The Neuronal Chondroitin Sulfate Proteoglycan Neurocan Binds to the Neural Cell Adhesion Molecules Ng-CAM/L1/NILE and N-CAM, and Inhibits Neuronal Adhesion and Neurite Outgrowth

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Abstract. We have previously shown that aggregation of microbeads coated with N-CAM and Ng-CAM is inhibited by incubation with soluble neurocan, a chondroitin sulfate proteoglycan of brain, suggesting that neurocan binds to these cell adhesion molecules (Grumet, M., A. Flaccus, and R. U. Margolis. 1993. J. Cell Biol. 120:815). To investigate these interactions more directly, we have tested binding of soluble 125I-neurocan to microwells coated with different glycoproteins. Neurocan bound at high levels to Ng-CAM and N-CAM, but little or no binding was detected to myelin-associated glycoprotein, EGF receptor, fibronectin, laminin, and collagen IV. The binding to Ng-CAM and N-CAM was saturable and in each case Scatchard plots indicated a high affinity binding site with a dissociation constant of ~1 nM. Binding was significantly reduced after treatment of neurocan with chondroitinase, and free chondroitin sulfate inhibited binding of neurocan to Ng-CAM and N-CAM. These results indicate a role for chondroitin sulfate in this process, although the core glycoprotein also has binding activity. The COOH-terminal half of neurocan was shown to have binding properties essentially identical to those of the full-length proteoglycan.

To study the potential biological functions of neurocan, its effects on neuronal adhesion and neurite growth were analyzed. When neurons were incubated on dishes coated with different combinations of neurocan and Ng-CAM, neuronal adhesion and neurite extension were inhibited. Experiments using anti-Ng-CAM antibodies as a substrate also indicate that neurocan has a direct inhibitory effect on neuronal adhesion and neurite growth. Immunoperoxidase staining of tissue sections showed that neurocan, Ng-CAM, and N-CAM are all present at highest concentration in the molecular layer and fiber tracts of developing cerebellum. The overlapping localization in vivo, the molecular binding studies, and the striking effects on neuronal adhesion and neurite growth support the view that neurocan may modulate neuronal adhesion and neurite growth during development by binding to neural cell adhesion molecules.

Interactions of cells with proteins in the extracellular matrix and on the surfaces of other cells are important for cell adhesion, cell migration, and transmembrane signalling. These processes are mediated by plasma membrane proteins that include cell adhesion molecules (CAMs) and receptors for other proteins such as extracellular matrix (ECM) proteins and growth factors. Whereas CAMs have been found to promote cell adhesion and cell migration, different ECM proteins may either promote or inhibit these processes in different situations (Hynes and Lander, 1992; Edelman and Crossin, 1991; Reichardt and Tomaselli, 1991; Schwab et al., 1993). For example, whereas fibronectin promotes adhesion and migration of many types of cells, tenascin/cytotactin can either support or inhibit neurite growth in culture, depending on the conditions (Crossin et al., 1990; Lochter et al., 1991).

Chondroitin sulfate proteoglycans have been found to be potent inhibitors of cell adhesion, cell migration, and neurite growth (Ruoslhti, 1989; Wight et al., 1992; Margolis and Margolis, 1993). They are found predominantly in the ECM of various tissues and their expression during development in regions such as neural crest cell pathways and the spinal cord is consistent with the concept that they may act as barriers for cell migration (Perris et al., 1991; Oakley and Tosney, 1991; Snow et al., 1990a, b; Pindzola et al., 1993). Ini-
tial investigations of potential functions of proteoglycans in the central nervous system were performed using proteoglycans isolated from other tissues from which they could be purified more easily than from brain (Snow et al., 1990a, 1991). Although the observed effects have been attributed primarily to the chondroitin sulfate component, our recent studies (Grumet et al., 1993) indicate that the core glycoproteins of neurocan and phosphacan (Maurel et al., 1994; previously designated the 3F8 proteoglycan) are potent inhibitors of neuronal adhesion. This emphasizes the importance of determining the primary structures of brain proteoglycans, since this structural information will facilitate more detailed investigations of their molecular mechanisms of action.

Neurocan (previously designated IDI) is an ~500-kD chondroitin sulfate proteoglycan of rat brain which is developmentally regulated with respect to its molecular size, concentration, carbohydrate composition, sulfation, and immunocytochemical localization (Rauch et al., 1991). A 5.2-kb composite sequence of overlapping cDNA clones contains an open reading frame of 1,257 amino acids which encodes a 136-kD protein (Rauch et al., 1992). The deduced amino acid sequence reveals a signal peptide followed by an Ig domain, tandem repeats characteristic of the hyaluronic acid-binding region of aggregating proteoglycans, and an RGDS sequence. The COOH-terminal portion has ~60% identity to regions in the COOH termini of two other proteoglycans, versican and aggrecan, whereas the central 595–amino acid portion of neurocan has no homology with other reported protein sequences. The proteoglycan isolated from adult brain with the IDI monoclonal antibody represents the COOH-terminal half of neurocan and is therefore designated neurocan-C. A probe corresponding to a region of neurocan having no homology with versican or aggrecan hybridized specifically with a single band at 7.5 kb on Northern blots of mRNA from both 4-d and adult rat brain. This indicates that neurocan-C of adult brain, containing a 68-kD core protein, is generated by a developmentally regulated in vivo proteolytic processing of the 136-kD species which is predominant in early postnatal brain (Rauch et al., 1992).

We have recently investigated interactions of neurocan with neurons and the neural CAMs, Ng-CAM, and N-CAM (Grumet et al., 1993). Both of these CAMs have been implicated in developmental processes in nervous tissue, including cell adhesion, neuronal migration, and axonal growth (Edelman and Crossin, 1991; Grumet, 1992; Doherty and Walsh, 1992). Ng-CAM and N-CAM show some structural similarities in that they are ~25% identical in amino acid sequence, they both contain extracellular regions with Ig-like and fibronectin-like domains, and they share at least one carbohydrate determinant that is recognized by monoclonal antibodies HNK-1 and NC-1 (Burgoon et al., 1991; Grumet et al., 1984b; Kruse et al., 1984). Each CAM can bind homophilically, as demonstrated by the ability of CAM-coated fluorescent beads (Covaspheres) to self-aggregate (Grumet and Edelman, 1988; Hoffman and Edelman, 1983). It was found that the aggregation of Covaspheres coated with either Ng-CAM or N-CAM was strongly inhibited by intact neurocan, and by the core glycoproteins resulting from chondroitinase treatment of both neurocan and neurocan-C (Grumet et al., 1993). Neurocan also inhibited adhesion of neurons to Ng-CAM when mixtures of these proteins were adsorbed to polystyrene dishes. On the other hand, in a short term binding assay that allows one to detect interactions of cells with proteins that inhibit cell spreading (Friedlander et al., 1988), direct adhesion of neurons to neurocan core protein could be demonstrated (Grumet et al., 1993).

These results indicate that neurocan can bind to neurons, and raise the possibility that Ng-CAM and N-CAM may be heterophilic ligands for neurocan. The ability of chondroitin sulfate proteoglycans of brain to inhibit cell adhesion to CAMs may be an important mechanism for modulating cell adhesion and migration in the nervous system. We have therefore directly studied neurocan binding to CAMs and other cell surface or ECM molecules, as well as its effects on neuronal adhesion and neurite outgrowth. Neurocan was chosen for these studies because it is one of the most abundant chondroitin sulfate proteoglycans of developing brain (Rauch et al., 1991) and its primary structure has recently been determined (Rauch et al., 1992).

**Materials and Methods**

**Proteins and Antibodies**

Ng-CAM and N-CAM were purified from 14-d embryonic chicken brains by immunoaffinity chromatography using monoclonal antibodies 10F6 and 3G2 that specifically recognize Ng-CAM, and monoclonal anti-N-CAM No. 1, respectively (Grumet and Edelman, 1988). Nerve growth factor-inducible large external glycoprotein (NILE/L1) was purified from 7-d postnatal rat brain using a combination of other anti-Ng-CAM monoclonal antibodies (2C2 and 19H3) that recognize the cytoplasmic region of Ng-CAM, which is highly conserved between chicken Ng-CAM (Burgoon et al., 1991), mouse L1 (Moos et al., 1988), and rat NILE (Prince et al., 1991). Because of extensive similarities between these proteins in structure, expression patterns, and function (Grumet, 1992; Sonderegger and Rathjen, 1992), we refer to them generally as Ng-CAM/LI/NILE, and we refer to Ng-CAM and LI/NILE when discussing experiments using the particular proteins from chick and rat, respectively.

The LI/NILE protein purified from detergent extracts of rat brain membranes contains two major components of Mr 200,000 and 140,000 and small amounts of a component of Mr 80,000. Polyclonal antibodies against human LI (kindly provided by Dr. John Hemperly, Becton Dickinson, Research Triangle Park, North Carolina) recognized the Mr 200,000 and 140,000 species on immunoblots, confirming that it is NILE/L1. The 5B8 monoclonal antibody used to purify N-CAM from 7-d postnatal rat brain and for immunocytochemistry was obtained from the Developmental Studies Hybridaoma Bank (maintained by the Department of Pharmacology and Molecular Science, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract NOI-HD-6-2915 from the NIH). This antibody recognizes cytoplasmic regions of N-CAM. When the rat N-CAM was resolved on SDS-PAGE and stained with Coomassie blue, the characteristic heterodisperse pattern of polysialylated N-CAM was observed.

Neurocan was isolated and analyzed as described previously (Rauch et al., 1991; Grumet et al., 1993). Briefly, brains of 7-d or 2- to 3-mo-old Sprague-Dawley rats were extracted with PBS, and proteoglycans were purified by ion exchange chromatography and gel filtration (Xiang et al., 1981). Neurocan was purified by immunoaffinity chromatography using the 1D1 monoclonal antibody (Rauch et al., 1991). Rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) was isolated by CsCl density gradient centrifugation (Pultz et al., 1979). For studies of the core proteins, proteoglycans were digested for 45–60 min at 37°C with protease-free chondroitinase ABC (Seikagaku America Inc., Rockville, MD) in 100 mM Tris-HCl buffer (pH 8.0 at 37°C) containing 30 mM sodium acetate, and completeness of digestion was confirmed by SDS-PAGE (Rauch et al., 1991); control treatments were performed at 37°C in the absence of enzyme.

Myelin-associated glycoprotein (MAG) is a recombinant form including the ectodomain (Pedraza et al., 1990) and epidermal growth factor receptor (a recombinant, soluble, extracellular, ligand-binding form; Lax et al., 1991) were kind gifts from Drs. J. L. Salzer and J. Schlessinger (New York University Medical Center, New York), respectively. Commercial reagents included laminin and type I and IV collagens (Collaborative Research, Bed-
ford, MA), fibronectin (New York Blood Center, New York, NY), and BSA (ICN Biomedical, Lisle, IL). Sturgeon notochord chondroitin sulfate, consisting of 80% chondroitin 4-sulfate and 20% chondroitin 6-sulfate, was obtained from Seikagaku America Inc. (Rockville, MD).

Monoclonal antibodies against chicken Ng-CAM (Grunet and Edelman, 1984) and the IDI monoclonal antibody to neurocan (Rauch et al., 1991) have been described previously. A monoclonal antibody (CL-300) to calbindin-D was obtained from Sigma Chemical Co. (St. Louis, MO). When IgG was used, it was precipitated from ascites fluid with ammonium sulfate and further purified on DE-52 columns.

Radioisogand Binding Assay

Proteoglycans were labeled to a specific activity of 2.5-6 × 10^18 cpm/mole with [35S] by the lactoperoxidase/glucose oxidase method using Enzymobeads (BioRad Laboratories, Richmond, CA). Typically, 50 μg of protein were labeled per reaction, and free iodine was removed by gel filtration on a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ). Binding assays were performed essentially as described by Zisch et al. (1992). 1-30 μg/ml of soluble proteins in binding buffer (16 mM Tris, pH 7.2, 50 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 0.02% NaN3) were adsorbed to removable Immunlon-2 wells (Dyantech, Chantilly, VA) by overnight incubation at room temperature. Unbound proteins were removed with three washes in binding buffer/0.02% Tween 20, and the wells were blocked by incubation with 1 mg/ml heat-treated BSA in binding buffer. Wells were then emptied and 50 μl/well of labeled proteins or mixtures of labeled and unlabeled proteins and the binding buffer containing 1 mg BSA/ml) were incubated for 2 h at room temperature. Unbound proteoglycan was removed by four washes with TBS (50 mM Tris, pH 7.2, 150 mM NaCl)/0.02% Tween 20, and radioactivity bound to wells was determined with a gamma counter. Some experiments were also performed in isotonic buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 0.02% NaN3). Scatchard plots were generated and dissociation constants were determined using the Macintosh version of the Ligand program (Munson and Rodbard, 1988).

Cells

Dissociated cells were prepared essentially as described previously (Brackenbury et al., 1981). In brief, 9-d chick embryo brains were treated with trypsin/EDTA (GIBCO BRL, Gaithersburg, MD) followed by trituration in DME (GIBCO BRL) containing 10% fetal calf serum and 50 μg/ml DNase I (Worthington Biochem. Corp., Freehold, NJ). The cells were washed twice with DME/ITS+ (Collaborative Research Inc., Waltham, MA) and once by centrifugation through a 3.5% BSA/PBS step gradient.

Substrates

Substrates for cell adhesion and neurite growth assays were arranged in a circular array (<1-cm diam) of 8 to 12 small circular regions of polystyrene dishes (Falcon 1007 and 1008; Becton Dickinson, Lincoln Park, NJ) that were coated with adsorbed proteins. Coated regions were prepared by incubating 1.5-μl droplets of protein solutions in a humidified chamber for 30 min (Friedlander et al., 1988). After removing the droplets by suction, the dishes were washed three times with PBS and blocked with 1% BSA. For double coats, the first solution was applied and washed as described above, except that no blocking step was performed. Following the third wash, the second solution was applied as described above, including the blocking step. Thus, protein in the second solution could bind both to the plastic and to the first adsorbed protein. For quantitative determination of protein binding to the substrate, radiolabeled proteins were incubated with dishes using the same materials and procedures used for the cell assays. After the final wash, the dishes were dried, their walls removed with pliers, and attached cells were counted under a microscope at 200× magnification.

Cell Adhesion Assays

250 μl of DME/ITS+ containing 6 × 10^5 cells were deposited in the central region of 35 mm polystyrene dishes that had been coated with proteins. Following incubation for 80 min at 37°C, unattached cells were removed by washing with PBS and the remaining cells were fixed with 3.5% formalin. Attached cells were counted under a microscope at 200× magnification.

Neurite Growth

10^4 brain cells were incubated for 2 d under the same conditions used for cell adhesion assays, and were fixed with formalin. Neurite length was defined as the distance between the furthest removed neurite tip and the cell body. Quantitation was done under phase contrast microscopy with the help of a measuring eyepiece.

Immunocytochemistry

The immunocytochemical localization of neurocan, NIL/LI, and N-CAM was performed on sagittal Vibratome sections of 7-d rat cerebellum using the IDI, 2C2, and 5B8 monoclonal antibodies, respectively. Rats were perfusion fixed with picric acid-parafomaldehyde-glutaraldehyde, and sections were stained with peroxidase-conjugated secondary antibody as described previously (Rauch et al., 1991).

Analytical Methods

Proteins were resolved on SDS-PAGE (Laemmli, 1970) and were either stained with Coomassie blue. Radioactive proteins were detected by autoradiography, and protein concentrations were determined using either the Lowry (for neurocan) or the Bradford protein assay (Bio-Rad Laboratories). The concentration of aggrecan was determined gravimetrically.

Results

Binding of Neurocan to Ng-CAM and N-CAM

Binding of neurocan to various cell surface proteins including neural CAMs and ECM proteins was determined using a radioligand binding assay (Fig. 1). Both rat neurocan and neurocan-C bind to chicken Ng-CAM and to its presumed rodent homologue NIL/LI (Grunet, 1992; Sonderegger and Rathjen, 1992). Neurocan also bound to N-CAMs from chicken and rat. These results suggest that the proteoglycan binding domains in Ng-CAM and N-CAM have been conserved during the evolution of avian and mammalian species.

To analyze the specificity of the binding, we tested whether neurocan binds to other cell surface proteins that were available to us including MAG and the epidermal growth factor receptor, as well as extracellular matrix components that can modulate neuronal adhesion or neurite outgrowth. Neurocan bound weakly to collagen I and not at all to MAG, collagen IV, or EGF receptor. Under our standard assay conditions (wells coated with proteins at a concentration of 1.25 μg/ml) neurocan did not bind to laminin but binding (9% of total counts) was detected when a higher laminin concentration (10 μg/ml) was used. The fraction of neurocan that bound specifically to Ng-CAM was consistently 20-25%, with a signal to background ratio as high as 65:1. The percent of neurocan bound to N-CAM was usually lower and varied to a greater extent between experiments, ranging from ~6-15% bound with a signal to background ratio of 18-20:1. By comparison, 125I-labeled aggrecan, a chondroitin sulfate proteoglycan isolated from rat chondrosarcoma, bound very weakly to Ng-CAM and N-CAM (Fig. 1), sug-
Figure 1. Binding of neurocan and aggrecan to CAMs and extracellular matrix proteins. Removable wells of 96-well plates were coated with unlabeled proteins (1.25 μg/ml) and incubated with 125I-labeled proteins (~160,000 cpm). The fraction of the input bound to the different substrates is given as a percentage. Non-specific binding to BSA was also determined, and specific binding (percent bound) is calculated as total minus non-specific binding. Specific activities in the experiments shown here were 2.5–2.9 × 10^6 cpm/mole. All values are means of duplicate determinations ±SEM. EGF-R, epidermal growth factor receptor.

suggesting that the interactions between neurocan and these neural CAMs depend upon structural domains in neurocan that are not present in this related proteoglycan, or are masked by its much larger number of chondroitin sulfate chains.

The radioligand binding assays described above were performed in buffers containing 50 mM NaCl. This hypotonic buffer yielded higher signal to background ratios than incubation in buffer with 150 mM NaCl. At physiological salt concentration, binding of neurocan to Ng-CAM and N-CAM was diminished by 35–60%, and the binding to collagen I was reduced to baseline levels (Fig. 2). These results suggest that in vivo neurocan may not bind to collagen I but that it could bind to the neural CAMs. Because the amounts of neurocan that we can isolate from rat brain are limited, we chose to perform most saturation and inhibition experiments using the hypotonic conditions.

To investigate the reversibility of binding of neurocan to Ng-CAM, a kinetic analysis of the dissociation of neurocan was performed. Labeled neurocan or neurocan-C were allowed to bind to Ng-CAM, free labeled molecules were removed, and the amount of bound proteoglycan was determined as a function of time under two conditions. Wells were incubated either in the presence of 5 μg/ml of unlabeled proteoglycan (Fig. 3 A) or in large amounts of buffer (Fig. 3 B). Binding was reversible under both conditions, although the time required to reduce the binding to half the initial value was shorter in the presence of unlabeled proteoglycan (t_{1/2} ≈ 20–30 min) than in the presence of excess buffer (t_{1/2} ≈ 4 h). Similar results were observed for the binding of neurocan to N-CAM (data not shown).

Figure 2. Binding of neurocan to CAMs and collagen I under hypotonic and isotonic conditions. The hypotonic and isotonic buffers were as described in Materials and Methods. Labeled proteoglycans were applied at an average of 160,000 cpm/well. Values are means of duplicate determinations; error bars represent mean deviations.

Figure 3. Reversibility of binding of neurocan to Ng-CAM. Binding of 125I-neurocan and 125I-neurocan-C to Ng-CAM-coated wells was performed as described in Materials and Methods. Following the removal of free proteoglycan, wells were incubated with 5 μg/ml unlabeled neurocan or neurocan-C in 100 μl of isotonic binding buffer (A), or in 40 ml of isotonic binding buffer without added proteoglycan (B). At the indicated times, the amounts of labeled proteoglycan remaining in the wells were determined. Experimental points correspond to specific binding and are averages (n = 2). Bars represent mean deviations.
Figure 4. Saturation curves and Scatchard plot analysis of 125I-labeled neurocan (A) and neurocan-C (B) binding to Ng-CAM. Binding values represent specific binding (total cpm bound minus cpm bound to BSA). Neurocan was tested at 0.5–50 ng/well (6.8 × 10^18 cpm/mole), and neurocan-C at 0.2–37 ng/well (4.8 × 10^18 cpm/mole). Points in the saturation curve are averages of duplicate determinations, and the error bars represent mean deviations. Values are the averages of duplicate determinations; error bars represent mean deviations. It should be noted that in terms of moles of chondroitin sulfate, neurocan (which contains only 20% by weight of chondroitin sulfate) is a much better inhibitor than aggrecan (~80% chondroitin sulfate) or free chondroitin sulfate chains (Fig. 6). The disaccharide composition of the sturgeon notochord chondroitin sulfate used in these inhibition experiments (80% chondroitin 4-sulfate and 20% chondroitin 6-sulfate) is identical to that of neurocan in early postnatal brain (Rauch et al., 1991). However, sulfated disaccharides had very little effect on the binding of neurocan, demonstrating that intact chondroitin sulfate chains are required to inhibit proteoglycan binding to CAMs. In the same assay, fibronectin also had very little effect. Using the same

Figure 6. Binding of 125I-labeled neurocan to Ng-CAM in the presence of other soluble molecules. Wells coated with Ng-CAM were incubated with 125I-labeled neurocan (160,000 cpm/well) in the presence of unlabeled neurocan (●), aggrecan (●), chondroitin sulfate (○), chondroitin sulfate disaccharides (△), and fibronectin ( ◊) at the concentrations indicated. Specific binding of neurocan to Ng-CAM in the absence of soluble molecules corresponds to 0% inhibition; background level of neurocan binding to BSA defines 100% inhibition. Values are the averages of duplicate determinations; error bars represent mean deviations. It should be noted that in terms of moles of chondroitin sulfate, neurocan (which contains only 20% by weight of chondroitin sulfate) is a much better inhibitor than aggrecan (~80% chondroitin sulfate) or free chondroitin sulfate chains.
set of soluble molecules as inhibitors, we observed similar patterns of inhibition for binding of neurocan to N-CAM and for binding of neurocan to Ng-CAM and to N-CAM (data not shown). The results suggest that chondroitin sulfate chains, which constitute ~20% by weight of neurocan, are important in the binding of neurocan to the neural CAMs.

To analyze more directly the role of chondroitin sulfate chains in interactions of neurocan with neural CAMs, we compared the binding of native and chondroitinase-treated neurocan to Ng-CAM (Fig. 7 A) and N-CAM (Fig. 7 B). Chondroitinase treatment reduced binding of neurocan and neurocan-C to Ng-CAM by ~70%, and to N-CAM by ~80%, in both hypotonic (Fig. 7) and isotonic buffers (data not shown). Heat-treated proteoglycans retained the ability to bind to the CAMs, although at levels that were lower than those of controls. However, chondroitinase treatment followed by heat treatment further reduced the binding to nearly background levels. Since it is known that glycosylation can protect proteins from denaturation (Oh-eda et al., 1990), the modest effect of heat treatment on the binding activity of the intact proteoglycan may be due to this phenomenon. These results are consistent with the involvement of chondroitin sulfate chains on neurocan in binding to Ng-CAM and N-CAM. Nevertheless, the data also indicate that the core protein of neurocan retains binding for Ng-CAM and N-CAM even in the absence of chondroitin sulfate chains.

Overlapping Localization of Neurocan, Ng-CAM, and N-CAM in Developing Brain

If the interactions between neurocan and neuronal CAMs are biologically significant, one would expect these molecules to appear in close proximity at certain times during development. The ability to isolate by immunoaffinity chromatography significant amounts of neurocan (using mAb ID1) as well as NILE/L1 (using anti-Ng-CAM mAbs 2C2 and 19H3) and N-CAM (using mAb 5B8) from 7-d postnatal rat brain is evidence that all these proteins are present at this stage of development (see Materials and Methods). To determine the histological localization of these proteins, specific monoclonal antibodies were used for immunoperoxidase staining of sections of early postnatal rat cerebellum. The staining with monoclonal antibodies against these three molecules was similar insofar as it was strongest in the molecular layer and in the deeper layers of the cerebellum (Fig. 8). Examination at higher magnification (Fig. 9) confirmed that strong staining was associated with regions containing bundles of axons such as in the molecular layer and fiber tracts. This pattern of staining is in sharp contrast to that observed with other antibodies such as to calbindin, which specifically labeled Purkinje cells and their dendrites projecting into the molecular layer (Fig. 9). This general staining pattern for neurocan, Ng-CAM, and N-CAM was also observed in 4-, 10-, and 14-d postnatal brain (data not shown), and is characteristic of several neural cell adhesion molecules (Rathjen and Schachner, 1984; Rathjen et al., 1987a, b; Pollerberg et al., 1985). Overlapping staining of these CAMs and neurocan was also seen prenatally (in E13 and E16 brain and spinal cord), whereas quite different staining patterns were observed using monoclonal antibodies against unrelated proteins such as glial fibrillary acidic protein (Flad, M., and R. K. Margolis, unpublished results).

Effects of Neurocan on Neuronal Adhesion to Ng-CAM

We previously showed, using a short-term (~1 min) centrifugation adhesion assay, that neurons attach to plastic coated with neurocan, whereas using a medium-term assay (~1 h) under the force of gravity, neurons do not attach to a neurocan substrate or to substrates containing both neurocan and Ng-CAM (Grumet et al., 1993). Although rat neurocan contains an RGDS sequence (Rauch et al., 1991), this sequence may not be functionally active insofar as a GRGDSP peptide (at 100 μg/ml) does not inhibit the binding of chick neurons to neurocan in the centrifugation assay (Flad, M., and R. K. Margolis, unpublished results).

Based on our finding that neurocan binds to Ng-CAM, we sequentially adsorbed two different proteins to polystyrene dishes and then tested the substrate for neuronal adhesion. To help interpret the results, we also determined the amounts of protein adsorbed to the dish for the various combinations of protein concentrations and coating orders used (see Materials and Methods). When individual proteins were adsorbed to the dish, neurons attached strongly to Ng-CAM and anti-Ng-CAM Ig, weakly to N-CAM, negligibly to BSA, and not at all to neurocan. Because of the weak neuronal adhesion to N-CAM, it was omitted from the double coating experiments. To analyze direct effects of neurocan on neurons, we used a monoclonal anti-Ng-CAM Ig as a per-
Figure 8. Immunoperoxidase staining of 7-d postnatal rat cerebellum with antibodies to Ng-CAM, neurocan, and N-CAM. (A) mAb 2C2, which recognizes a cytoplasmic region that is highly conserved between avian Ng-CAM and mammalian NILE/LI; (B) mAb 1D1 to neurocan; (C) mAb 5B8 to N-CAM. No staining was seen with supernatant from a hybridoma that does not produce antibodies to brain antigens. Whereas all three antibodies stained long processes in the fiber tracts, antibodies against neurocan also intensely stained some regions (B, parallel arrows) surrounding the fiber tracts which were strongly stained with anti-Ng-CAM mAb 2C2. The anti-N-CAM monoclonal antibody recognizes the cytoplasmic region of N-CAM, and therefore revealed only the larger N-CAM species whose expression pattern in developing cerebellum parallels that of Ng-CAM/LI/NILE (Pollerberg et al., 1985). EGL, external granule cell layer; ML, molecular layer; FT, fiber tract. Bar, 200 μm.

Figure 9. High magnification views of immunoperoxidase staining of 7-d postnatal rat cerebellum. Staining was performed as described in Fig. 8 and photographed at high magnification to compare staining in different lamina of the cerebellum using antibodies to Ng-CAM/LI/NILE (2C2), neurocan (1D1), N-CAM (5B8), and calbindin (CL-300). The staining for Ng-CAM/LI/NILE, neurocan, and N-CAM is most intense in the axon-rich molecular layer and fiber tract (the developing white matter). Purkinje cell bodies and their dendritic trees and axonal projections are labeled by antibodies to calbindin. IGL, internal granule cell layer; PC, Purkinje cells. Bar, 10 μm.

missive substrate that does not interact with neurocan. Neurons bound well to dishes coated with anti-Ng-CAM Ig (Table I) even after blocking with a large excess of BSA (Materials and Methods). Dishes coated first (protein 1) with BSA and then (protein 2) with anti-Ng-CAM Ig were also able to support neuronal attachment. Coating first with anti-Ng-CAM Ig and then with neurocan inhibited neuronal adhesion. The inhibition was most apparent at lower densities of bound anti-Ng-CAM Ig. In addition, substrates coated first with neurocan and then with anti-Ng-CAM Ig did not support neuronal adhesion. Inhibition occurred even though anti-Ng-CAM Ig was present at densities that supported significant levels of neuronal attachment in the absence of neurocan. For example, neurons adhered to anti-Ng-CAM (0.25 ng/mm²) when it was coated following BSA, but no adhesion was detected when even greater amounts of anti-Ng-CAM (0.42 ng/mm²) were coated following neuro-
Table I. Adhesion of Cells to Dishes Coated with Neurocan and anti-Ng-CAM Ig

| Protein 1 | Concentration 1 | Density 1 | Protein 2 | Concentration 2 | Density 2 | Attached cells |
|-----------|----------------|----------|-----------|----------------|----------|---------------|
|           | µg/ml          | ng/mm²   |           | µg/ml          | ng/mm²   | cells/mm²     |
| Anti-Ng-CAM | 30          | 1.50 ± 0.22 | BSA       | 33          | ND       | 535 ± 32      |
| Anti-Ng-CAM | 10          | 0.73 ± 0.00 | BSA       | 33          | ND       | 286 ± 53      |
| BSA       | 33          | ND       | Anti-Ng-CAM | 30          | 0.55 ± 0.06 | 305 ± 73      |
| BSA       | 33          | ND       | Anti-Ng-CAM | 10          | 0.25 ± 0.01 | 221 ± 52      |
| Anti-Ng-CAM | 30          | 1.50 ± 0.22 | Neurocan  | 33          | 0.41 ± 0.00 | 386 ± 0       |
| Anti-Ng-CAM | 10          | 0.73 ± 0.00 | Neurocan  | 33          | 0.76 ± 0.06 | 0 ± 0         |
| Neurocan  | 33          | 1.20 ± 0.04 | Anti-Ng-CAM | 30          | 0.42 ± 0.01 | 1 ± 1         |
| Neurocan  | 33          | 1.20 ± 0.04 | Anti-Ng-CAM | 10          | 0.17 ± 0.03 | 0 ± 0         |

Cell adhesion assays were performed using substrates consisting of sequentially incubated soluble proteins 1 and 2 at concentrations 1 and 2, respectively. The resulting surface densities of adsorbed protein (densities 1 and 2) were determined in parallel experiments using the same substrate, proteins, protein concentrations and liquid volume as in the cell adhesion assays. To determine density 1, duplicate samples of different concentrations of labeled protein 1 (anti-Ng-CAM and neurocan) were spotted on the dish, and bound radioactive was measured as described in Materials and Methods. Identical values of density 1 appear in the table when they were based on the same experimental point. Data not included in the table indicated that the amount of bound radioactive of protein 1 was not lowered by applying a second coat with unlabeled protein. To determine density 2, duplicate spots of unlabeled protein 1 were prepared first, after which labeled protein 2 was added and the bound radioactivity was measured. In the parallel cell adhesion assays with unlabeled proteins, dissociated brain cells from 9-d chick embryos were added to substrates, and the number of attached cells after an 80-min incubation period were determined as indicated in Materials and Methods. The number of cells that attached to the BSA-coated background was 13 ± 8. Data represent averages (n = 2) ± mean deviations.

can (Table I). Because neurocan does not bind to anti-Ng-CAM (data not shown), these results suggest that neurocan interacts directly with the cell surface.

These experiments also indicated that the surface density of 125I-neurocan on dishes coated with different proteins including anti-Ng-CAM Ig (Table I) and BSA (data not shown) was inversely related to the amount of protein already adsorbed onto the dish. In contrast, the level of binding of 125I-neurocan to dishes coated first with Ng-CAM was higher and did not vary much from the level of adsorption to untreated dishes (Table II), consistent with the interpretation that neurocan binds to Ng-CAM but not to BSA and anti-Ng-CAM Ig.

When similar substrates were tested for neuronal adhesion, strong inhibition was found with those that were coated first with Ng-CAM and then with high concentrations of neurocan (Table II). In contrast to the results with anti-Ng-CAM substrates, changing the order of the adsorptions (adding Ng-CAM after neurocan) allowed for high levels of neuronal binding (Table II). This occurred even though the surface density of Ng-CAM was relatively low. Qualitatively similar results were obtained for both neurocan and neurocan-C, although neurocan was a somewhat more potent inhibitor.

Effects of Neurocan on Neurite Outgrowth

A critical aspect of neuronal development is the growth of processes. We therefore studied the effects of neurocan on neurite growth in culture using double-coated substrates prepared essentially as described above for the neuronal adhesion experiments. A major difference in these experiments was that the non-adherent cells were not removed by washing after 80 min of incubation and many cells eventually adhered to substrates even in the presence of neurocan. Neurons extended numerous processes on surfaces coated with either Ng-CAM or anti-Ng-CAM Ig followed by BSA, whereas neurite extension was dramatically diminished on surfaces coated with either Ng-CAM or anti-Ng-CAM Ig followed by

Table II. Adhesion of Cells to Dishes Coated with Neurocan and Ng-CAM

| Protein 1 | Concentration 1 | Density 1 | Protein 2 | Concentration 2 | Density 2 | Attached cells |
|-----------|----------------|----------|-----------|----------------|----------|---------------|
|           | µg/ml          | ng/mm²   |           | µg/ml          | ng/mm²   | cells/mm²     |
| Ng-CAM    | 50            | 2.69 ± 0.23 | BSA       | 33            | ND       | 395 ± 9       |
| Ng-CAM    | 17            | 0.75 ± 0.04 | BSA       | 33            | ND       | 474 ± 3       |
| BSA       | 33            | ND       | Ng-CAM    | 50            | 2.54 ± 0.18 | 427 ± 11      |
| BSA       | 33            | ND       | Ng-CAM    | 17            | 0.87 ± 0.00 | 392 ± 20      |
| Ng-CAM    | 50            | 2.69 ± 0.23 | Neurocan  | 33            | 1.22 ± 0.03 | 89 ± 2        |
| Ng-CAM    | 17            | 0.75 ± 0.04 | Neurocan  | 33            | 1.23 ± 0.02 | 37 ± 19       |
| Neurocan  | 33            | 1.2 ± 0.04 | Ng-CAM    | 50            | 1.26 ± 0.18 | 460 ± 2       |
| Neurocan  | 33            | 1.2 ± 0.04 | Ng-CAM    | 17            | 0.38 ± 0.01 | 393 ± 7       |
| Ng-CAM    | 50            | 2.69 ± 0.23 | Neurocan-C | 33            | 1.18 ± 0.02 | 183 ± 15      |
| Ng-CAM    | 17            | 0.75 ± 0.04 | Neurocan-C | 33            | 1.21 ± 0.03 | 95 ± 15       |
| Neurocan-C | 33            | 1.15 ± 0.02 | Ng-CAM    | 50            | 1.05 ± 0.01 | 475 ± 26      |
| Neurocan-C | 33            | 1.15 ± 0.02 | Ng-CAM    | 17            | 0.25 ± 0.03 | 529 ± 14      |

The number of cells adhering to the BSA-coated background was 20 ± 3. Data represent averages (n = 2) ± mean deviations. See Table I for methods.
neurocan (Fig. 10). In quantitative experiments, the neurite length histograms for both Ng-CAM and anti-Ng-CAM substrates showed a significant fraction of neurites longer than 20 μm and a decreasing proportion of neurites with greater lengths (Fig. 11). In contrast, neurons grown on neurocan plus either Ng-CAM or anti-Ng-CAM had neurites mostly in the 0–20-μm range, with only relatively small numbers of longer neurites. These results demonstrate that neurocan is a potent inhibitor of neurite growth both on proteins to which it can bind (e.g., Ng-CAM) and on those to which it does not bind (e.g., anti-Ng-CAM Ig).

Discussion

We have characterized several functional properties of neurocan, which accounts for >20% of the soluble chondroitin sulfate proteoglycans in developing rat brain. The major observations of the present study are: (a) that neurocan binds with high affinity to Ng-CAM and N-CAM, two of the most prevalent neural CAMs that play key roles in cell adhesion, neuronal migration, and axonal growth during development; (b) that these three molecules are coexpressed during critical stages of cerebellar histogenesis; and (c) that neurocan inhibits neuronal adhesion and neurite growth.

Binding of Neurocan to Neural CAMs

Neurocan bound with high affinity to Ng-CAM and N-CAM, but not to other cell surface proteins except for laminin, which bound much more weakly. The potential biological relevance of this lower affinity interaction is unclear because, in contrast to Ng-CAM and N-CAM which are prevalent during brain development, there is very little laminin in brain (Jacobson, 1991). However, one must also consider the potential relevance of such interactions in the peripheral nervous system, where certain ECM proteins are more abundant.

The observation that the apparent dissociation constant of binding of neurocan-C to Ng-CAM and N-CAM is comparable to that obtained using the full-length proteoglycan suggests that neurocan-C (which represents the COOH-terminal half of neurocan) contains at least one binding site for these neural CAMs. This region of neurocan contains several domains, some of which have been implicated in binding of other proteins, including two EGF-like repeats, a lectin-like domain, and a complementary regulatory protein-like sequence (Rauch et al., 1992). Neurocan-C contains a single 32-kD chondroitin 4-sulfate chain that is linked at serine-944, whereas three additional potential chondroitin sulfate
attachment sites (only two of which are used) are present in the NH2-terminal portion of neurocan. The observation that attachment sites (only two of which are used) are present in the NH2-terminal portion of neurocan. The observation that attachment sites (only two of which are used) are present in the NH2-terminal portion of neurocan. The observation that attachment sites (only two of which are used) are present in the NH2-terminal portion of neurocan. The observation that attachment sites (only two of which are used) are present in the NH2-terminal portion of neurocan.

Expression and Function of Chondroitin Sulfate Proteoglycans in Brain

In agreement with evidence for a functional role of chondroitin sulfate proteoglycans during neural development (Snow et al., 1990a, 1991; Brittis et al., 1992; Margolis et al., 1991), our studies also suggest a role for chondroitin sulfate in the function of neurocan (Figs. 6 and 7). Although little

is known regarding the mechanism of inhibition by these chondroitin sulfate proteoglycans, our findings suggest that Ng-CAM and N-CAM may be specific neuronal receptors for neurocan. In future studies it will be important to analyze the functions of different domains that are present in nervous tissue proteoglycans (Margolis and Margolis, 1993), and the molecular cloning of cDNAs for neurocan, phosphacan (Maurel et al., 1994), and other chondroitin sulfate proteoglycans of brain will facilitate such studies.

Proteoglycans that have previously been identified in non-neural tissues may also be present in the brain. For example, aggrecan, which was first identified in cartilage, is present in rat brain (Milev, P., R. U. Margolis and R. K. Margolis, unpublished results) and the primary structure of its core protein has regions that are homologous to domains in neurocan (Rauch et al., 1992). However, the glycosylation of cartilage aggrecan, which has been found to inhibit neurite growth (Snow et al., 1990a, 1991), differs considerably from that of neurocan (Rauch et al., 1991), and there are also significant differences between aggrecan from chick brain and cartilage (Krueger et al., 1992). In contrast to reports that aggrecan has effects on neurons (Snow and Letourneau, 1993), our (Grumet et al., 1993) and other (Katoh-Semba and Oohira, 1993) studies indicate little or no effect of cartilage aggrecan. One important difference is that significant inhibition of neurite growth required levels of aggrecan that are at least an order of magnitude greater than those used in our experiments (Grumet et al., 1993; and Table II). The dependence of the effects of cartilage aggrecan on the presence of chondroitin sulfate chains may be due to the involvement of these charged glycans in proteoglycan binding, as we showed here for neurocan. Moreover, in contrast to neurocan-C and neurocan, which contain only 1–3 chondroitin sulfate chains, cartilage aggrecan contains up to 100 of these chains per molecule. In view of structural similarities and differences between proteoglycans from different tissues, and the potential functions of chondroitin sulfate, it will be interesting to examine the effects of brain aggrecan on neural cells.

The immunolocalization studies in cerebellum demonstrate that neurocan, Ng-CAM/L1/NILE, and N-CAM are prevalent during brain development and have extensively overlapping distributions. Developing cerebellar neurons express Ng-CAM/L1/NILE and N-CAM in vitro (Grumet et al., 1984a; Rathjen and Rutishauser, 1984; Stalcup and Beasley, 1985), and in situ hybridization studies demonstrate that neurocan mRNA is present in cerebellar granule cells (Milev et al., 1993; and Engel, M., R. U. Margolis, and R. K. Margolis, unpublished results). Granule cells may therefore be major contributors to the high levels of neurocan that appear in the molecular layer of developing cerebellum.

Effects of Neurocan on Neurons

The cellular assays employed in this study were designed to investigate potential mechanisms by which neurocan influences neuronal behavior in the presence of proteins that promote neuronal adhesion and neurite outgrowth. When anti-Ng-CAM antibodies were used as a permissive substrate, neurocan inhibited neuronal adhesion and neurite growth. Because neurocan does not bind to anti-Ng-CAM,
the results imply that neurocan inhibited neuronal adhesion and neurite growth by interacting directly with the cell surface. The interpretation of the effects of neurocan on the adhesion of neurons to Ng-CAM itself is more complex due to potential interactions of neurocan with both substrate-bound Ng-CAM and the neuronal plasma membrane. When Ng-CAM was used as a permissive substrate, inhibition occurred only when neurocan was added after Ng-CAM, and not when neurocan was coated before Ng-CAM. This effect was seen even when the amounts of adsorbed neurocan were essentially the same in both cases (Table II).

Neurocan is also a potent inhibitor of neurite outgrowth. It was important to perform such experiments because in contrast to cell adhesion, which is usually defined in terms of various in vitro assays, neurite growth is a fundamental cellular process that can be analyzed directly both in culture and in situ. In the future, it will be important to determine how various “inhibitory” proteins (Schwab et al., 1993) interact with cells and modulate different aspects of neuronal behavior. These modulations may also include growth control, insofar as a mixture of chondroitin sulfate proteoglycans from brain was recently reported to inhibit progression of the cell cycle in PC12 cells (Katoh-Semba and Oohira, 1993).

The overlapping localization of neurocan with Ng-CAM/LI/NILE and N-CAM, together with the effects of mixtures of neurocan and Ng-CAM on cells, raise the possibility of at least two opposing but not exclusive modes of action that may occur in vivo: (a) binding of neurocan to CAMs and other cell surface proteins results in inhibition of neuronal adhesion and axonal migration, consistent with the hypothesis that proteoglycans act as barriers to neuronal penetration (Snow et al., 1990a,b; Perris et al., 1991; Oakley and Tosney, 1991; Pindzola et al., 1993); and (b) CAMs neutralize the inhibitory effects of neurocan by directly binding to it and/or directly by promoting neuronal adhesion and axonal migration. Inasmuch as there is no direct relationship between the ability of particular proteins to promote neuronal adhesion and neurite growth (Lemmon et al., 1992; Calof and Lander, 1991), it is possible that the ability of axons to migrate may depend on the balance between adhesive effects of CAMs and the inhibitory effects of molecules such as neurocan. For example, cytotactin/tenascin, which inhibits neurite outgrowth from dorsal root ganglia on fibronectin and laminin substrates (Crossin et al., 1990), is able to enhance neurite growth when neurons are cultured on polylysine (Lochter et al., 1991). Hence, a “repulsive” protein could reduce neurite outgrowth on a moderately adhesive substrate (e.g., fibronectin or laminin) by lowering the adhesivity, yet it could enhance outgrowth on a strongly adhesive substrate (e.g., polylysine) by lowering adhesivity to a level that is more permissive for migration. This raises the possibility that various patterns of cell migration may be differentially affected as a result of local differences in the composition of cell surface receptors. A classical example of differences in the migratory behavior of different parts of the same neuron can be found in the developing cerebellum, where granule cells extend axons (parallel fibers) that fasciculate along the growth surface of the molecular layer (rich in neurocan and tenascin), but only their cell bodies migrate radially across this layer (Jacobson, 1991).

Although it is possible that neurocan inhibits the function of CAMs by proteolytically inactivating them, we have not detected any change in the electrophoretic mobility of Ng-CAM following incubation in physiological buffers with neurocan (Grumet et al., 1993). A more likely mechanism of action is that neurocan binds to the cell surface either to block an adhesion molecule and/or to generate a signal to the cell. In support of this possibility is the finding that 125I-neurocan binds to neurons, and Fab' fragments of antibodies against Ng-CAM and N-CAM inhibit this binding (Milev, P., D. R. Friedlander, M. Grumet, and R. U. Margolis, unpublished observations). Moreover, recent studies indicate that increases in intracellular calcium levels follow binding of ligands to these CAMs at the surface of neurons (Schuch et al., 1989; Von Bohlen und Halbach et al., 1992; Doherty and Walsh, 1992) as well as binding of chondroitin sulfate proteoglycans to growth cones (Snow, D. M., P. Atkinson, T. Hassinger, S. B. Kater, and P. C. Letourneau, 1993. Soc. Neurosci. Abstr. 19:876), and suggest that binding of neurocan to neural CAMs may generate signals that influence cell behavior.

It is clear that additional studies are needed to define more precisely the molecular mechanisms of action of neurocan and other chondroitin sulfate proteoglycans in the nervous system. Nevertheless, our results indicate that whether a particular region of tissue will or will not be favorable for cell adhesion and axonal growth will depend not only on the relative amounts of the CAMs and proteoglycans, but also on their sequences of expression and organization during development.

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