. A, chondroitin sulfate was adsorbed to a PVC 96-well microtiter plate at a concentration of 20 µg/well in TBS, pH 8, and incubated with increasing concentrations of N-CAM. B, tissue-derived neurocan was adsorbed to a PVC 96-well microtiter plate at 1 µg/well and incubated with N-CAM, which had been preincubated with increasing concentrations of chondroitin sulfate.

whereas chondroitinase digestion consistently destroyed the ability of neurocan and the recombinant fragments to interact with N-CAM (results not shown). To find out whether these observations reflect a direct binding of N-CAM to chondroitin sulfate chains or a perturbation of core protein-related binding sites by the chondroitinase treatment, microtiter plates were coated with chondroitin sulfate from bovine trachea. Significant binding of N-CAM could be observed to immobilized chondroitin sulfate (Fig. 5A). The interaction of N-CAM with immobilized tissue-derived native neurocan could be inhibited by chondroitin sulfate in a concentration-dependent fashion (Fig. 5B). The ability of N-CAM to interact with chondroitin sulfate chains from three different sources, rat brain, 293 cells, and bovine trachea, indicated a general affinity of N-CAM for this type of glycosaminoglycan structures.

**Inhibition of N-CAM Binding to Covalently Immobilized Neurocan Core Proteins by Recombinant Neurocan Fragments**—The demonstration of the binding of N-CAM to chondroitin sulfate chains in the enzyme-linked immunosorbent assay reflects a considerable contribution of the glycosaminoglycan chains to the neurocan/N-CAM interaction, whereas the BIAcore studies show the significant contribution of the neurocan core protein. To be able to evaluate the contribution of both components in the same system, an indirect assay was designed, where the ability of tissue-derived neurocan and of the recombinant fragments to inhibit the binding of N-CAM to covalently immobilized neurocan core protein was observed. In these experiments, two general principles became apparent. The ability of all proteoglycans to inhibit N-CAM binding was considerably higher than the activity of the corresponding core proteins (Figs. 6 and 7). This effect was most pronounced with fragment D925, where the inhibitory activity was reduced from 98% to 1% after chondroitinase digestion. Furthermore, the ability of the core proteins of neurocan fragments containing major parts of the central region to inhibit N-CAM binding was considerably higher than the inhibitory activity of fragments with no or only a small contribution of the central region. This effect was especially apparent with fragment 952P, which showed an inhibitory activity (74%) that was even higher than the activity of tissue-derived neurocan (60%) (Figs. 6 and 7). A fragment that could not be used in this assay system was fragment 359H, representing the N-terminal globular domain. This fragment was the only one that showed affinity to immobilized neurocan core protein, whereas fragment 773M and neurocan, which both contain this domain as well, did not show any interaction. Binding of fragment 359H was increased by preincubation of the immobilized neurocan core protein with hyaluronan (results not shown).

**Inhibition of N-CAM-mediated Covasphere Aggregation**—To correlate the N-CAM binding properties of the recombinant fragments with their inhibitory activities regarding homophilic N-CAM interaction, which had already been observed for some of the fragments in the BIAcore system, the Covasphere aggregation assay was used. Covaspheres are fluorescently labeled plastic particles of 0.4 µm in diameter, which can be coated with protein by covalent linkage. Due to the homophilic N-CAM
interaction, Covaspheres coated with N-CAM aggregate (Fig. 8). Since, in this system, multiple interactions of N-CAM molecules are possible, it should be more representative for the situation in tissue, where many N-CAM molecules are integrated in the same membrane. It has been shown previously that the N-CAM-mediated aggregation of Covaspheres can be inhibited by brain-derived neurocan and neurocan-C and their core proteins, but not by various other extracellular matrix molecules like tenascin, fibronectin, and the cartilage-derived proteoglycan aggrecan (3). Similar to neurocan-C, the recombinant fragment L639 inhibited the aggregation in its native and chondroitinase ABC-treated form (Figs. 7 and 8). Recombinant fragment 773M, which has a 160-amino acid overlap with fragment L639, was also able to inhibit the N-CAM-Covasphere aggregation in its native and chondroitinase-treated form, whereas fragment D925 was only active in the presence of its chondroitin sulfate chains (Figs. 7 and 8). Recombinant fragment T950 showed no inhibitory activity. Fragment 952P, which was most active in the N-CAM binding assay, showed no inhibitory activity in the aggregation assay (Figs. 7 and 8). Recombinant fragment T950 showed no inhibitory activity. Fragment 952P, which was most active in the N-CAM binding assay, showed no inhibitory activity in the aggregation assay (Figs. 7 and 8). Recombinations of this fragment with either the N- or C-terminal domain (359H and T950) or both were not sufficient to regain inhibitory activity (results not shown). These results were well reproducible throughout at least five individual experiments. With free chondroitin sulfate from bovine trachea, aggregates were observed in most of the experiments, although sometimes less and smaller compared to the BSA control (Fig. 8).

**DISCUSSION**

Fragments of the brain-derived chondroitin sulfate proteoglycan neurocan produced in a mammalian cell line have been used to investigate those parts of this proteoglycan involved in interactions with the neural cell adhesion molecule N-CAM.

The results obtained in three different assay systems can be summarized as follows. 1) N-CAM has the ability to bind to all major components of the neurocan molecule, the N-terminal globular domain, the mucin-like central region, the chondroitin sulfate chains, and the C-terminal globular domain. 2) These components are, however, not able to inhibit N-CAM-mediated Covasphere aggregation when they are separated from each other. 3) Only certain covalently linked combinations of these components, generally consisting of at least one extended and one globular structure, were able to inhibit the homophilic N-CAM aggregation.

The recombinant fragments that were used in this study had been analyzed previously and compared to tissue-derived neurocan by rotary shadowing electron microscopy, their electrophoretic migration behavior on SDS-PAGE, and for their substitution with sialic acid residues, oligomannosidic glycans, and the HNK-1 epitope (10). The major observed difference was the presence of oligomannosidic glycans on the recombinantly expressed C-terminal fragments T950 and D925. However, this modification is not likely to induce significant N-CAM binding activity, since fragment 359H, in accordance with the situation in vivo also modified with these saccharide structures, showed actually (with a \(K_D\) value of 100 nM) the weakest affinity to N-CAM of all immobilized fragments. Fragment L639, like tissue-derived neurocan-C not modified with oligomannosidic glycans (10), showed in the BIAcore assay very similar N-CAM binding kinetics as the core protein of tissue-derived neurocan, the core protein of fragment 773M, and fragment T950, which are all modified with these oligosaccharides (10). The calculated \(K_D\) values for these proteins were all in the range of 25–33 nM. These values are significantly higher than the \(K_D\) value of 0.4 nM for tissue-derived neurocan obtained by a Scatchard analysis of the binding of \(^{125}\text{I}\)-labeled neurocan proteoglycan to N-CAM-coated microtiter plates (4). This difference might reflect the considerably reduced ability of the \(^{125}\text{I}\)-labeled neuro-
can core protein to bind to N-CAM also observed in that study (4), indicating a significant participation of the chondroitin sulfate chains in this interaction. Whereas heparan sulfate binding to N-CAM has already been considered to play a role in N-CAM-mediated neural cell adhesion for some time (14), the participation of chondroitin sulfate became apparent only recently (4,15). The present results render all chondroitin sulfate proteoglycans potential ligands for N-CAM.

The observation that fragment T950 showed an affinity for N-CAM similar to that of the core proteins of fragment L639 and 773M in the BIAcore binding study, but was much less effective in the inhibition studies, might indicate the presence of more than one neurocan binding site within N-CAM. For an efficient inhibition of the homophilic N-CAM interaction, more than one site might have to be occupied simultaneously by the same molecule. The endogenous proteolytic fragment, neurocan-C, would actually be perfectly suited for such inhibitory activity, since it has retained all features essential for this inhibition. Even the loss of additional parts of the central region or of the glycosaminoglycan chains would not eliminate its inhibitory activity. The loss of the N-terminal half of neurocan containing the hyaluronan binding region should enable neurocan-C, no longer contained in aggregates with hyaluronan, to reach even more efficiently its target molecule (Fig. 9). Therefore, this fragment might be considered as a processing rather than as a degradation product.

Although no $K_D$ value could be determined for fragment 952P, the BIAcore inhibition results indicate a considerable ability of the central region of neurocan to interact with N-CAM. Another brain-derived proteoglycan with the ability to interact with and to inhibit the aggregation of neural cell adhesion molecules with and without chondroitin sulfate chains is phosphacan (16, 17). Although phosphacan and neurocan have no significant similarities in their amino acid sequences, the amino acid composition throughout the C-terminal two thirds of phosphacan reveals, however, the same high content of serine, threonine, and proline (35%) (16) as is found in the central region of neurocan (35%) (2). In neurocan, this part of the molecule has an extended shape and is modified with many sialic acid residues, both characteristic features of mucins (10). Since it is likely that the C-terminal part of phosphacan exhibits similar structural features, it might be possible that the interaction of both core proteins with N-CAM depends, in part, on a mucin-like character of certain protein domains. Specific functions of mucin-like domains have mainly been investigated in lymphocyte-endothelial interactions (18–20). It might be quite possible that similar recognition and guiding mechanisms are involved in the homing of leucocytes and the targeting of neurons and growth cones. The selectin-mediated rolling of leucocytes requires very rapid formation and dissociation of adhesion contacts (21). During development, fast formations and dissociation of contacts should also be important for the filopodia of growth cones to achieve rapid extension of axons. The $k_e$ and $k_d$ constants for the neurocan-N-CAM interaction obtained with the BIAcore evaluation program do not indicate extraordinarily high turnover rates, but they are at least as high as the values observed for the homophilic N-CAM interaction (Table I). A second feature dis-

![Fig. 8. Effects of recombinant neurocan fragments on aggregation of N-CAM-coated Covaspheres. a, N-CAM-coated Covaspheres with 10 μg/ml BSA. b, BSA-coated Covaspheres. c, N-CAM-coated Covaspheres with 10 μg/ml chondroitinase-treated recombinant fragment 773M. d, N-CAM-coated Covaspheres with 10 μg/ml chondroitinase-treated recombinant fragment L639. e, N-CAM-coated Covaspheres with 10 μg/ml chondroitinase-treated recombinant fragment D925. f, N-CAM-coated Covaspheres with 10 μg/ml chondroitinase-treated recombinant fragment 952P. h, N-CAM-coated Covaspheres with 10 μg/ml chondroitin sulfate.

Neurocan/N-CAM Interactions 27309
played by lymphocyte receptors containing mucin-like domains is their simultaneous interaction with different receptors via different domains (19). Due to its domain organization and the observed interactions with other ligands, in particular hyaluronan, L1/Ng-CAM, and tenascins, neurocan could exhibit similar abilities, binding to several molecules via different domains. Neurocan would therefore be well suited to serve as a substrate in pathfinding activities of growing axons and migrating cells during development.

Acknowledgments—We thank Dr. Rupert Timpl for support and discussions, Drs. Richard Margolis and Karlheinz Mann for reading manuscripts, Dr. Martin Grumet for the batch number of functional Covaspheres, and Gerlinde Kulbe for expert technical assistance.

REFERENCES

1. Edelman, G. M., and Crossin, K. L. (1991) Annu. Rev. Biochem. 60, 155–190
2. Rauch, U., Karthikeyan, L., Maurel, P., Margolis, R. U., and Margolis R. K. (1992) J. Biol. Chem. 267, 19356–19547
3. Grumet, M., Flaccus, A., and Margolis, R. U. (1993) J. Cell Biol. 120, 815–824
4. Friedlander, D. R., Milev, P., Karthikeyan, L., Margolis, R. K., Margolis, R. U., and Grumet, M. (1994) J. Cell Biol. 125, 669–680
5. Hoffman, S., Sorkin, B. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A., and Edelman, G. M. (1982) J. Biol. Chem. 257, 7720–7729
6. Rao, Y., Wu, X.-F., Yip, P., Gariepy, J., and Siu, C.-H. (1993) J. Biol. Chem. 268, 20630–20638
7. Rutishauser, U., Walanabe, M., Silver, J., Troy, P. A., and Vines, E. R. (1985) J. Cell Biol. 101, 1842–1849
8. Rauch, U., Gao, P., Janetzko, A., Flaccus, A., Hilgenberg, L., Tekotte, H., Margolis, R. K., and Margolis, R. U. (1991) J. Biol. Chem. 266, 14785–14801
9. Iozzo, R. V., and Murdoch, A. D. (1996) FASEB J. 10, 586–614
10. Retzlaff, C., Wiedemann, H., Kulbe, G., and Rauch, U. (1996) J. Biol. Chem. 271, 17107–17115
11. Mayer, U., Nischl, R., Pischl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y., and Timpl, R. (1990) EMBO J. 9, 1879–1885
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Maurel, P., Rauch, U., Flad, M., Margolis, R. K., and Margolis, R. U. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2512–2516
14. Cole, G. J., Loewy, A., and Glaser, L. (1986) Nature 320, 445–447
15. Storms, S. D., Anvekar, V. M., Adams, L. D., and Murray, B. A. (1996) Exp. Cell Res. 223, 385–394
16. Milet, P., Friedlander, D. R., Sakurai, T., Karthikeyan, L., Flad, M., Margolis, R. K., and Margolis, R. U. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2512–2516
17. Milev, P., Friedlander, D. R., Sakurai, T., Karthikeyan, L., Flad, M., Margolis, R. K., Grumet, M., and Margolis, R. U. (1994) J. Cell Biol. 127, 1703–1715
18. Imai, Y., Singer, M. S., Fennie, C., Laskey, L. A., and Rosen, S. D. (1991) J. Cell Biol. 113, 1213–1221
19. Berg, E. L., McEvoy, L. M., Berlin, C., Bargatz, R. F., and Butcher, E. C. (1993) Nature 366, 685–698
20. Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., and Larsen, G. R. (1993) Cell 72, 1179–1186
21. Van der Merwe, P. A., and Barclay, A. N. (1994) Trends Biochem. Sci. 19, 354–358

Fig. 9. Neurocan-C is released from hyaluronan aggregates and interacts with N-CAM, thereby inhibiting N-CAM-mediated cell adhesion.