Functional Characterization of Chondroitin Sulfate Proteoglycans of Brain: Interactions with Neurons and Neural Cell Adhesion Molecules

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Abstract. Ng-CAM and N-CAM are cell adhesion molecules (CAMs), and each CAM can bind homophilically as demonstrated by the ability of CAM-coated beads (Covaspheres) to self-aggregate. We have found that the extent of aggregation of Covaspheres coated with either Ng-CAM or N-CAM was strongly inhibited by the intact 1D1 and 3F8 chondroitin sulfate proteoglycans of rat brain, and by the core glycoproteins resulting from chondroitinase treatment of the proteoglycans. Much higher concentrations of rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) core proteins had no significant effect in these assays. The 1D1 and 3F8 proteoglycans also inhibited binding of neurons to Ng-CAM when mixtures of these proteins were adsorbed to polystyrene dishes.

Direct binding of neurons to the proteoglycan core glycoproteins from brain but not from chondrosarcoma was demonstrated using an assay in which cell-substrate contact was initiated by centrifugation, and neuronal binding to the 1D1 proteoglycans was specifically inhibited by the 1D1 monoclonal antibody. Different forms of the 1D1 proteoglycan have been identified in developing and adult brain. The early postnatal form (neurocan) was found to bind neurons more effectively than the adult proteoglycan, which represents the C-terminal half of the larger neurocan core protein. Our results therefore indicate that certain brain proteoglycans can bind to neurons, and that Ng-CAM and N-CAM may be heterophilic ligands for neurocan and the 3F8 proteoglycan. The ability of these brain proteoglycans to inhibit adhesion of cells to CAMs may be one mechanism to modulate cell adhesion and migration in the nervous system.

There is increasing evidence to indicate that glycosaminoglycans and proteoglycans are involved in modulating cell interactions in developing nervous tissue. In some of the initial studies in this area it was found that soluble glycosaminoglycans or glycosaminoglycan-coated substrata modify the adhesion and neurite extension of cultured PC12 cells (Akeson and Warren, 1986; Damon et al., 1988) and dorsal root ganglia (Carbonetto et al., 1983; Verna et al., 1989). More recent studies indicate that chondroitin sulfate and keratan sulfate proteoglycans may be components of barriers for axonal migration (Snow et al., 1990a, b, 1991; McKeon et al., 1991; Oakley and Tosney, 1991; Cole and McCabe, 1991; Britiss et al., 1992), and that chondroitin/keratan sulfate proteoglycans can inhibit neural crest cell migration (Perris and Johansson, 1990). In addition, studies of spatial and temporal changes in the distribution of proteoglycans during avian neural crest development showed that in general, chondroitin/keratan sulfate proteoglycans are abundant in regions where neural crest cells are absent, and that their segmental distribution inversely correlates with migration pathways of neural crest cells (Perris et al., 1991).

Although there is in certain cases a degree of chemical specificity for the effects observed in vitro, it is not clear to what extent they are due to factors such as modification of the surface charge of the substrate, alteration of the binding of growth promoting or extracellular adhesive molecules produced by the cultures, or a modulation of binding mediated by cell adhesion molecules (CAMs) in the plasma membrane. In tissues, sulfated glycosaminoglycans occur as covalent complexes with a core protein in the form of proteoglycans (Margolis and Margolis, 1989), and both the glycosaminoglycan and protein components have been suggested to affect cell behavior (Perris and Johansson, 1990; Snow et al., 1990b, 1991; Britiss et al., 1992).

Other cell surface molecules that have been implicated in adhesion during neural development include various CAMs that are members of the Ig superfamily (reviewed in Edelman, 1987; Grumet, 1991; Rathjen and Jessel, 1991). N-CAM and Ng-CAM are two of the CAMs that are expressed on neurons and have been implicated in various aspects of cell adhesion, cell migration, and neurite fasciculation that correlate with their general patterns of expression during development (Edelman and Cunningham, 1990; Rutishauser, 1991; Walsh and Doherty, 1991; Grumet, 1992). Each CAM can mediate homophilic binding (Hoffman and Edelman, 1983; Grumet and Edelman, 1988) and

1. Abbreviations used in this paper: CAM, cell adhesion molecule; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM.
has at least one heterophilic ligand (Reyes et al., 1990; Gru- met, M., and G. M. Edelman, 1992. Soc. Neurosci. Abstr. 18:1326). Although these neural CAMs are functionally and structurally distinct, they share features including certain carbohydrate structures (Grumet et al., 1984a), and both contain related Ig-like and fibronectin-like domains (Cunningham et al., 1987; Burgoon et al., 1991).

In view of evidence that extracellular proteoglycans may act as repulsive molecules which modulate cell-cell and cell-matrix interactions, it is important to determine whether purified brain proteoglycans can affect the behavior of brain cells and, in particular, interfere with mechanisms of cell adhesion. Recently, the biochemical properties and immunocytochemical localization of five developmentally regulated chondroitin sulfate proteoglycans of brain have been characterized using specific mAbs, and one of these has been cloned and its primary structure determined (Rauch et al., 1991, 1992). To study the potential roles of individual proteoglycans in nervous tissue histogenesis, we have examined the ability of two of these proteoglycans, IDI (now named neurocan; Rauch et al., 1992) and SF8, to affect actions of N-CAM and Ng-CAM, and have compared their effects to those of a nonnervous tissue (rat chondrosarcoma) chondroitin sulfate proteoglycan (aggrecan). In addition to affecting CAM binding, we found that the brain proteoglycans supported binding of neurons and perturbed cell adhesion to Ng-CAM.

Materials and Methods

Proteins and Antibodies

Ng-CAM and N-CAM were purified from 14-d embryonic chicken brains by immunoaffinity chromatography using specific mAbs (Grumet and Edelman, 1988). Analysis of the proteins on SDS-PAGE showed that Ng-CAM consisted of a major component of 135 kD and lesser amounts of the 200 and 80 kD species (Grumet and Edelman, 1988), and N-CAM migrated as related Ig-like and fibronectin-like domains (Cunningham et al., 1987; Burgoon et al., 1991).

Chondroitin sulfate proteoglycans were extracted with PBS from the brains of 7- to 10-d-old or 2- to 3-mo-old Sprague-Dawley rats, and purified by ion exchange chromatography and gel filtration (Kiang et al., 1988). The IDI and SF8 proteoglycans were then isolated by immunoaffinity chromatography using mAbs coupled to CNBr-activated Sepharose 4B (Rauch et al., 1991). Analysis of the proteins on SDS-PAGE after chondroitinase treatment showed that the IDI proteoglycan from adult brain contained a 150-kD core glycoprotein, whereas the IDI proteoglycan from 7-d brain showed an additional major band at 245 kD. Because the COOH-terminal half of the proteoglycan which can be isolated from adult brain with the IDI mAb is not the only neurocan-derived proteoglycan present at this age, we will refer to this as the adult IDI proteoglycan, and reserve the designation of neurocan for the larger form of the proteoglycan found in early postnatal brain. In both cases, the IDI proteoglycan was accompanied by a 45-kD link protein (identified with the 8A4 mAb; Caterson et al., 1985). The core glycoprotein obtained by chondroitinase treatment of the SF8 proteoglycan from either early postnatal or adult brain migrated on SDS-PAGE as a single band at 400 kD (Rauch et al., 1991). Chondroitin sulfate proteoglycan aggregates (containing hyaluronic acid and link protein) were also extracted from a transplantable rat chondrosarcoma (Choi et al., 1971), and isolated by CsCl density gradient centrifugation (Falke 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. Ratios of 0.5 and 1.5 mU chondroitinase/μg proteoglycan protein were used for the IDI and SF8 proteoglycans, respectively, whereas the chondrosarcoma proteoglycan, which contains a much higher concentration of chondroitin sulfate, was digested with chondroitinase in a ratio of 4 mU/μg dry weight. Completeness of digestion was confirmed by SDS-PAGE, which demonstrated that the large native proteoglycan which did not enter the separating gel was converted to discrete core glycoprotein bands after enzyme treatment (Rauch et al., 1991).

To test the sensitivity of proteoglycans to proteolysis, solutions containing 0.1 mg/ml proteoglycan were treated with 10 μg/ml of trypsin for 1 h at 37°C and the reaction was terminated by addition of 20 μg/ml of soybean trypsin inhibitor.

Polyclonal rabbit antibodies against chicken Ng-CAM were prepared as previously described (Grumet et al., 1984b). mAbs specific for the IDI and SF8 proteoglycans have previously been described (Rauch et al., 1991), and the Ig fraction was obtained after precipitation with ammonium sulfate at 45% of saturation.

Other proteoglycans included Engelbreth-Holm-Swarm laminin (Collaborative Research, Bedford, MA), human plasma fibronectin (New York Blood Center, New York, NY), BSA (ICN Biomedical, Lisle, IL), and deoxyribonuclease I (Worthington Biochemical Corp., Freehold, NJ).

Cowasphere Aggregation

Proteins (50 μg) were covalently coupled to 200 μl of 0.5-μm Cowaspheres (Duke Scientific Corp., Palo Alto, CA), washed twice in PBS containing 1 mg/ml BSA/10 mM NaNO3, and resuspended in the original volume of buffer (200 μl) (Grumet and Edelman, 1988); Cowaspheres as supplied by the manufacturer were at a concentration of 850-cm2 surface area/ml. Quantitative measurements indicated that under these conditions >20% of the Ng-CAM protein was bound to the Cowaspheres. For Cowasphere aggregation experiments, pre-existing aggregates in the bead preparations were first dissociated by sonication for 10-20 s, and 6-μl aliquots (containing >0.2 μg protein) were mixed with 54 μl of PBS containing the indicated amounts of proteins. After a 30-min incubation on ice, the samples were resorbed and aggregation was monitored at 25°C. The appearance of superthreshold aggregates of Cowaspheres was measured using a Coulter Counter (model ZF) fitted with a 100-μm aperture, set at amplification = 0.17, aperture current = 0.33, threshold 10-100; these settings allowed detection of particles >4 μm as described previously (Grumet and Edelman, 1988; Hoffman and Edelman, 1987). Superthreshold aggregates were measured in samples of 20 μl that were diluted into 20 ml of PBS. Microscopic observations of Cowasphere preparations that had been measured in the Coulter Counter indicated that populations containing aggregates of 5-10 Cowaspheres were subthreshold, inasmuch as they gave measurements that were not significantly greater than the background levels for buffer alone (range of 300-1,000). It was difficult to resolve the numbers of individual Cowaspheres present in larger aggregates.

Gravity Cell Binding Assay

Neurons were prepared from 9-d chicken embryo brains (Grumet and Edelman, 1988) and cell binding to protein-coated substrates was performed using a gravity assay (Friedlander et al., 1988). Briefly, 2 μl of proteins in PBS were placed in a circular array near the center of a polystyrene petri dish. After 30 min, the dishes were washed three times with PBS containing 1 mg/ml BSA. After 1-h incubation, the BSA-blocking solution was removed and replaced with 2 × 106 cells in 0.5 ml of Eagle's minimal essential medium with spinner salts (Gibco Laboratories, Grand Island, NY) containing 50 μg/ml deoxyribonuclease I, and incubated for 1 h at 37°C. The unbound cells were removed by four gentle washes in PBS and a fifth wash in medium; in some experiments, the cells were fixed with 1% glutaraldehyde. The dishes were observed by phase contrast microscopy, and cells were counted in four fields (2.2 mm2) within the protein-coated area.

Centrifugation Cell Binding Assay

Proteins were adsorbed for 1 h to U-shaped wells of 96-well polyvinyl chloride microtiter plates, which were then washed, and blocked with BSA (Friedlander et al., 1988). 200 μl of a cell suspension containing 5 × 104 cells was placed in each well and the plate was centrifuged at ~250 g for 2 min at room temperature. The pattern of cells in each well was observed under dark field illumination (Friedlander et al., 1988), and reflects a balance between the centrifugal force and the adhesivity of the substrate. On nonadhesive substrates, the cells were centrifuged into a pellet at the bottom of the well, and as the adhesivity of the substrate increased, more cells bound to the substrate along the wall of the well which could be detected in a circular area with a measurable diameter. On strongly adhesive substrates, cells were distributed more or less uniformly on the well (see Fig. 9).
Immunofluorescent Staining

After gravity binding assays, dishes were treated with 3.7% formaldehyde/PBS and 0.1 M glycine/PBS, each for 10 min. After blocking for 30 min in PBS containing 4% goat serum, indirect staining (Grumet et al., 1988) and N-CAM (Hoffman and Edelman, 1983) were used to detect aggregates larger than a given size and was shown to be highly dependent on the concentration of Covaspheres in suspension. It was previously determined that at a concentration of about 85 cm$^2$ of bead surface area/ml, the appearance of aggregates began to reach a plateau level after about 1 h of incubation (Grumet and Edelman, 1988). Therefore, to explore potential interactions between proteoglycans and CAMs, we used this concentration of beads to test the effects of various proteins and proteoglycans on the extent of aggregation of Ng-CAM-Covaspheres after 2 h of incubation.

The appearance of aggregates of fluorescent Covaspheres was observed by fluorescence microscopy and was quantitated using a Coulter Counter (see Materials and Methods). Whereas control proteins including BSA and fibronectin did not inhibit aggregation of the Ng-CAM–coated beads (Grumet and Edelman, 1988), two distinct chondroitin sulfate proteoglycans from 7-d postnatal rat brain inhibited aggregation (Fig. 1). The aggregation of Ng-CAM–Covaspheres was measured using a Coulter Counter to detect aggregates larger than a given size and was shown to be highly dependent on the concentration of Covaspheres in suspension. It was previously determined that at a concentration of about 85 cm$^2$ of bead surface area/ml, the appearance of aggregates began to reach a plateau level after about 1 h of incubation (Grumet and Edelman, 1988). Therefore, to explore potential interactions between proteoglycans and CAMs, we used this concentration of beads to test the effects of various proteins and proteoglycans on the extent of aggregation of Ng-CAM–Covaspheres after 2 h of incubation.

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Results

Inhibition of CAM–Covasphere Aggregation by Proteoglycans

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Based on these results and the possible complications due to the presence of highly charged glycosaminoglycans on the native proteoglycans, we focused primarily on effects produced by the chondroitinase-treated proteoglycans.

The 3F8 proteoglycan inhibited aggregation of Ng-CAM–Covaspheres both at 10 and 30 μg/ml (Fig. 2 b). The ID1 proteoglycan from 7-d rat brain also strongly inhibited aggregation at 10 μg/ml (Fig. 2 c). However, at 30 μg/ml some aggregation could be detected by fluorescence microscopy (Fig. 2 d) and using the Coulter Counter (Fig. 3). In contrast to the fairly uniform effects on aggregation of Ng-CAM–Covaspheres in the presence of the 3F8 proteoglycan, the effects of the 7-d 1D1 proteoglycan at 30 μg/ml were much more heterogeneous; for example, microscopic observations showed that in addition to single Ng-CAM–Covaspheres and small aggregates (Fig. 2 d), large aggregates were also detected in some experiments (data not shown). This effect of the 7-d 1D1 proteoglycan was somewhat variable between experiments and increased with time. In certain experiments, measurement of superthreshold aggregates showed little or no difference between the 7-d 1D1 and 3F8 proteoglycans at 2 h of incubation but large aggregates were observed after 24 h of incubation with the chondroitinase-treated 1D1 proteoglycan but not with the other brain proteoglycans tested, which showed persistent inhibition (Table I, and microscopic observations, data not shown). These effects may be related to the ability of the 7-d 1D1 proteoglycan to self-aggregate (see Discussion). It is also important to note that the counter detects particles larger than a certain size but does not provide a weighted average that would be needed to determine the percentage of Covaspheres present in the aggregates.
Figure 2. Effects of proteoglycans on aggregation of Ng-CAM-Covaspheres. Green-fluorescing Ng-CAM-Covaspheres after incubation for 2 h at 25°C in the presence of 10 μg/ml of BSA (a), 30 μg/ml 3F8 proteoglycan (b), 10 μg/ml 7-d 1D1 proteoglycan (c), 30 μg/ml 7-d 1D1 proteoglycan (d), 30 μg/ml adult 1D1 proteoglycan (e), and 80 μg/ml aggrecan (f), were photographed under a fluorescence microscope as described in Materials and Methods. In contrast to the adult 1D1 and the 7-d 3F8 proteoglycans which strongly inhibited the aggregation at 30 μg/ml, we observed that the size distribution of aggregates varied considerably when the 7-d 1D1 proteoglycan was tested at 30 μg/ml, and panel d represents a typical sample. Bar, 100 μm.

Therefore, in this mode of operation the Coulter Counter may provide an indication of aggregation that is an underestimate of the percentage of Covaspheres in aggregates, particularly when larger aggregates have formed.

Different forms of the 1D1 proteoglycan are present in early postnatal and adult brain (Rauch et al., 1991), and the adult form showed dose-dependent inhibition when tested over a range of concentrations (Fig. 3). Unlike the early postnatal form of this proteoglycan, the adult form did not potentiate aggregation of Ng-CAM-Covaspheres when tested at 30 μg/ml (Figs. 2 e, and 3). In contrast to the brain proteoglycans, aggrecan had no effect on the Ng-CAM-Covaspheres even when tested at concentrations up to 80 μg/ml (Fig. 2 f). It is unlikely that the proteoglycans inhibited Covasphere aggregation by a trivial mechanism such as proteolysis of Ng-CAM because we found that incubation of the 1D1 and 3F8 proteoglycans with Ng-CAM for 1 h at 37°C had no effect on the molecular sizes of the Ng-CAM components when resolved by SDS-PAGE (data not shown).

To determine whether the proteoglycans could affect other CAMs, a similar series of experiments was performed using N-CAM-coated beads. The aggregation of N-CAM-Covaspheres was inhibited strongly in the presence of 3 μg/ml of the native 1D1 and 3F8 proteoglycans (Fig. 4). In contrast, native aggrecan had little or no effect at the same concentration but showed some inhibition when tested at higher concentrations (Fig. 4). The observation that native aggrecan could inhibit aggregation of Covaspheres coated with N-CAM but did not affect Covaspheres coated with Ng-CAM (Fig. 1) may be attributed to electrostatic interactions between negatively charged polysialic acid on N-CAM and negatively charged sulfate and uronic acid residues on aggrecan chondroitin sulfate. This possibility is consistent with the observations that chondroitinase-treated aggrecan did not inhibit aggregation of N-CAM-Covaspheres (Fig. 5). In addition, chondroitinase treatment did not significantly modify the inhibitory effects of the 1D1 and 3F8 brain proteoglycans (Fig. 5).

Table I. Effects of High Concentrations of Proteoglycans on Aggregation of Ng-CAM Covaspheres

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Chondroitinase treatment</th>
<th>Superthreshold aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>34,860 ± 2,789</td>
</tr>
<tr>
<td>Neurocan</td>
<td>-</td>
<td>356 ± 233</td>
</tr>
<tr>
<td>Neurocan</td>
<td>+</td>
<td>434 ± 144</td>
</tr>
<tr>
<td>7-d 3F8</td>
<td>-</td>
<td>494 ± 130</td>
</tr>
<tr>
<td>7-d 3F8</td>
<td>+</td>
<td>459 ± 23</td>
</tr>
</tbody>
</table>

Ng-CAM-Covaspheres (6 μl in a final volume of 60 μl) were preincubated for 15 min on ice with untreated or chondroitinase-treated proteoglycans at 30 μg/ml, sonicated, and the appearance of superthreshold aggregates was measured at the times indicated using a Coulter Counter as described in Materials and Methods. Numbers of superthreshold aggregates are averages of duplicates ± mean deviations.
Figure 4. Inhibition of N-CAM–Covasphere aggregation by native brain proteoglycans. The appearance of superthreshold aggregates of Covaspheres coated with N-CAM was measured after 2 h; ID1 proteoglycan from 7-d brain (■), 3F8 proteoglycan from 7-d brain (●), and rat chondrosarcoma aggrecan (▲). Data represent means (n = 3) ± the standard error of the percent of the control levels of superthreshold aggregates detected. The mean level of superthreshold particles in control samples was 25,622 ± 2,651.

were maximal at ~3–10 μg/ml. In a typical assay (60 μl) at this concentration of proteoglycans, the amount of proteoglycan in solution was ~0.2–0.6 μg and the amount of Ng-CAM on the Covaspheres was ~0.3 μg (see Materials and Methods), suggesting that the brain proteoglycans can perturb homophilic CAM binding at approximately stoichiometric levels with the CAM. In contrast, aggrecan had no significant effect at several fold higher concentrations (Figs. 2, 3, and 5).

Figure 5. Inhibition of N-CAM–Covasphere aggregation by chondroitinase-treated brain proteoglycans. The appearance of superthreshold aggregates of Covaspheres coated with N-CAM was measured after 2 h of incubation with chondroitinase-treated proteoglycans (PG). Data represent means (n = 3) ± the standard error of the percent of the control levels of superthreshold aggregates detected. The mean level of superthreshold particles in control samples was 19,993 ± 2,190. (○) 7-d ID1 PG; (□) 7-d 3F8 PG; (△) Adult ID1 PG; (▲) Aggrecan.

Neuronal Adhesion to Ng-CAM Is Inhibited in the Presence of Proteoglycans

To determine whether the brain proteoglycans could affect cell adhesion, we tested the binding of cells to substrates containing mixtures of CAMs and various chondroitinase-treated proteoglycans. Although substrate bound N-CAM weakly promoted cell adhesion, the 8D9 antigen (Ng-CAM) was found to be a potent promoter of neuronal adhesion (Lagenaur and Lemmon, 1987). We obtained similar results (data not shown), and Ng-CAM was therefore used in the present studies. When mixtures containing equal amounts of Ng-CAM and aggrecan were adsorbed to petri dishes, binding of neurons could be detected, but much lower levels of binding were detected when equivalent amounts of neurocan were used (Fig. 6, a and c). To verify that the proteoglycans were not simply preventing the binding of Ng-CAM to the substrates, the dishes were immunofluorescently stained for Ng-CAM after the binding assay. Ng-CAM was detected in regions adsorbed with mixtures containing aggrecan (Fig. 6 b) or neurocan (Fig. 6 d), as well as with mixtures contain-
Figure 7. Binding of neurons to Ng-CAM substrates is inhibited in the presence of native brain proteoglycans. Attachment of neurons prepared from 9-d chicken embryo brains to dishes coated with mixtures containing 10 μg/ml of Ng-CAM and 10 μg/ml of proteoglycans was measured in gravity binding assays as described in Materials and Methods. Data represent means (n = 4) ± the standard error of cells bound to 2.2 mm² of the dish.

Reducing the 3F8 proteoglycan and the adult brain 1D1 proteoglycan (data not shown). Preliminary experiments also indicated that when Ng-CAM was first adsorbed to dishes and proteoglycans were then applied, neurocan inhibited neuronal binding whereas aggrecan had little or no effect.

To analyze the relative effectiveness of the different proteoglycans, the binding of neurons to mixtures of Ng-CAM and various proteoglycans was measured using different ratios of proteoglycan to Ng-CAM. When equal amounts of native (non-chondroitinase-treated) proteoglycans were mixed with Ng-CAM and adsorbed to petri dishes, aggrecan produced a relatively small inhibition of neuronal binding (Fig. 7). In contrast, equivalent amounts of neurocan and the 3F8 proteoglycan strongly inhibited (>80%) neuronal binding (Fig. 7). In similar experiments using chondroitinase-treated aggrecan, little or no effect on neuronal binding was detected (Fig. 8), suggesting that the high concentration of chondroitin sulfate in aggrecan as compared to the brain proteoglycans may make native aggrecan an inappropriate control in certain assays. To avoid the potential complications due to the presence of chondroitin sulfate, we focused on comparisons between the proteoglycan core proteins. When mixtures containing 10 μg/ml of Ng-CAM and various concentrations of proteoglycan were adsorbed to dishes, the 1D1 and 3F8 proteoglycans were treated with chondroitinase. Bar, 0.35 mm.

Figure 8. Binding of neurons to Ng-CAM substrates is inhibited in the presence of chondroitinase-treated brain proteoglycans. Attachment of neurons prepared from 9-d chicken embryo brains to dishes coated with mixtures containing a final concentration of 10 μg/ml of Ng-CAM and the indicated amounts of chondroitinase-treated 1D1 proteoglycan from 7-d (∇) and adult brain (○), 3F8 proteoglycan from 7-d brain (○), and rat chondrosarcoma aggrecan (△) was measured in gravity binding assays as described in Materials and Methods. Data represent means (n = 4) ± the standard error of cells bound to 2.2 mm² of the dish.

Figure 9. Binding of neurons to proteins in the centrifugation assay. Substrates in 96-well plates were prepared by incubation with the indicated concentrations of different proteins. Neurons were added to the wells and binding was performed as described in Materials and Methods. The wells were visualized by dark field microscopy using a 4× objective and the cells appeared as bright spots. The speckled pattern of cells on Ng-CAM-coated wells represents strong binding to the substrate, whereas the cells formed pellets at the bottom of BSA-coated wells, indicating a lack of binding to the well. The arrow in the panel representing treatment with 5 μg/ml of adult 1D1 proteoglycan indicates the diameter of the binding region (see Table II). All proteoglycans were treated with chondroitinase.
Table II. Attachment of Neurons to Protein-coated Substrates in the Centrifugation Assay

<table>
<thead>
<tr>
<th>Protein applied (µg/ml)</th>
<th>Diameter (mm)</th>
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<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>7-d 1D1</td>
<td>1.07 ± 0.15</td>
</tr>
<tr>
<td>7-d 3F8</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td>Adult 1D1</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Ng-CAM</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>BSA</td>
<td>0.18 ± 0.08</td>
</tr>
</tbody>
</table>

Centrifugation assays were performed after incubating 96-well plates with protein solutions at the concentrations indicated, as described in the legend to Fig. 9. The diameter in micrometers of the binding region (for example, see diagonal arrow in Fig. 9, adult 1D1 at 5 µg/ml) was measured under the microscope using an internal calibrated reticle; the mean diameters and the standard errors were calculated for triplicate samples. All proteoglycans were treated with chondroitinase. Diameters >2.5 mm were difficult to resolve because they represented binding along the near vertical sides of the U-shaped wells.

proteoglycans produced concentration-dependent inhibition of neuronal binding (Fig. 8). Although all of the brain proteoglycans inhibited strongly when applied at 10 µg/ml, the 1D1 proteoglycan from 7-d brain was slightly more effective than the adult form at equivalent concentrations. In contrast to the ability of the brain proteoglycans to inhibit neuronal adhesion to substrates prepared using mixtures containing Ng-CAM, preliminary experiments indicated that these proteoglycans had little or no effect on the binding of neurons to substrates that were prepared with mixtures containing laminin instead of Ng-CAM; neuronal binding was inhibited by <20% of control when mixtures containing approximately equal amounts of laminin and the 1D1 or 3F8 proteoglycans were adsorbed to dishes (data not shown). These results suggest that the proteoglycans selectively affect only certain adhesive systems.

**Binding of Neurons to Brain Proteoglycans**

The results described above indicate that the brain proteoglycans can interact with CAMs and can inhibit adhesion of cells to a CAM. To determine whether cells can bind directly to proteoglycans, we used a centrifugation assay that has previously been used to demonstrate binding of cells to cytotactin, an extracellular molecule that can inhibit cell adhesion and migration (Friedlander et al., 1988; Crossin et al., 1990). Because of its very short duration, the centrifugation assay reflects more closely the strength of the initial molecular interactions than the gravity assay, which is highly sensitive to the ability of the immobilized molecule to allow cell spreading. Thus, proteins that bind to cells but cause a "repulsive" reaction (as appears to be the case with certain brain proteoglycans; see above) may demonstrate cell binding in the centrifugation assay even though no binding can be observed in the gravity assay. In the centrifugation assay, cells pelleted to the bottom of U-shaped wells that were treated with non-adhesive molecules. In contrast, cells bound to differing degrees to wells that were coated with various amounts of different proteins. For example, neurons pelleted on BSA-coated wells and bound tightly to wells treated with Ng-CAM (Fig. 9).

To determine the relative abilities of different proteins to promote neuronal binding in the centrifugation assay, wells were treated with various concentrations of the proteins. When proteins were applied at 20 µg/ml, neurons bound more or less evenly across the wells treated with 7-d 1D1 and 3F8 proteoglycans, and bound to a smaller region in wells treated with the adult 1D1 proteoglycan, indicating that cells bound more weakly to the adult 1D1 proteoglycan. In contrast, the pattern of neurons observed after centrifugation on wells treated with aggrecan was indistinguishable from that obtained with BSA, indicating that neurons did not bind to aggrecan. The ability of each brain proteoglycan to promote binding was diminished when lower concentrations were used (Fig. 9 and Table II).

To analyze the degree of binding, we measured the diameter of the region in which the binding was observed to be relatively uniform (for example, see arrow in Fig. 9, adult 1D1 at 5 µg/ml). The diameter of the binding region was 0 on non-adhesive substrates and increased to ~2.5 mm on the most adhesive substrates (Table II). When the proteoglycans were tested at various concentrations, little difference was observed between the effectiveness of the 1D1 and 3F8 proteoglycans from 7-d brain, and the adult form of 1D1 was consistently found to be less effective (Table II). Neurons bound strongly to Ng-CAM and they did not bind to aggrecan or to BSA in this assay.

The specificity of neuronal binding to the proteoglycans was analyzed using mAbs that specifically recognize the 1D1 or 3F8 proteoglycans (Table III). The 1D1 mAb partially inhibited neuronal binding to the 1D1 proteoglycan and had no effect on binding to the 3F8 proteoglycan suggesting that this mAb binds to the 1D1 proteoglycan at or near a region that is important for cell binding. The specificity of binding is consistent with previous observations that the 1D1 and 3F8 mAbs recognize distinct antigenic determinants on different brain proteoglycans (Rauch et al., 1991). The 3F8 antibody had no effect on binding to the 1D1 proteoglycan but it increased the degree of binding to wells coated with the 3F8 proteoglycan. This increase in binding to wells coated first with 3F8 proteoglycan and then with the 3F8 mAb raised the possibility that the chick brain cells could bind directly to the 3F8 mAb. In support of this possible mechanism, preliminary experiments indicated that chick brain cells bound to wells that had been treated only with 3F8 Ig, but not with control mAbs.

Table III. Effects of mAbs on Attachment of Neurons to Protein-coated Substrates in the Centrifugation Assay

<table>
<thead>
<tr>
<th>Protein on Substrate</th>
<th>Control</th>
<th>1D1</th>
<th>3F8</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-d 1D1</td>
<td>2.83 ± 0.15</td>
<td>1.13 ± 0.18 (60)</td>
<td>2.64 ± 0.13 (7)</td>
</tr>
<tr>
<td>Adult 1D1</td>
<td>0.65 ± 0.04</td>
<td>0.36 ± 0.09 (44)</td>
<td>0.59 ± 0.23 (10)</td>
</tr>
<tr>
<td>3F8</td>
<td>1.30 ± 0.63</td>
<td>1.43 ± 0.09 (0)</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BSA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ng-CAM</td>
<td>&gt;2.5</td>
<td>&gt;2.5</td>
<td>&gt;2.5</td>
</tr>
</tbody>
</table>

Centrifugation assays were performed as described in the legend to Table II using 20 µg/ml solutions of the indicated proteins. After removing the unbound proteins, the wells were washed three times and then incubated for 1 h with a 1:100 dilution of ascites fluid; the plates were washed three times and cell binding was performed as described in Materials and Methods. Numbers in parenthesis represent percent inhibition.
Discussion

The initial functional characterization of neurocan and the 3F8 proteoglycan reported here indicates that these brain proteoglycans: (a) can inhibit homophilic binding that is mediated by N-CAM and Ng-CAM; (b) can inhibit binding of neurons to Ng-CAM substrates; and (c) can bind directly to neurons. Although it was possible to demonstrate binding of neurons to brain proteoglycans using the centrifugation assay, the exact physiological relevance of this assay will require further investigation. However, this assay has been instrumental in demonstrating that other large multidomain proteins such as cytotactin have different regions that possess either positive or negative effects on cell binding (Friedlander et al., 1988; Prieto et al., 1992). In any case, these observations on neurocan and the 3F8 proteoglycan, which have been isolated from early postnatal rat brain and show dramatic changes in their patterns of expression during brain development (Rauch et al., 1991), suggest that proteoglycans may affect cell behavior during neural development by interacting with CAMs.

Several recent studies have implicated chondroitin sulfate and keratan sulfate in cell interactions, based partly on the ability of cartilage proteoglycans to inhibit cell adhesion and neurite growth on substrates containing either laminin or N-CAM (Snow et al., 1990b, 1991; Perris and Johansson, 1990). We have also obtained evidence for interactions between brain proteoglycans and N-CAM, but in contrast, we found that neurocan and the 3F8 proteoglycan had little or no effect on neuronal adhesion to laminin. Moreover, both brain proteoglycans are potent inhibitors of adhesion to Ng-CAM.

The specific inhibitory effects that we observed with the brain proteoglycans can be attributed primarily to the core glycoproteins. Although a number of previous studies indicate the involvement of the glycosaminoglycan portion of these molecules (Perris and Johansson, 1990; Snow et al., 1990b, 1991; Brittis et al., 1992), it appears that the glycosaminoglycan chains do not contribute significantly to the cytotactin/tenascin-binding properties of a chondroitin sulfate proteoglycan of embryonic chicken brain (Hoffman et al., 1988) and are not required for the effects of chondroitin sulfate proteoglycans of brain on neurite outgrowth (Iijima et al., 1991; Oohira et al., 1991). Results from studies utilizing cartilage proteoglycans, in which only the native proteoglycan (containing glycosaminoglycan chains) but not the core protein was found to have effects on neurite outgrowth or other aspects of cell behavior, might be due merely to a high negative charge density. In our studies, for example, adhesion of neurons to CAMs was inhibited to a relatively small degree (20-35%) by native (but not chondroitinase-treated) aggrecan (Figs. 4 and 7), but these effects may have little or no biological significance in view of the fact that chondrosarcoma aggrecan contains ~100 chondroitin sulfate chains as compared to the three to four chains in neurocan and the 3F8 proteoglycan. These brain proteoglycans differ significantly from aggrecan (in size, primary structure, degree and type of substitution with glycosaminoglycans, and N- and O-glycosidic oligosaccharides, sulfation, etc.), and they probably have different binding specificities.

Immunocytochemical studies have demonstrated that like the total population of soluble chondroitin sulfate proteoglycans of brain whose localization has previously been examined using polyclonal antibodies (Aquino et al., 1984a, b), staining of the 1D1 proteoglycan in 7-d rat cerebellum is generally most intense in the prospective white matter and absent from the external granule cell layer; in contrast, staining of the 3F8 proteoglycan is most intense in the molecular layer (Rauch et al., 1991). A common feature of both proteoglycans is that they are predominantly expressed in deeper layers of the developing cerebellum and appear to form a border between the external granule cell layer and the molecular layer. In contrast, N-CAM and Ng-CAM are expressed on many cerebellar neurons at the same stages of development and each CAM is distributed in a pattern that crosses the border formed by the proteoglycans (Grumet et al., 1984a; Daniloff et al., 1986). In the vicinity of this border, radially migrating granule cells project parallel fibers that contribute to the growth of the molecular layer during development, and the granule cells then migrate across the molecular layer to reside in the internal granule cell layer (Rakic, 1971). The abundance of the ID1 and 3F8 proteoglycans as well as other adhesion molecules in the molecular layer may modulate cell adhesion and provide signals to influence the behavior of the granule cells. For example, the inhibitory effects of the proteoglycans may prevent the parallel fibers from entering the molecular layer, thereby restricting them to migrate horizontally.

Recent studies (Miller, B., A. M. Sheppard, and A. L. Pearlman. 1992. Soc. Neurosci. Abstr. 18:778) showed that the ID1 and 3F8 proteoglycans are also present in the subplate region of the developing mouse cerebral cortex in regions that were previously found to contain chondroitin sulfate and fibronectin (Sheppard et al., 1991). Based on correlations between the patterns of protein expression and axonal migration during development, it has been suggested that these molecules may play a role in defining a destination for migrating axons that form the cortical plate, and in delineating pathways for early axonal extension (Sheppard et al., 1991).

Our studies raise the possibility that neural proteoglycans bind to CAMs, and further analysis to explore potential mechanism(s) of interaction between these molecules will be facilitated by their structural characterization. In 7-d brain, the ID1 proteoglycan consists of a major component containing a core glycoprotein with an apparent molecular size on SDS-PAGE of 245 kD after enzymatic removal of the three ~22 kD chondroitin 4- and 6-sulfate chains. A ID1 proteoglycan with a 150-kD core glycoprotein is also present at 7 d. By 2-3-wk postnatal this becomes the major species, and is essentially the only form present in adult brain. Peptide maps of both core proteins indicated that the 150-kD core glycoprotein is a part of the 245-kD core glycoprotein, because all peptides generated from it could also be found in the larger species (Rauch et al., 1991). The composite sequence of overlapping cDNA clones covering the entire ID1 proteoglycan (neurocan) encodes a protein of 1,257 amino acids (Rauch et al., 1992). The deduced amino acid sequence revealed a 22 amino acid signal peptide followed by an immunoglobulin domain, tandem repeats characteristic of the hyaluronic acid-binding region of aggregating proteoglycans, and an RGDS sequence. The COOH-terminal portion (amino acids 951-1,215) has ~60% identity to regions in the COOH termini of the fibroblast and cartilage proteoglycans, versican and aggrecan (Zimmermann and Ruoslahti, 1989; Doege et al., 1987), including two EGF-
like domains, a lectin-like domain, and a complement regulatory protein-like sequence. It is noteworthy that cytectactin/tenascin, which contains many EGF-like domains, can also inhibit cell adhesion (Jones et al., 1988). The central 595 amino acid portion of neurocan has no homology with other reported protein sequences. Northern blots demonstrated that a single message of \approx 7.5 \text{ kb} was detected in either 4-d or adult brain (but not in other rat tissues tested), indicating that the adult form of this proteoglycan is derived by in vivo proteolytic processing from the larger species present in early postnatal brain, and that neurocan is a new, and possibly nervous tissue-specific, member of the versican/aggrekan proteoglycan family.

The larger early postnatal 1D1 proteoglycan (neurocan) has the ability to form aggregates with hyaluronic acid, and a 45-kD link protein which stabilizes these aggregates copurifies with the proteoglycan (Rauch et al., 1991). Since link protein binds to the hyaluronic acid–binding domain of the proteoglycan core protein as well as to hyaluronic acid, it is possible that under appropriate conditions it may serve to cross-link neurocan bound to CAMs on the beads, and thereby induce aggregation. This may account for the observation that aggregates of CAM-coated Covaspheres were detected (see Figs. 2 and 3) in the presence of the 7-d form of the proteoglycan, but not the adult form (which does not contain a hyaluronic acid–binding region). The observation that the larger form of the ID1 proteoglycan is more potent in binding neurons suggests that in addition to regions in the COOH-terminal half of the molecule which may be involved in binding to the neural CAMs, the NH2-terminal half may contain additional sites important for proteoglycan–cell interactions.

In addition to neurocan, we have studied the effects on Ng-CAM and N-CAM interactions of the \approx 500-kD 3F8 chondroitin sulfate proteoglycan, which in both early postnatal and adult brain shows a core glycoprotein with an apparent molecular size of 400 kD after removal of the \approx 28 kD chondroitin sulfate chains. Like neurocan, the 3F8 proteoglycan contains \approx 20% by weight of chondroitin sulfate. Link protein is not associated with immunoaffinity purified 3F8 proteoglycan, which also does not aggregate with hyaluronic acid (Rauch et al., 1991).

For comparison with these rat brain chondroitin sulfate proteoglycans, we have also examined the effects on Ng-CAM and N-CAM interactions of a cartilage chondroitin sulfate proteoglycan (aggrecan) isolated from the transplantable Swarm rat chondrosarcoma. This proteoglycan has a 221-kD core protein (Doeege et al., 1987) to which are attached \approx 100 chondroitin sulfate chains as well as N- and O-glycosidically linked oligosaccharides. Aggrecan also has a hyaluronic acid binding region near its NH2 terminus that can form link protein stabilized aggregates with hyaluronic acid. It is unlikely that the associated link protein is responsible for effects of neurocan on CAMs, inasmuch as the chondroitinase-treated chondrosarcoma aggrecan, which also contains link protein (Faltz et al., 1979; Caterson and Baker, 1979) had no effect in our assays, whereas the 3F8 proteoglycan which does not contain link protein had strong effects.

HNK-1 epitopes that are present on both forms of the ID1 proteoglycan and on the early postnatal 3F8 proteoglycan (Rauch et al., 1991) are probably not involved in mediating interactions with the neural CAMs, insofar as: (a) the chondrosarcoma proteoglycan core protein, which contains HNK-1 epitopes (Margolis et al., 1987), had no effects in our assays; and (b) the adult 3F8 proteoglycan, which is HNK-1 negative (Rauch et al., 1991), was as effective as the HNK-1-positive early postnatal form of this brain proteoglycan (data not shown). HNK-1 epitopes are also present on several CAMs including Ng-CAM and N-CAM (Grumet et al., 1984; Schachner et al., 1990), and their potential functions in cell adhesion need to be explored further (Kühenmend et al., 1988). It is also unlikely that chondroitin sulfate chains are involved because chondroitinase-treated proteoglycans bound neurons and had strong inhibitory effects.

Our observations indicate a certain degree of selectivity in the effects of neurocan and the 3F8 proteoglycan on Ng-CAM and N-CAM, and suggest that they may bind to these CAMs. More direct evidence for binding between Ng-Cam and neurocan has been obtained in preliminary experiments which indicated that green-fluorescing Covaspheres coated with Ng-CAM coaggregated specifically with red-fluorescing Covaspheres coated with neurocan. The observation that the proteoglycans can affect binding mediated both by Ng-CAM and N-CAM suggests either that the proteoglycans bind to a common site on Ng-CAM and N-CAM, or that each CAM has a unique binding site for the proteoglycans. N-CAM and Ng-CAM share certain structural features including the presence of HNK-1 epitopes (Grumet et al., 1984b), and both contain Ig-like and fibronectin-like domains. However, the degree of amino acid sequence identity between chicken N-CAM and Ng-CAM is only 22% (Burgoon et al., 1991). Although most antibodies distinguish between them, they may share certain carbohydrate or protein domains that mediate interactions with proteoglycans. It will obviously be important to determine the nature of binding sites on the CAMs and the proteoglycans that mediate interactions between these molecules, and cDNA constructs are being prepared to express specific domains of neurocan and Ng-CAM to explore potential interactions. Further investigation of the interactions between proteoglycans and neural CAMs may thereby provide clues to understanding molecular mechanisms of cell repulsion (Keynes, 1991; Sage and Bornstein, 1991).

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References


Grumet et al. Interactions of Proteoglycans with Neurons and CAMs

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