# Interactions of the Chondroitin Sulfate Proteoglycan Phosphacan, the Extracellular Domain of a Receptor-type Protein Tyrosine Phosphatase, with Neurons, Glia, and Neural Cell Adhesion Molecules

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***Abstract.*** Phosphacan is a chondroitin sulfate proteoglycan produced by glial cells in the central nervous system, and represents the extracellular domain of a receptor-type protein tyrosine phosphatase (RPTPζ/β). We previously demonstrated that soluble phosphacan inhibited the aggregation of microbeads coated with N-CAM or Ng-CAM, and have now found that soluble $^{125}$I-phosphacan bound reversibly to these neural cell adhesion molecules, but not to a number of other cell surface and extracellular matrix proteins. The binding was saturable, and Scatchard plots indicated a single high affinity binding site with a $K_d$ of ~0.1 nM. Binding was reduced by ~15% after chondroitinase treatment, and free chondroitin sulfate was only moderately inhibitory, indicating that the phosphacan core glycoprotein accounts for most of the binding activity.

Immunocytochemical studies of embryonic rat spinal cord and early postnatal cerebellum demonstrated that phosphacan, Ng-CAM, and N-CAM have overlapping distributions. When dissociated neurons were incubated on dishes coated with combinations of phosphacan and Ng-CAM, neuronal adhesion and neurite growth were inhibited. $^{125}$I-phosphacan bound to neurons, and the binding was inhibited by antibodies against Ng-CAM and N-CAM, suggesting that these CAMs are major receptors for phosphacan on neurons. C6 glioma cells, which express phosphacan, adhered to dishes coated with Ng-CAM, and low concentrations of phosphacan inhibited adhesion to Ng-CAM but not to laminin and fibronectin. Our studies suggest that by binding to neural cell adhesion molecules, and possibly also by competing for ligands of the transmembrane phosphatase, phosphacan may play a major role in modulating neuronal and glial adhesion, neurite growth, and signal transduction during the development of the central nervous system.

THERE is increasing evidence that chondroitin sulfate proteoglycans play important roles in modulating cell adhesion, cell migration, and other developmental processes (Ruoslahti, 1989; Wight et al., 1992; Margolis and Margolis, 1993). We have previously demonstrated that two chondroitin sulfate proteoglycans of brain, neurocan (Rauch et al., 1992) and phosphacan (Maurel et al., 1994), interact with the neural cell adhesion molecules, Ng-CAM and N-CAM (Grumet et al., 1993; Friedlander et al., 1994). N-CAM and Ng-CAM are widely expressed in nervous tissue during periods of development and regeneration, and are involved in adhesion and migration of neurons and axons (Edelman, 1984; Rathjen and Jessel, 1991; Grumet, 1992). Both are members of the Ig-superfamily, containing extracellular Ig-like and fibronectin-like domains, membrane-spanning regions, and cytoplasmic regions. Each of these neural cell adhesion molecules (CAMs)[1] can bind homophilically through a $Ca^{++}$-independent mechanism, as demonstrated by the ability of CAM-coated beads (Covaspheres) to self-aggregate, and the aggregation of Covaspheres coated with either Ng-CAM or N-CAM is strongly inhibited by intact phosphacan and by the core glycoprotein resulting from chondroitinase treatment. Higher concentrations of rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) core protein have little effect in these assays. Phosphacan also inhibits binding of neurons to Ng-CAM when mixtures of these proteins are adsorbed to polystyrene dishes (Grumet et al., 1993).

Phosphacan is developmentally regulated with respect to its sulfation, carbohydrate composition and oligosaccharide

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1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; RPTP, receptor protein tyrosine phosphatase.

structure, and localization in nervous tissue (Rauch et al., 1991). It accounts for ~40% of the total soluble chondroitin sulfate proteoglycan protein in rat brain, has a size of ~500 kD estimated by gel filtration under dissociative conditions, and a core glycoprotein with an apparent molecular size of 400 kD on SDS-PAGE. The proteoglycan contains an average of four 28 kD chondroitin sulfate chains, in which approximately two-thirds of the disaccharides are 4-sulfated in 7-d brain, increasing to >96% chondroitin 4-sulfate in adult brain. A structurally related chondroitin/keratan sulfate proteoglycan (phosphacan-KS) with the same molecular size was also isolated from brain using the 3H1 monoclonal antibody to rat brain keratan sulfate. The concentration of phosphacan-KS increases over fivefold during postnatal brain development, and this change is accompanied by a developmental decrease in the branching and/or sulfation of the keratan sulfate chains (Rauch et al., 1991).

Cloning of phosphacan (previously designated the 3F8 proteoglycan) revealed a sequence of 1616 amino acids deduced from a 4.8-kb open reading frame (Maurel et al., 1994). The signal peptide is followed by an $NH_2$-terminal domain of 255 amino acids homologous to carbonic anhydrases, and one fibronectin type III-like domain. The entire amino acid sequence deduced from the cDNA clones corresponds to the extracellular domain of a human receptor-type protein tyrosine phosphatase, RPTP$\zeta/\beta$ (Krueger and Saito, 1992; Levy et al., 1993), with which it has 76% identity, and the proteoglycan appears to represent an mRNA splicing variant of the larger transmembrane protein (Maurel et al., 1994). A third probable mRNA splice variant differs by a deletion in the extracellular region of an 860–amino acid sequence adjacent to the membrane (Levy et al., 1993). The 30 $NH_2$-terminal amino acids of the 3H1 chondroitin/keratan sulfate proteoglycan from rat are identical to those of the 3F8 proteoglycan, and internal tryptic peptide sequences also matched those found in sequenced peptides of the 3F8 proteoglycan and amino acid sequences deduced from the cDNA clones. It was therefore concluded that the 3H1 chondroitin/keratan sulfate proteoglycan and the 3F8 chondroitin sulfate proteoglycan, which we have collectively named phosphacan, represent glycosylation and possible extracellular splicing variants of a receptor-type protein tyrosine phosphatase (Maurel et al., 1994). The structural features of this family of enzymes and their potential physiological roles in signal transduction and cell cycle regulation have been reviewed (Brautigan, 1992; Walton and Dixon, 1993).

We have now performed biochemical studies which demonstrate directly that phosphacan binds with high affinity to N-CAM and Ng-CAM/L1/NILE, cellular studies to examine its effects on neurons and glia, and by immunocytochemistry we have shown an overlapping localization of phosphacan with these neural CAMs during central nervous system development. Phosphacan, which is synthesized by glia (Milev et al., 1993; Canoll et al., 1993), bound to neurons, and inhibited adhesion of neurons and glia, and process outgrowth from these cells. Our results indicate that soluble phosphacan may modulate cell interactions and other developmental processes in nervous tissue through heterophilic binding to CAMs, and possibly also by competition for ligands shared with cell surface receptor protein tyrosine phosphatase (RPTP)-$\zeta/\beta$.

## Materials and Methods

### Proteins and Antibodies

Chicken Ng-CAM, rat NILE, and chicken and rat N-CAM were purified from 14-d embryonic chicken brains and 7-d postnatal rat brains by immunoaffinity chromatography, and the 2C2 and 5B8 monoclonal antibodies that recognize the NILE glycoprotein and cytoplasmic regions of rat N-CAM, respectively, were also used for immunocytochemistry as described (Friedlander et al., 1994). Because of extensive similarities between chicken Ng-CAM, rat NILE and mouse L1 in structure, expression patterns, and function (Grumet, 1992; Sonderegger and Rathjen, 1992), we refer to them generally as Ng-CAM/L1/NILE, and we refer to Ng-CAM and L1/NILE when discussing experiments using the particular proteins from chick and rat, respectively.

Phosphacan and phosphacan-KS (core protein = 173 kD) were isolated from PBS extracts of brains of 7-d or 2–3-month-old Sprague-Dawley rats by ion exchange chromatography, gel filtration, and immunoaffinity chromatography using the 3F8 and 3H1 monoclonal antibodies as described previously (Rauch et al., 1991). Rat chondrosarcoma chondroitin sulfate proteoglycan (aggrecan) was isolated by CsCl density gradient centrifugation (Faltz et al., 1979). Chondroitinase treatment of proteoglycans, SDS/PAGE, immunoblotting, and protein assays were performed as described previously (Friedlander et al., 1994).

Basic fibroblast growth factor receptor (a recombinant soluble form representing the three Ig-like domains; Dionne et al., 1990) was a kind gift from Dr. J. Schlessinger, and myelin-associated glycoprotein and epidermal growth factor receptor were obtained as described previously (Friedlander et al., 1994). Commercial reagents included laminin and type I and IV collagens (Collaborative Research, Bedford MA); fibronectin (New York Blood Center, NY); BSA (ICN Biomedical, Lisle, IL); collagen II (Chemicon, Temecula, CA); collagens III, V, VI, human merosin, and mouse vitronectin (GIBCO/BRL, Gaithersburg, MD); and human thrombospondin-1 (Telios, San Diego, CA). Human tenascin was a generous gift from Dr. Mario Bourdon. Sturgeon notochord chondroitin sulfate was obtained from Seikagaku America Inc. (Rockville, MD).

Monoclonal antibodies against chicken Ng-CAM and polyclonal antibodies against Ng-CAM, N-CAM, and chick brain membranes were prepared as previously described (Grumet and Edelman, 1984). 3F8, a monoclonal antibody specific for phosphacan, has been described (Rauch et al., 1991). A polyclonal antiserum to rat phosphacan was raised in rabbits by immunization with the 3F8 proteoglycan, and showed a staining pattern identical to that seen with the 3F8 monoclonal antibody on both immunoblots of brain proteoglycans and when used for immunocytochemistry. When Ig was used, it was precipitated from ascites fluid with ammonium sulfate and further purified on DE-52 columns.

### Radioligand-binding Assay

Proteoglycans were labeled to a specific activity of 0.5–1.3 × $10^{19}$ cpm/mol with $^{125}I$ by the lactoperoxidase/glucose oxidase method and binding assays were performed in removable Immulon-2 wells in either 16 mM Tris, pH 7.2/50 mM NaCl/2 mM $CaCl_2$/2 mM $MgCl_2$/0.02% $NaN_3$ (binding buffer), or in isotonic buffer (50 mM Tris, pH 7.2/150 mM NaCl/2 mM $CaCl_2$/2 mM $MgCl_2$/0.02% $NaN_3$), as described previously (Friedlander et al., 1994). Heat treated BSA in binding buffer (1 mg/ml) was used for blocking. Scatchard plots were generated and the $K_d$ was determined using the Macintosh version of the Ligand program (Munson and Rodbard, 1980).

### Cells

Dissociated neurons for cell adhesion and neurite outgrowth studies were prepared as described previously (Brackenbury et al., 1981; Friedlander et al., 1994). Immunofluorescence microscopy demonstrated that >90% of adherent cells prepared in this manner and used for cell adhesion assays (see below) could be identified as neurons based on their staining by antibodies to Ng-CAM (Grumet and Edelman, 1988). Brain cells for centrifugation and radioligand binding assays were prepared from 9–10-d chick embryo brains by trypsinization (20 μg/ml, 15 min, 37°C) in Eagle's minimal essential medium with Spinner salts, containing 0.02 mg/ml DNase I and 1 mM EDTA. Primary cultures of skin fibroblasts were prepared as described (Grumet and Edelman, 1988), and removed from dishes with 5 mM EDTA/PBS. For neurite outgrowth studies, cells were incubated in

DME/Ham's F12/ITS$^+$. C6 glioma cells were grown in DME/10% FCS. For adhesion assays, cells were removed from tissue culture dishes by treating briefly with trypsin/EDTA, and washed with medium containing 10% FCS. The cells were then washed with DME/ITS$^+$ and by centrifugation through a 3.5% BSA/PBS step gradient.

For endo-$\beta$-galactosidase treatment, $5 \times 10^5$ cells were washed with enzyme buffer (50 mM sodium acetate, 80 mM NaCl, pH 5.9) and resuspended in 100 $\mu$l buffer containing 10 mU of *Bacteriodes fragilis* endo-$\beta$-galactosidase (EC3.2.1.103; Oxford Glycosystems, Rosedale, NY). After incubation for 2.5 h at 37°C, the cells were counted, centrifuged through 3.5% BSA in SMEM, resuspended in medium, and cell debris was removed by low speed centrifugation.

## Substrates

Substrates for cell adhesion and neurite growth assays were prepared by incubating 1.5-$\mu$l droplets of protein solutions on polystyrene dishes (Falcon 1007 and 1008, Becton Dickinson, Lincoln Park, NJ) (Friedlander et al., 1988, 1994). For adhesion assays, protein solutions contained 5 $\mu$g/ml BSA as a carrier protein. After removing the droplets by suction, the dishes were washed three times with PBS and blocked with 1% BSA. For quantitative determination of protein binding to the dishes, radiolabeled proteins were incubated with dishes using the same materials and procedures employed for the cell assays. After the final wash, the dishes were dried, their walls removed with pliers, and the bottoms of the dishes were exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) to determine the relative amounts of radioactivity in the central region of each spot by using interactive software (ImageQuant, Molecular Dynamics). Absolute values of bound protein were obtained by comparing the relative values with the total radioactivity adsorbed to a similar set of spots that were dried completely, without prior washing (Friedlander et al., 1994).

## Centrifugation Cell Adhesion 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). 100 $\mu$l of a cell suspension containing $5 \times 10^4$ cells was placed in each well and the plates were centrifuged at ~250 *g* for 2 min at room temperature. The pattern of cells in the wells reflects a balance between the centrifugal force and the adhesivity of the substrate. On non-adhesive substrates, the cells form a pellet at the bottom of the well, and as the adhesivity of the substrate is increased, more cells adhere to the substrate along the wall of the well, and appear as a ring. On strongly adhesive substrates, cells are distributed more or less uniformly on the well (see Fig. 5).

## Gravity Cell Adhesion Assays

250 $\mu$l of DME/ITS$^+$ containing $6 \times 10^5$ cells were deposited in the central region of 35-mm polystyrene dishes that had been coated with proteins. After 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

$10^5$ 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 on phase contrast micrographs.

## Binding of Labeled Phosphacan to Neurons

E9 chick brain neurons and chick fibroblasts were prepared as described above and resuspended in cold cell binding buffer (2% BSA in SMEM/20 mM Hepes/2 mM $CaCl_2$, pH 7.2). Aliquots of cell suspensions (300 $\mu$l; $1.5 \times 10^7$ cells/ml) were distributed in 1.5 ml screw-cap tubes. Unlabeled proteins in binding buffer were added, and the cells were allowed to stand for 30 min at 4°C. Labeled proteins were then added and incubated for 2 h at room temperature with end-over-end mixing at 15 rpm. Cells were pelleted at 350 *g* for 3 min and then resuspended and washed twice with cell-binding buffer. The radioactivity bound to the cells was measured with a gamma-counter.

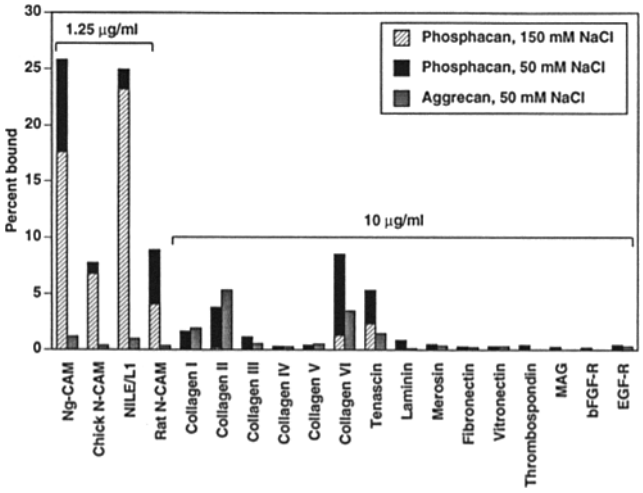


*Figure 1.* Binding of phosphacan and aggrecan to CAMs, extracellular matrix proteins, and growth factor receptors. Ng-CAM, chick N-CAM, rat NILE/L1, and rat N-CAM were coated in removable wells at a concentration of 1.25 $\mu$g/ml, and all other proteins were coated at 10 $\mu$g/ml. Labeled proteins were used at ~160,000 cpm/well. The percent bound represents specific binding (total cpm bound minus cpm bound to wells coated with BSA). All values are means of duplicate determinations. *MAG*, myelin associated glycoprotein; *bFGF-R*, basic fibroblast growth factor receptor; *EGF-R*, epidermal growth factor receptor. Total bar height (*hatched* + *solid*) corresponds to binding of phosphacan in 50 mM NaCl.

# Results

## Binding of Phosphacan to Adhesion Molecules

A radioligand-binding assay was used to analyze the specificity of binding of $^{125}$I-phosphacan to various cell adhesion molecules, extracellular matrix proteins, and other cell surface proteins. Binding was most pronounced to chicken Ng-CAM (26% bound; signal to background ratio 80:1) and N-CAM (8% bound; signal to background ratio 20:1), and to their rat homologues, NILE/L1 and N-CAM, respectively. Phosphacan also bound, but at much lower levels, to collagens I, II, VI, and tenascin, and higher amounts of immobilized protein were required for its detection (Fig. 1). Despite the lower level of phosphacan binding to tenascin, this interaction was nevertheless of high affinity ($K_d$ ~3 nM; Grumet et al., 1994). Very little or no binding was detected to collagens III, IV, and V, laminin, merosin, fibronectin, vitronectin, thrombospondin, myelin-associated glycoprotein, and to extracellular domains of the receptors for epidermal growth factor and basic fibroblast growth factor. For comparison, we also measured the binding of an unrelated chondroitin sulfate proteoglycan, rat cartilage aggrecan. There was very low binding of aggrecan to the CAMs, although aggrecan did bind to collagens I, II, and VI, and to tenascin.

Binding assays involving cell adhesion and extracellular matrix molecules are often carried out in hypotonic buffers to allow higher signal to background ratios (Zisch et al., 1992; Streit et al., 1993; Friedlander et al., 1994), and buffers containing 50 mM NaCl were used in our initial assays. Under physiological salt concentration, binding of phosphacan to chicken Ng-CAM and N-CAM and to NILE/L1 was re-

duced by 8–32%, and binding to rat N-CAM was reduced by 50%. Phosphacan binding to tenascin and collagen VI was also observed in isotonic buffers, although at a lower level (Fig. 1). In contrast, binding to collagens I and II was at a background level in isotonic buffers, indicating that phosphacan is not a likely ligand for these collagens in vivo. Most of the saturation and inhibition experiments described below were performed under hypotonic conditions to conserve material.

Reversibility of binding of phosphacan to Ng-CAM and N-CAM was demonstrated by a kinetic analysis of its dissociation (Friedlander et al., 1994). Labeled phosphacan was allowed to bind to the CAMs, 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 or in a large volume of buffer. 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 ($t_{1/2}$ ~15 min) than in the presence of excess buffer ($t_{1/2}$ ~2.5 h).

Binding of phosphacan to chicken Ng-CAM was saturable (Fig. 2). When the data were plotted according to the procedure of Scatchard (1949), a straight line was obtained, indicating a single class of binding sites. Binding to chicken and rat N-CAMs and to NILE/L1 was also saturable and the data yielded linear Scatchard plots. The highest affinities for

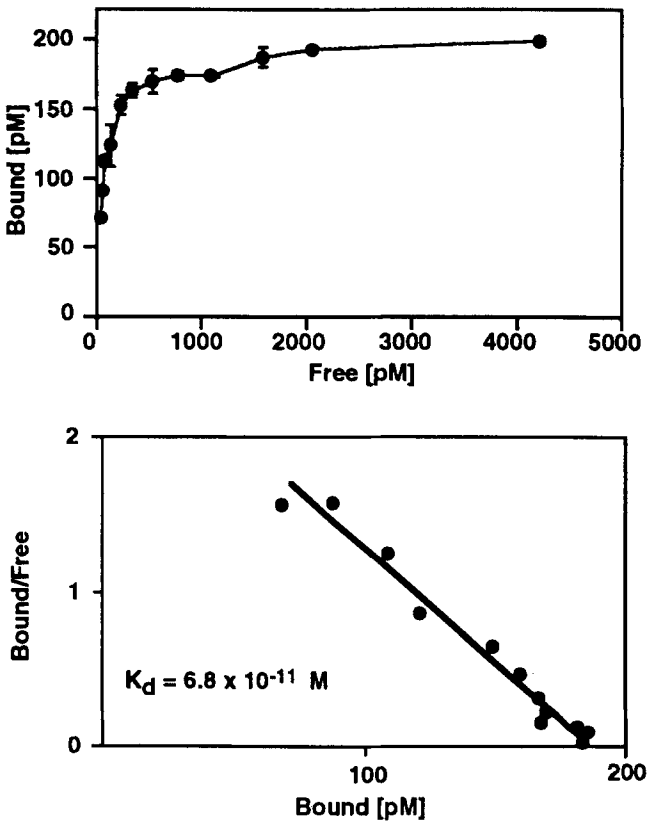


*Figure 2.* Saturation curve and Scatchard plot for the binding of $^{125}$I-phosphacan to Ng-CAM. Binding values represent specific binding as defined in the legend to Fig. 1. Phosphacan was tested at 0.5–37 ng/well (1.2 × 10$^{19}$ cpm/mol). Points in the saturation curve are averages of duplicate determinations ± SEM.

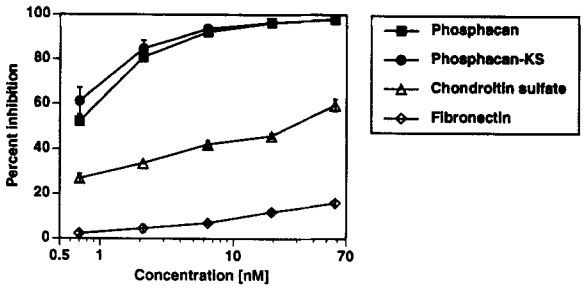


*Figure 3.* Inhibition of $^{125}$I-phosphacan binding to Ng-CAM by other soluble molecules. Wells coated with Ng-CAM were incubated with $^{125}$I-phosphacan (~160,000 cpm/well) in the presence of unlabeled phosphacan (■), phosphacan-KS (●), chondroitin sulfate (△), and fibronectin (◇) at the concentrations indicated. The molar concentration of chondroitin sulfate was based on an estimated average chain size of 30 kD. Specific binding of phosphacan to Ng-CAM in the absence of soluble molecules corresponds to 0% inhibition; background level of phosphacan binding to BSA defines 100% inhibition. Values are the averages of duplicate determinations ± SEM.

binding of phosphacan were to chicken Ng-CAM ($K_d$ = 0.07 nM) and to its mammalian homologue NILE/L1 ($K_d$ = 0.14 nM). The dissociation constants obtained for the binding of phosphacan to N-CAM were 0.3 nM and 3 nM for the chicken and rat proteins, respectively. Binding of phosphacan to Ng-CAM under isotonic conditions was also saturable and the Scatchard plot was linear with a $K_d$ = 0.13 nM.

The specificity of binding of phosphacan to Ng-CAM was also studied by inhibition experiments. Unlabeled phosphacan inhibited binding of labeled phosphacan to Ng-CAM in a concentration-dependent manner (Fig. 3). Half-maximal binding of labeled phosphacan at a total concentration of 0.52 nM was obtained in the presence of 0.7 nM unlabeled ligand. The chondroitin/keratan sulfate proteoglycan recognized by the 3H1 monoclonal antibody (phosphacan-KS) is identical to phosphacan in its protein sequence, but differs in its pattern of glycosylation (Rauch et al., 1991; Maurel et al., 1994). Despite differences in their concentrations of chondroitin sulfate, keratan sulfate, and other glycans, phosphacan and the four glycoforms of phosphacan-KS were all potent inhibitors of binding of phosphacan to Ng-CAM, with $IC_{50}$ values between 0.5 and 2 nM (Fig. 3, and data not shown). After treatment with chondroitinase ABC, phosphacan and phosphacan-KS had $IC_{50}$ values similar to those of their native counterparts (data not shown), in agreement with other data showing that binding of phosphacan to Ng-CAM is not dependent on the presence of chondroitin sulfate (see below, Fig. 4). Free chondroitin sulfate inhibited binding of phosphacan to Ng-CAM to a much lesser extent than did phosphacan itself, while fibronectin had only a slight effect (Fig. 3).

To study more directly the relative contribution of the phosphacan core protein and chondroitin sulfate to CAM binding, labeled phosphacan was treated with chondroitinase ABC and binding of native proteoglycan and core protein to Ng-CAM and N-CAM were compared. After chondroitinase treatment, binding of phosphacan to Ng-CAM was reduced by 23% as compared to native proteoglycan, and by 15% when compared to proteoglycans incubated with buffer alone (Fig. 4). Similar data were obtained for binding to N-CAM

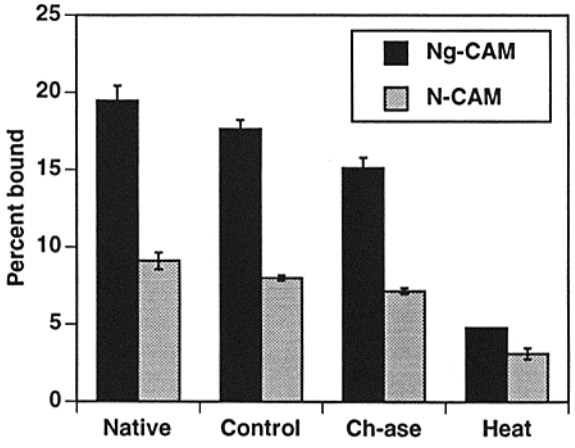


*Figure 4.* Effects of chondroitinase and heat treatment on the binding of phosphacan to Ng-CAM and N-CAM. Control treated phosphacan was incubated at 37°C for 2 h in chondroitinase buffer, and heat treatment was for 15 min at 95°C. Chondroitinase treatment (*Ch-ase*) and the radioligand-binding assay were performed as described in Materials and Methods. $^{125}$I-labeled phosphacan was used at 55,000 cpm per well. Values are averages of duplicate determinations, and error bars represent mean deviations.

(25% and 14% reduction, respectively). These results indicate that the phosphacan core protein plays a major role in binding to neural CAMs. This conclusion is supported by the finding that heat treatment of phosphacan abolished much of its binding activity (Fig. 4). Also consistent with our conclusion that the chondroitin sulfate chains on phosphacan are probably not essential or sufficient for binding to CAMs is the finding that cartilage aggrecan, which has a much higher concentration of chondroitin sulfate than phosphacan, does not bind significantly to these neural CAMs (see Fig. 1).

### *Binding of Phosphacan to Cells*

The solid-phase binding data indicate that phosphacan binds with high affinity to Ng-CAM and N-CAM. It was therefore of interest to examine the binding of phosphacan to cells that express these neural CAMs. For this purpose, $^{125}$I-phosphacan (0.13 nM) was incubated with suspensions of brain cells that express both of these CAMs (Grumet et al., 1984) and with skin fibroblasts (both prepared from 9-d chick embryos), and the binding of labeled phosphacan was determined. Typically, 7–8% of the label bound to neurons (Table I), and binding was inhibited by unlabeled ligand in a concentration-dependent manner. The level of binding was reduced from 8% to <3% by addition of 30 nM unlabeled ligand, suggesting that the binding of phosphacan to ligands on neurons is specific. A much lower percent of phosphacan bound to chick fibroblasts which do not express detectable amounts of Ng-CAM and N-CAM, and 30 nM of unlabeled phosphacan had little effect on this binding, indicating that the binding of fibroblasts is of lower affinity or nonspecific. Saturation experiments were not performed because of the relatively large amounts of phosphacan that would be required to determine nonspecific binding to cells. Chondroitinase treatment of phosphacan reduced its binding to neurons by only 25%, suggesting that most of the neuronal-binding activity is attributable to the core protein. The phosphacan-KS glycosylation variant bound to neurons at the same level as phosphacan (Table I).

Chick neurons may contain several cell surface molecules that bind phosphacan. Therefore, to determine whether Ng-CAM and N-CAM on neurons contribute to the binding of phosphacan, Fab' fragments of anti-Ng-CAM, anti-N-CAM, and anti-brain membrane polyclonal IgGs were used in inhibition experiments. Whereas binding of phosphacan to neurons was not significantly inhibited in the presence of nonimmune rabbit Fab', anti-Ng-CAM, anti-N-CAM, and anti-membrane Fab' at the same concentrations inhibited binding by 91, 77, and 90%, respectively (Table I). The combination of anti-Ng-CAM Fab' plus anti-N-CAM Fab' reduced binding to approximately the same level as that obtained with unlabeled phosphacan used at a concentration of 30 nM, and which is likely to represent the level of nonspecific binding. These results suggest that Ng-CAM and N-CAM

*Table I. Binding of $^{125}$I-Phosphacan to Chick Neurons and Fibroblasts*

| Cell type | Ligand | Unlabeled protein | Percent bound | Percent inhibition |
|---|---|---|---|---|
| Neurons | Phosphacan | | 7.9 ± 0.7 | 0 |
| Neurons | Phosphacan | Phosphacan 30 nM | 2.9 ± 0.1 | 100 |
| Neurons | Phosphacan | Phosphacan 10 nM | 5.0 ± 0.1 | 58 |
| Neurons | Phosphacan | Phosphacan 3.3 nM | 6.0 ± 0.3 | 39 |
| Neurons | Phosphacan | Phosphacan 1.1 nM | 7.2 ± 0.1 | 14 |
| Neurons | Phosphacan | Normal rabbit Fab' | 7.5 ± 0.9 | 9 |
| Neurons | Phosphacan | anti-Ng-CAM Fab' | 3.3 ± 0.5 | 91 |
| Neurons | Phosphacan | anti-N-CAM Fab' | 4.1 ± 0.7 | 77 |
| Neurons | Phosphacan | anti-Ng-CAM + anti-N-CAM Fab' | 2.4 ± 0.2 | 110 |
| Neurons | Phosphacan | anti-membrane Fab' | 3.4 ± 0.9 | 90 |
| Neurons | Phosphacan-KS | | 7.5 ± 0.2 | |
| Neurons | Phosphacan/chase | | 5.9 ± 0.4 | |
| Neurons | Phosphacan/chase | Phosphacan/chase 30 nM | 2.4 ± 0.2 | |
| Fibroblasts | Phosphacan | | 3.4 ± 0.3 | |
| Fibroblasts | Phosphacan | Phosphacan 30 nM | 2.3 ± 0.3 | |

Binding assays were performed as described in Materials and Methods. The concentration of labeled phosphacan, phosphacan-KS, or chondroitinase-treated phosphacan (Phosphacan/Chase) was 0.13 nM (300,000 cpm per tube), and the concentration of antibody Fab' was 700 μg/ml. Normal rabbit serum was from a pool obtained from four nonimmunized rabbits. Values are averages of 3–6 samples, and errors represent mean deviations. Calculation of the % inhibition was based on the assumption that the level of binding detected in the presence of 30 nM unlabeled phosphacan represents background binding.

account for a major fraction of phosphacan binding sites on neurons.

### *Adhesion of Brain Cells in a Centrifugation Assay*

We previously reported that using a centrifugation assay, embryonic chick brain cells, which are predominantly Ng-CAM positive neurons, attached to substrates coated with the phosphacan (3F8 proteoglycan) core glycoprotein (Grumet et al., 1993). We have now extended these studies to native phosphacan and to the various forms of phosphacan-KS which contain keratan sulfate in addition to chondroitin sulfate glycosaminoglycan chains.

Both native phosphacan and the core protein resulting from chondroitinase treatment promoted neuronal adhesion, and chondroitinase treatment increased adhesion when the wells were coated with proteoglycan at a concentration of 10 μg/ml (Fig. 5). Four forms of phosphacan-KS have been isolated from 7-d and adult rat brain using a 3H1 monoclonal antibody immunoaffinity column eluted with 0.5 M NaCl and with pH 11.5 buffer (Rauch et al., 1991). The phosphacan-KS species differ from one another in their concentrations of chondroitin sulfate (12–23% by weight) and keratan sulfate (5–19%), which vary reciprocally, and in their concentration of glycoprotein-type oligosaccharides (20–31%). All four forms of phosphacan-KS supported adhesion of E9 chick brain neurons in the centrifugation assay (Fig. 5 *A* and data not shown). Although some differences in their relative efficacies were seen, there was no clear correlation between keratan sulfate content and binding activity.

Chick brain cells also attached strongly to wells coated with the 3H1 monoclonal antibody to rat brain keratan sulfate (Rauch et al., 1991), and this attachment was almost completely abolished by treatment of the cells with endo-β-galactosidase, which causes depolymerization of the keratan sulfate chains (Fig. 5 *B*). In control experiments, brain cells did not bind to substrates coated with monoclonal anti-fibronectin, and endo-β-galactosidase treatment had no effect on cell adhesion to anti–Ng-CAM monoclonal antibody (data not shown), demonstrating that the lack of adhesion to mAb 3H1 was not due to nonspecific factors such as proteolytic activity in the endo-β-galactosidase preparation. The presence of cell surface keratan sulfate on embryonic chick brain cells was also supported by our finding that control-incubated cells used for the centrifugation assay were stained by the 3H1 monoclonal antibody, whereas staining was abolished by treatment with endo-β-galactosidase (data not shown). These results are consistent with our finding that phosphacan and phosphacan-KS, which are produced by glia (Milev et al., 1993; Canoll et al., 1993; and unpublished results), both bind to neurons (see above), and indicate that chick brain cells isolated by our procedure contain cell surface keratan sulfate (most likely in the form of a membrane-bound proteoglycan homologous to rat phosphacan-KS, which contains all of the detectable rat brain keratan sulfate; Rauch et al., 1991).

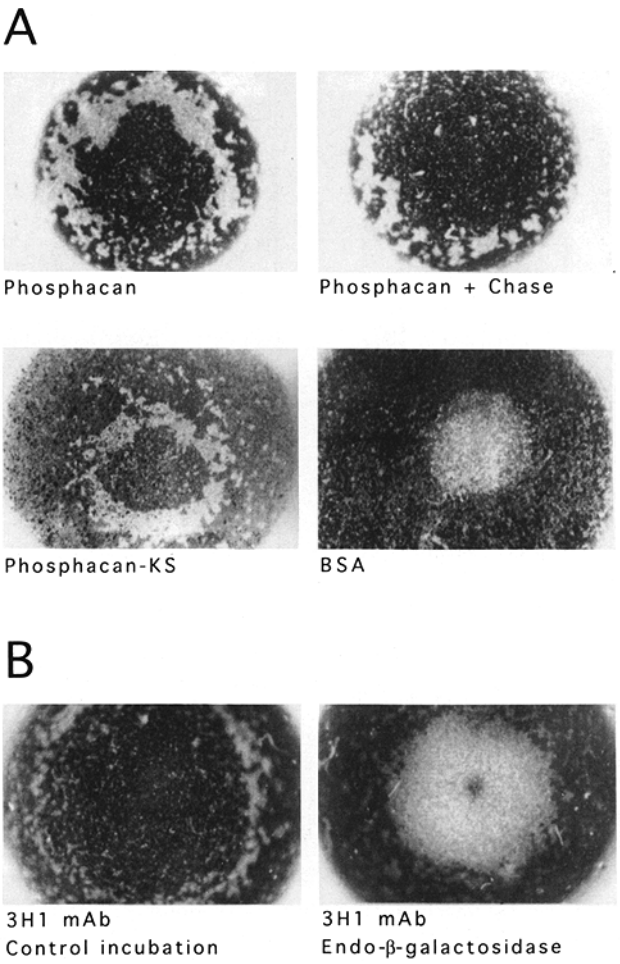


*Figure 5.* Adhesion of brain cells in the centrifugation assay. Substrates in 96-well plates were prepared by incubation with different proteins at a concentration of 10 μg/ml or 1:1,000 dilutions of ascites. Cells (>90% neurons) were added to the wells and assays were performed as described in Materials and Methods. The wells were visualized by dark-field microscopy using a 2.5× objective and the cells appeared as bright spots. (*A*) Adhesion to phosphacan. Cells adhered at intermediate levels (*large ring pattern*) to wells coated with native and chondroitinase (Chase)-treated phosphacan, to a lesser extent (*small ring pattern*) to phosphacan-KS, and not at all to BSA (*disc pattern*). (*B*) Adhesion to 3H1 mAb. Cells were incubated in buffer alone (control incubation) or in buffer containing endo-β-galactosidase, and were tested for adhesion to 3H1-coated wells. Whereas control cells adhered to the substrate, endo-β-galactosidase–treated cells did not adhere.

### *Overlapping Localization of Phosphacan, Ng-CAM/L1/NILE, and N-CAM in the Developing Central Nervous System*

In order for the observations that phosphacan binds to Ng-CAM and N-CAM, and to neurons that express these CAMs on their surface, to be biologically meaningful, it is essential that phosphacan and the neural CAMs be present together during nervous tissue development. At the whole tissue level we know that phosphacan, Ng-CAM/L1/NILE, and N-CAM are present in 7-d rat brain, since they were all isolated from brain by immunoaffinity chromatography using monoclonal antibodies (3F8, 2C2/19H3, and 5B8, respectively). To compare the localization of these proteins, monoclonal antibodies were used for immunoperoxidase staining of early postnatal rat cerebellum (Fig. 6). The general pattern of staining was similar for all three molecules insofar as it was strongest in the molecular layer and fiber tracts, weaker in the internal granule cell layer, and essentially absent in the external granule cell layer except for some staining of Bergmann glia

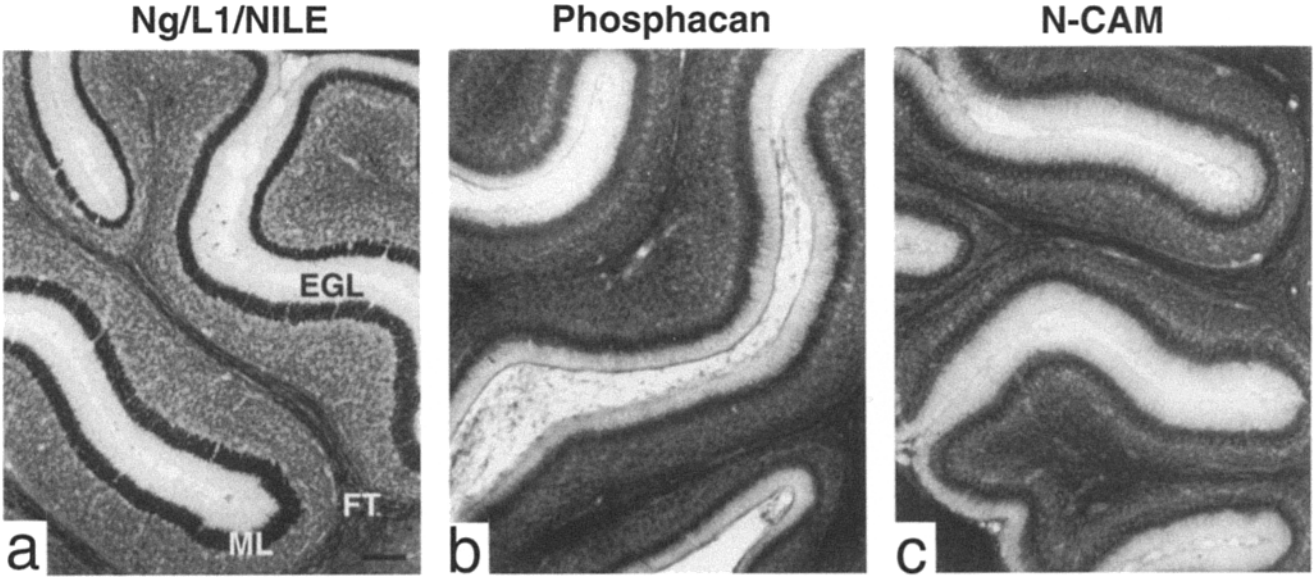


*Figure 6.* Immunoperoxidase staining of 7-d postnatal rat cerebellum with antibodies to Ng-CAM, phosphacan, and N-CAM. Rats were perfusion-fixed with picric acid-paraformaldehyde-glutaraldehyde, and sagittal Vibratome sections were stained with the 2C2 monoclonal antibody to Ng-CAM/NILE/L1 (*a*), the 3F8 monoclonal antibody to phosphacan (*b*), and the 5B8 monoclonal antibody to N-CAM (*c*), followed by peroxidase-conjugated second anfibody as described previously (Rauch et al., 1991). The 2C2 mAb recognizes a cytoplasmic region that is highly conserved between avian Ng-CAM and mammalian NILE/L1, and the 5B8 mAb to N-CAM recognizes the cytoplasmic region of N-CAM. *EGL*, external granule cell layer; *ML*, molecular layer; *FT*, fiber tract. Bar, 100 μm.

fibers. Similar staining patterns for these three proteins were observed in rat cerebellum between postnatal days 4 and 14.

A comparison of their distribution patterns in rat embryos was also performed by double label indirect immunofluorescence, using polyclonal antibodies to phosphacan together with monoclonal antibodies to either Ng-CAM or N-CAM. In E13 and E16 embryos, staining for phosphacan was observed in neural tissues including brain, retina, spinal cord, and peripheral ganglia such as the dorsal root ganglia. N-CAM and Ng-CAM/L1/NILE were observed in these same regions as well as in certain other locations as described previously (Beasley and Stallcup, 1987). In the spinal cord at E16 (Fig. 7), staining for phosphacan (Fig. 7, *a* and *c*) was strongest in the developing ventral, lateral, and dorsal columns and in the roof plate, which at this stage extends from the central canal to the dorsal surface of the spinal cord (Snow et al., 1990). On the same section, the staining for Ng-CAM/L1/NILE (Fig. 7 *b*) was similar to that of phosphacan insofar as it was strongest in the developing columns, but it differed by its absence from the roof plate and the prospective grey matter. In contrast, N-CAM (Fig. 7 *d*) was found in all these regions, being more generally distributed throughout the spinal cord. No significant staining of E16 brain or spinal cord was detected using the 3H1 monoclonal antibody, consistent with the fact that the keratan sulfate epitopes recognized by this antibody occur only in the postnatal rat central nervous system (Meyer-Puttlitz, B., and R. K. Margolis, unpublished results).

### *Effects of Phosphacan on Neuronal Adhesion and Neurite Outgrowth*

We have previously shown that neurons adhere to plastic coated with phosphacan core protein in a short-term centrifugation assay, but do not adhere to a phosphacan or to a mixed phosphacan and Ng-CAM substrate in a medium-term adhesion assay performed without centrifugation (Grumet et al., 1993). Based on the result that phosphacan binds to Ng-CAM, we analyzed the adhesion of neurons to dishes that had been treated sequentially with Ng-CAM and phosphacan, covering a wide range of concentrations for both molecules. Phosphacan inhibited neuronal adhesion to Ng-CAM, and this inhibition was concentration dependent for given amounts of Ng-CAM (Fig. 8). Higher concentrations of phosphacan were required to inhibit adhesion at higher concentrations of Ng-CAM. Comparable inhibitory effects on neuronal adhesion to Ng-CAM were observed for chondroitinase-treated phosphacan (data not shown). Quantitation of the surface density of the adsorbed proteins indicated that adsorption of Ng-CAM increased linearly with the concentration of soluble Ng-CAM, whereas the adsorption of phosphacan tended to saturate. Moreover, consistent with the observation that phosphacan binds to Ng-CAM with high affinity, phosphacan adsorbed more efficiently to dishes precoated with higher concentrations of Ng-CAM (data not shown).

In view of the inhibitory effects of phosphacan on Ng-CAM mediated neuronal adhesion, it was important to study the effects of phosphacan on neurite extension. Neurons were added to double-coated dishes similar to those used for the adhesion studies. In contrast with the procedures used for the cell adhesion assays, weakly adhering cells were not removed by washing after 80 min, and eventually cells attached to the substrate even when phosphacan was present (Fig. 9 *C*, *E*, and *G*). Neurons plated on dishes coated with BSA after Ng-CAM extended numerous long processes, reaching as far as ~280 μm (Fig. 9, *A* and *B*). In contrast, neurons that bound to dishes coated with phosphacan after Ng-CAM extended fewer processes and these processes were shorter (Fig. 9, *C–F*). Whereas strong inhibition of neurite growth

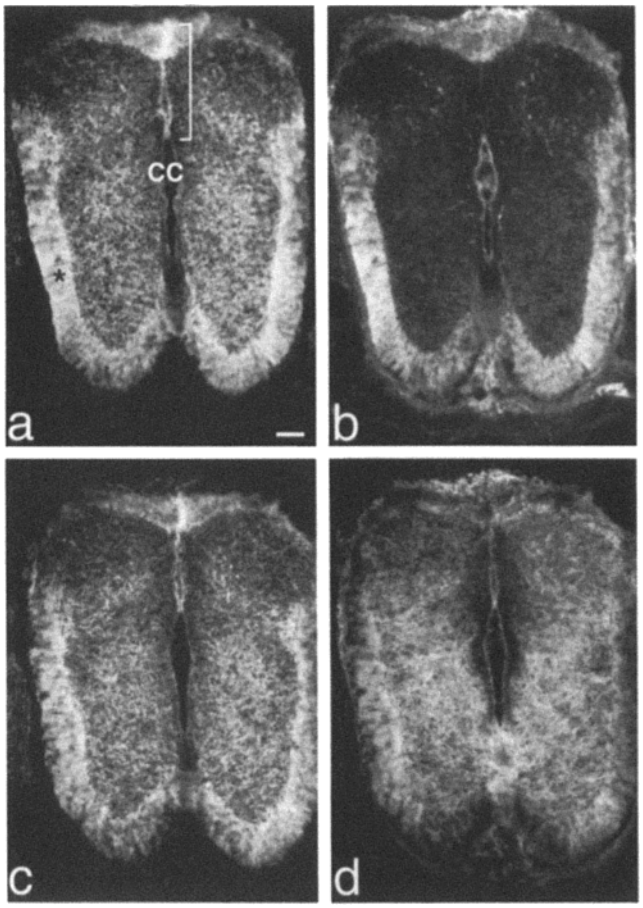


*Figure 7.* Double label immunofluorescence staining of cryostat sections of E16 rat spinal cord using polyclonal antibodies to phosphacan (*a* and *c*), and the 2C2 and 5B8 monoclonal antibodies to Ng-CAM/L1/NILE (*b*) and N-CAM (*d*), respectively. The extent of the roof plate at this stage of development is indicated by a vertical bracket; *cc*, central canal; *, lateral columns. Note that staining by monoclonal antibodies seen in connective tissue surrounding the spinal cord and outlining the central canal in *b* and *d* is nonspecific, since it was also seen using the rhodamine-labeled anti-mouse IgG alone in the absence of primary antibody. Bar, 100 μm.

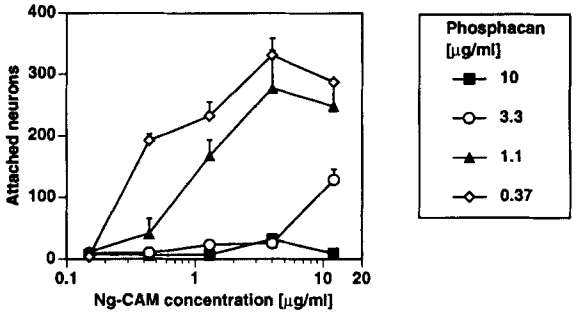


*Figure 8.* Inhibition by phosphacan of neuronal adhesion to Ng-CAM. Adhesion assays were performed using dishes that were incubated first with Ng-CAM, and then with phosphacan at 0.37 (◇), 1.1 (▲), 3.3 (○), and 10 (■) μg/ml. Dissociated brain cells from 9-d chick embryos were added to substrates, and the numbers of attached cells after an 80-min incubation period were determined as described in Materials and Methods. The number of cells that attached to the BSA-coated substrate was 10 ± 4. Data represent averages ($N = 2$) ± mean deviations. Immunofluorescent staining of the bound cells with antibodies to Ng-CAM indicated that >90% were neurons.

was observed at 30 μg/ml (Fig. 9, *C* and *D*), ~100-fold lower concentrations of phosphacan still had significant effects on neurite growth. This effect was primarily seen as a decrease in the proportion of cells with longer neurites (Fig. 9, *E* and *F*). Inhibition of neurite growth was found to be dependent on phosphacan concentration over a wide range (Fig. 10). At the highest concentration of phosphacan that was tested (30 μg/ml), the mean length of the neurites growing on Ng-CAM/phosphacan was less than one fifth the value observed in controls. This inhibition can be attributed primarily to the phosphacan core protein, insofar as it also was found to be a potent inhibitor at both high and low concentrations (Figs. 9 and 10).

Because we found that phosphacan can bind both to Ng-CAM (used here as a substrate) and to neurons, it was of interest to determine whether phosphacan had direct effects on neurons. For this purpose we used monoclonal antibodies against Ng-CAM as a substrate because they are potent promoters of neurite growth (Friedlander et al., 1994). Inhibitory effects of phosphacan were also found for neurite outgrowth on dishes coated with anti-Ng-CAM monoclonal antibodies (data not shown). Because phosphacan did not bind to these antibodies, one can conclude that phosphacan can interfere with Ng-CAM-mediated neurite growth by interacting directly with the cell surface.

### *Effects of Phosphacan on Adhesion of C6 Glioma Cells*

The studies described above relate to potential functions of extracellular phosphacan which is purified from PBS extracts of brain. However, analysis of mRNAs encoding phosphacan in rat brain indicate that two transmembrane forms of the protein also exist (Maurel et al., 1994), and all three messages are present in rat C6 glioma cells, with the short (6.4 kb) transmembrane form predominating (Flad, M., and R. K. Margolis, unpublished results). Because immunostaining of C6 glioma cells with polyclonal antibodies to phosphacan demonstrated cell surface immunoreactivity, these cells were used to analyze the effects of ligands for phosphacan.

When dishes were coated with different proteins, C6 glioma cells adhered and extended processes on Ng-CAM, fibronectin, and laminin, but not on BSA, suggesting that C6 cells express receptors for these adhesion proteins. Because Ng-CAM binds soluble phosphacan with high affinity, we determined whether phosphacan could inhibit adhesion of C6 cells to these and other adhesion molecules. In double-coating experiments, adhesion of C6 glioma cells to Ng-CAM was inhibited when phosphacan was used as the second protein (Fig. 11). In contrast, a second coating with phosphacan did not inhibit adhesion of C6 glioma cells to fibronectin or laminin (Fig. 11), neither of which bound phosphacan in physiological buffers (see Fig. 1). Although C6 cells express low levels of NILE/L1, similar results were obtained using human glioma cells (U251 MG) which do not express detectable levels of NILE/L1 (Sakurai, T., and M. Grumet, unpublished results). Quantitative analysis of the adhesion of C6 glioma cells with increasing concentrations of phosphacan indicated that adhesion to Ng-CAM was extremely sensitive to phosphacan whereas adhesion to laminin and fibronectin

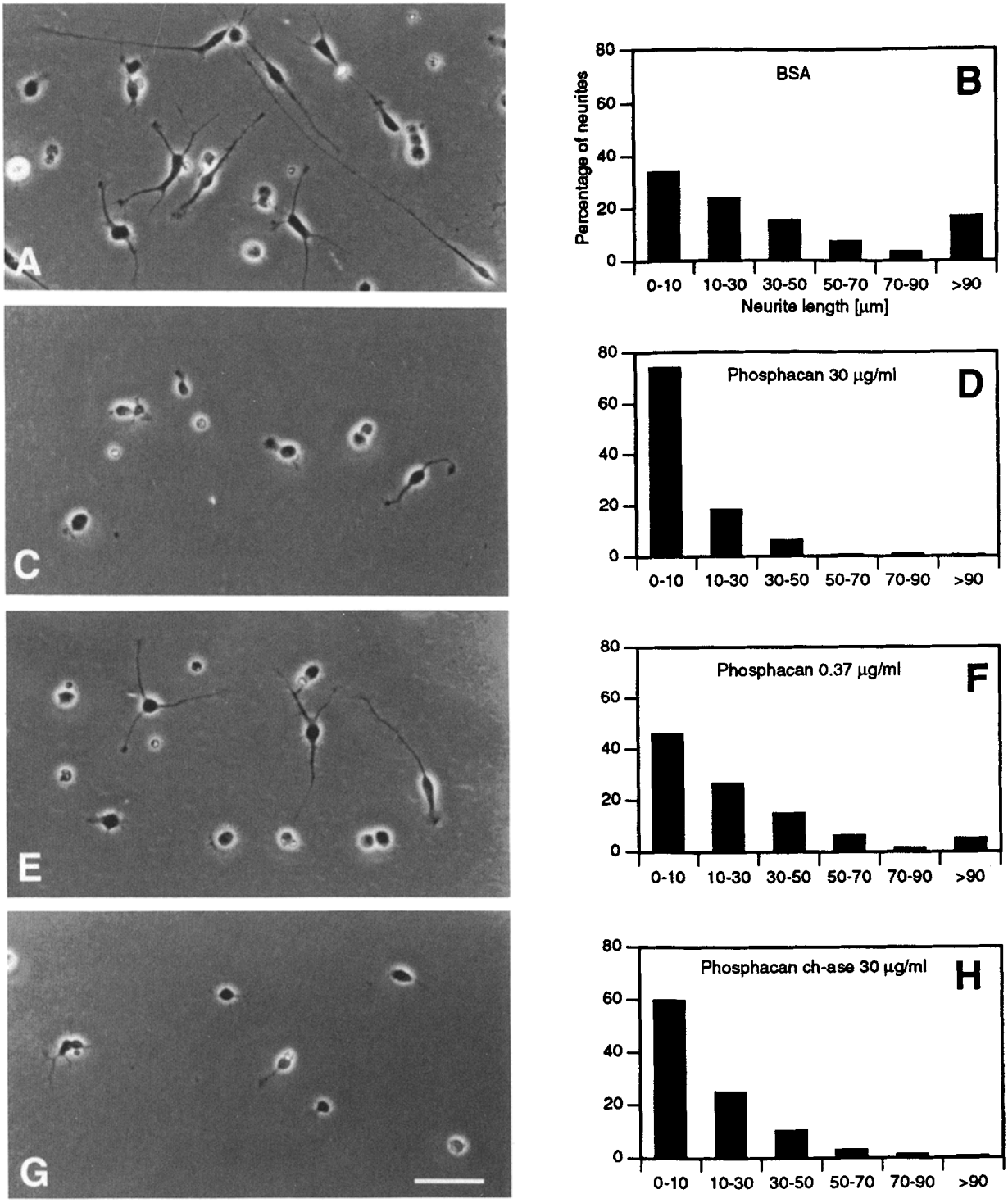


*Figure 9.* Inhibition by phosphacan of neurite growth on Ng-CAM. Substrates were prepared by incubation first with 30 μg/ml Ng-CAM, followed by incubation with BSA (*A* and *B*), phosphacan (*C–F*), or chondroitinase-treated phosphacan (*G* and *H*), as described in Materials and Methods. All proteins were used at 30 μg/ml except for the measurements shown in *E* and *F*, where the concentration was 0.37 μg/ml. Brain cells from 9-d chick embryos were added to the substrates, and the cells were fixed after 40 h in culture and photographed under phase microscopy. The histograms were obtained by counting the number of neurites with lengths falling within the indicated ranges. An average of 266 neurons were analyzed for each histogram. Bar, 50 μm.

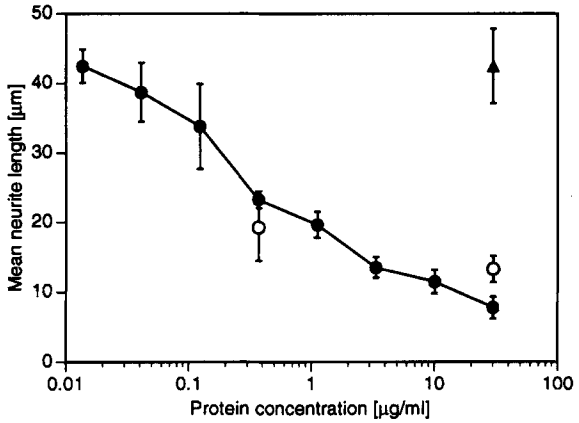


*Figure 10.* Inhibition of neurite outgrowth by phosphacan. Brain cells from 9-d chick embryos were plated on substrates coated sequentially with Ng-CAM and either BSA (▲), phosphacan (●), or chondroitinase-treated phosphacan (○), and neurite growth was analyzed as described in the legend to Fig. 9. Values represent averages of two experiments and error bars show the mean deviation.

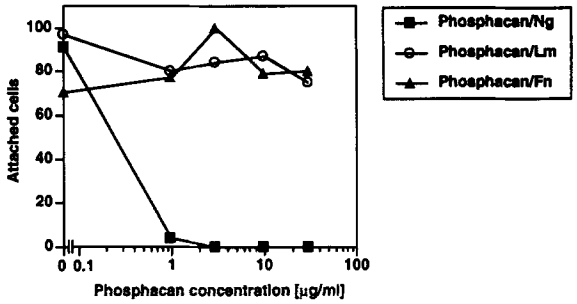


*Figure 12.* Inhibition by phosphacan of adhesion of C6 glioma cells to Ng-CAM. Substrates were prepared and adhesion of C6 glioma cells was performed as described in the legend to Fig. 11, except that phosphacan was tested over the indicated range of concentrations. Attached cells were counted under the microscope in one field at 200×, and the average of duplicate samples is shown. Phosphacan was applied as a second coat after Ng-CAM (■), laminin (○), and fibronectin (▲).

was unaffected even when phosphacan was used at much higher concentrations (Fig. 12). These results are consistent with the observation that phosphacan binds to Ng-CAM with high affinity (see Fig. 2), and suggest that soluble phosphacan can inhibit adhesion and interaction of C6 glioma cells with Ng-CAM.

## Discussion

Several biological activities of phosphacan were explored in our investigations, including binding to neural CAMs and neurons, and effects on cell adhesion and neurite outgrowth in culture. It is interesting that most of the effectiveness of phosphacan with respect to these properties appears to reside in the core glycoprotein, with a relatively minimal contribution of the chondroitin sulfate chains, and it has previ-

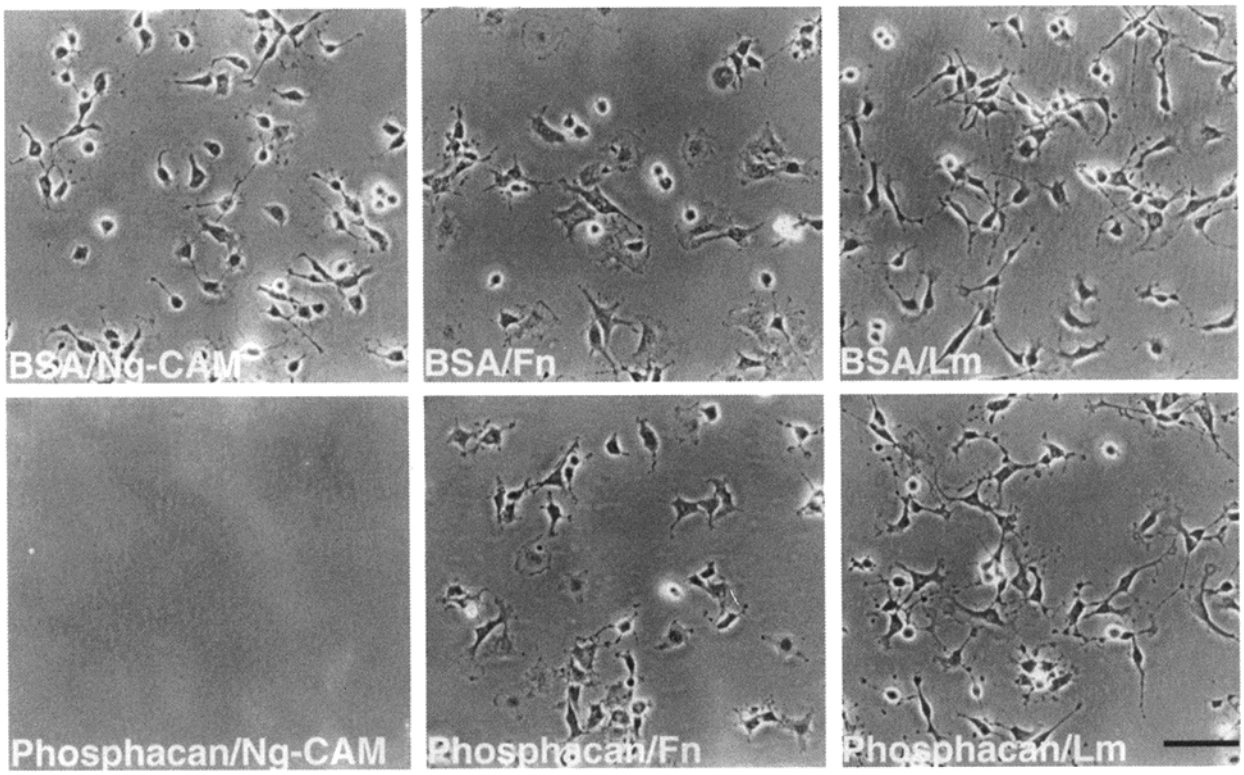


*Figure 11.* Adhesion of C6 glioma cells to Ng-CAM, fibronectin, and laminin, and inhibition by phosphacan. Substrates were coated as described in Materials and Methods first with 10 µg/ml of Ng-CAM, 50 µg/ml fibronectin, or 7.4 µg/ml laminin, and then with 30 µg/ml of BSA or phosphacan. After incubation with cells for 90 min at 37°C, unattached cells were removed by washing gently with PBS, and the remaining cells were fixed with 3.5% formalin and photographed. The concentrations of Ng-CAM, fibronectin, and laminin used for coating were based on control experiments indicating that they produced comparable levels of adhesion of C6 glioma cells.

ously been reported that a mixture of chondroitinase-treated chondroitin sulfate proteoglycans from brain inhibited neurite outgrowth from a mutant PC12 cell line (Oohira et al., 1991). These findings stand in contrast to the properties of neurocan, a structurally unrelated chondroitin sulfate proteoglycan of nervous tissue (Rauch et al., 1992) whose activity in similar assays was considerably more dependent on the presence of chondroitin sulfate (Friedlander et al., 1994). Although other recent studies (Brittis et al., 1992; Snow and Letourneau, 1992) have also indicated the importance of chondroitin sulfate in modulating axonal growth, the effectiveness of the core glycoproteins indicates that they contribute significantly to these and other cellular processes.

The fact that phosphacan represents the entire extracellular domain of a receptor-type protein tyrosine phosphatase (Maurel et al., 1994) suggests that some of the interactions we have observed may be equally applicable to the transmembrane protein, which is also synthesized in the form of a chondroitin sulfate proteoglycan (Barnea et al., 1994; Shitara et al., 1994). Using probes based on conserved sequences in their phosphatase domains, many transmembrane protein tyrosine phosphatases have now been cloned, but with the exception of homophilic interactions between phosphatases containing immunoglobulin-like domains (Gebbink et al., 1993; Brady-Kalnay et al., 1993; Sap et al., 1994), no cell surface ligands for these putative receptors have been reported up to now. Our findings indicate that the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM are high affinity ligands for one of these phosphatases. The binding of receptor-type phosphatases to neural CAMs could be affected by soluble phosphacan, which may modulate cell interactions and other developmental processes in nervous tissue by competition for ligands of the transmembrane phosphatase. These heterophilic ligands may also include other cell surface proteins, and extracellular matrix molecules such as tenascin (Grumet et al., 1994).

The use of nucleic acid and antibody probes to phosphacan and RPTP$\zeta/\beta$ has revealed expression in glial precursor cells and radial glia during development (Milev et al., 1993; Canoll et al., 1993; Engel, M., R. U. Margolis, and R. K. Margolis, unpublished results). Moreover, glioma cell lines (Krueger and Saito, 1992) and human glial tumors (Friedlander, D., and M. Grumet, unpublished results) also express significant levels of phosphacan. Being a secreted proteoglycan, phosphacan may inhibit or modulate cell adhesion and neurite growth by binding to receptors on neurons, including those on their growth cones, or to extracellular matrix proteins. Receptor forms of this molecule on glial cells may also be involved in neuron-glia interactions by serving as a mediator of transmembrane signaling. In any case, binding of Ng-CAM to this glial proteoglycan provides additional evidence that Ng-CAM mediates neuron-glia adhesion and interactions (Grumet, 1992).

In the present study we have used two types of assays to analyze effects of phosphacan on cells in culture. In a short term ($\sim$1 min) centrifugation assay, neurons bound to phosphacan, whereas in longer term (>1 h) gravity adhesion and neurite growth assays, phosphacan inhibited interactions of cells on substrates coated with Ng-CAM. Attachment of cells in the longer term assays involves interactions of the coated proteins with the cell surface to induce cellular responses such as cell spreading. In contrast, the short term centrifugation assay does not require cellular responses for adhesion, and it probably provides only a measure of the initial interaction between the cell membrane and the coated protein. Thus, the lack of cell adhesion and neurite growth in the presence of phosphacan in the gravity assay is likely to be due to inability of the cells to establish stable contacts after initial binding to the substrate.

In considering potential functions of phosphacan in vivo, it is important to note that it is present in certain regions where neural CAMs are prevalent such as in the developing columns of the spinal cord, and that it is also found in other regions such as the roof plate where Ng-CAM/L1 is not present or is at very low levels. Because neurons and their processes do not cross certain boundaries such as the roof plate (Snow et al., 1990), it is possible that phosphacan may contribute to this repulsion as a consequence of its inhibitory effects on neuronal adhesion and neurite growth. On the other hand, in regions containing both neural CAMs and phosphacan, it is likely that phosphacan acts to modulate local CAM-mediated adhesion, and the sequence of expression of the proteins as well as their relative concentrations will influence the migration of cells and processes. In this regard, it is noteworthy that both phosphacan and its core glycoprotein had effects on neurite growth even when tested at relatively low concentrations (Fig. 10). Whereas the secreted form of phosphacan has inhibitory effects on both neurons and glia, membrane-anchored forms appear to be restricted to astroglial cells and therefore may function as cellular receptors for neural CAMs that modulate glial behavior.

The properties of a mouse brain chondroitin sulfate proteoglycan, which is expressed by astrocytes and has been named astrochondrin, have recently been described (Streit et al., 1993). Astrochondrin consists of three proteoglycans with HNK-1 positive core glycoproteins having apparent molecular sizes of 380, 360, and 260 kD on SDS-PAGE. It binds to laminin and collagen type IV but not to N-CAM, L1, or tenascin, and antibodies to astrochondrin reduced granule cell migration and astrocytic process formation on certain substrates. The primary structure of astrochondrin has not yet been described, and the relationship between the three core glycoproteins remains unclear although similarities in their proteolytic peptides have been interpreted as indicating that the three core proteins are closely related. In view of its different binding properties it appears unlikely that astrochondrin is related to phosphacan.

The presence in nervous tissue of a significant portion of phosphacan molecules that contain sulfated poly(*N*-acetyllactosaminyl) oligosaccharides raises the question of the biological significance of these keratan sulfate chains, whose biosynthesis and fine structure are developmentally regulated in brain (Rauch et al., 1991). One possibility is that they may modulate the binding of phosphacan and the membrane tyrosine phosphatase to particular ligands. Although in our assays phosphacan, phosphacan-KS, and their core glycoproteins all had generally similar abilities to bind to neurons, neural cell adhesion molecules, and to tenascin (Grumet et al., 1994), the detection of functional differences, possibly involving additional ligands, may require other types of assays. It is especially interesting in this connection that another receptor-type protein tyrosine phosphatase, the leukocyte common antigen CD45, also contains in its extracellular domain both *N*- and *O*-glycosidically linked

poly(*N*-acetyllactosaminyl) oligosaccharides (Childs et al., 1983; Sato et al., 1993). In the CD45 system, the isoforms generated by alternative splicing within the extracellular region are each expressed on lymphocytes at distinct differentiation stages and on T cells having distinct functions (Kincaide, 1987; Gillitzer and Pilarski, 1990; Bottomly, 1988; Powrie and Mason, 1988). Although the significance of CD45 isoform switching in cellular function is not yet clear, it has been shown that poly(*N*-acetyllactosaminyl) oligosaccharides on CD45 participate in natural killer cell-mediated cytotoxicity against susceptible YAC-1 lymphoma targets (Gilbert et al., 1988). The involvement of poly(*N*-acetyllactosaminyl) oligosaccharides in leukocyte binding suggests that the developmental regulation of their sulfated forms, which we have previously demonstrated in phosphacan (Rauch et al., 1991), may serve a similar function by modulating its binding to various ligands at different stages of nervous tissue histogenesis, and may also affect interactions and the activity of the related transmembrane tyrosine phosphatase.

Our finding that two chondroitin sulfate proteoglycans of brain (phosphacan and neurocan) with no obvious primary structural similarity both bind with high affinity to tenascin (Grumet et al., 1994) and to neural cell adhesion molecules (Ng-CAM and N-CAM) having only a limited amount (~25%) of amino acid sequence identity, but not to a large number of other cell surface and extracellular matrix proteins, raises interesting questions concerning the biochemical basis and functional role of these interactions. Since the structures and properties of the different oligosaccharides present on both the proteoglycans and CAMs remain to be determined, it is possible that the binding involves interactions with oligosaccharides which would not be evident from a comparison of amino acid sequences. Whether this is in fact the case should become clearer from studies of both non-glycosylated and glycosylated domains of these proteins, whose expression in bacterial and eukaryotic systems is currently in progress. However, since both the proteoglycans and CAMs are large multidomain proteins, it would also not be surprising if different amino acid sequences could serve to mediate heterophilic binding to different proteins. The multiple interactions of chondroitin sulfate proteoglycans and neural CAMs suggests a degree of biological redundancy, such as has been indicated by recent gene knockout studies of tenascin, N-CAM, and other proteins (Erickson, 1993; Tomasiewicz et al., 1993; Cremer et al., 1994). Because it is likely that individual proteoglycans interact with a broad range of cell surface and extracellular matrix molecules to mediate specific developmental processes at particular anatomical sites and stages of nervous tissue histogenesis, it will be important to determine whether additional proteins bind to phosphacan and whether they colocalize with it during development.

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