# The Interaction of the Retina Cell Surface N-Acetylgalactosaminylphosphotransferase with an Endogenous Proteoglycan Ligand Results in Inhibition of Cadherin-mediated Adhesion

Janne Balsamo,* Heidemarie Ernst,‡ Mary K. B. Zanin,‡ Stanley Hoffman,‡ and Jack Lilien*

*Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202; and ‡Department of Medicine, Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425-2229

*Abstract.* We have previously shown that the binding to cells of a monoclonal antibody directed against the chick neural retina N-acetylgalactosaminylphosphotransferase (GalNAcPTase) results in inhibition of cadherin-mediated adhesion and neurite outgrowth. We hypothesized that the antibody mimics the action of an endogenous ligand. Chondroitin sulfate proteoglycans (CSPGs) are potential ligands because they inhibit adhesion and neurite outgrowth and are present in situ at barriers to neuronal growth. We therefore assayed purified CSPGs for their ability to inhibit homophilic cadherin-mediated adhesion and neurite outgrowth, as well as their ability to bind directly to the GalNAcPTase. A proteoglycan with a 250-kD core protein following removal of chondroitin sulfate chains (250-kD PG) inhibits cadherin-mediated adhesion and neurite outgrowth whether presented as the core protein or as a proteoglycan monomer bearing chondroitin sulfate. A proteoglycan with a 400-kD core protein is not inhibitory in either core protein or monomer form. Treatment of cells with phosphatidylinositol-specific phospholipase C, which removes cell surface GalNAcPTase, abolishes this inhibitory effect. Binding of the 250-kD core protein to cells is competed by the

anti-GalNAcPTase antibody 1B11, suggesting that 1B11 and the 250-kD core protein bind to the same site or in close proximity. Moreover, soluble GalNAcPTase binds to the immobilized 250-kD core protein but not to the immobilized 400-kD core protein. Concomitant with inhibition of cadherin mediated adhesion, binding of the 250-kD core protein to the GalNAcPTase on cells results in the enhanced tyrosine phosphorylation of β-catenin and the uncoupling of N-cadherin from its association with the cytoskeleton. Moreover, the 250-kD PG is present in embryonic chick retina and brain and is associated with the GalNAcPTase in situ. We conclude that the 250-kD PG is an endogenous ligand for the GalNAcPTase. Binding of the 250-kD PG to the GalNAcPTase initiates a signal cascade, involving the tyrosine phosphorylation of β-catenin, which alters the association of cadherin with the actin-containing cytoskeleton and thereby inhibits adhesion and neurite outgrowth. Regulation of the temporal and spatial expression patterns of each member of the GalNacPTase/250-kD PG interactive pair may create opportunities for interaction that influence the course of development through effects on cadherin-based morphogenetic processes.

T HE complex patterns of cell movements during morphogenesis are presumably guided by, and reflected in, the temporal and spatial patterns of expression of adhesion molecules. However, adhesion molecule function may also be regulated to create temporal and spatial patterns guiding morphogenetic rearrangements. One potential regulator of adhesion molecule function is a cell surface N-acetylgalactosaminylphosphotransferase (Gal-

NAcPTase).[1] Binding of certain monoclonal antibodies to the cell surface GalNAcPTase indirectly inhibits N- and E-cadherin mediated adhesion (Balsamo et al., 1991; Bauer et al., 1992). Furthermore, this perturbation results in the inhibition of neurite outgrowth in vitro, dependent on each of three distinct adhesive mechanisms; cadherin, β1 integrin, or G4 (the chicken homologue of L1, a member of the immunoglobulin/N-CAM family) (Gaya-

Address all correspondence to Jack Lilien, Dept. of Biological Sciences, Wayne State University, 5047 Gullen Mall, Detroit, MI 48202. Tel.: (313) 577-2876. Fax: (313) 577-6891.

1. *Abbreviations used in this paper.* GalNAcPTase, N-acetylgalactosaminylphosphotransferase; ECL, enhanced chemiluminescence; GPI, glycophosphatidylinositol; NCD-2, anti–N-cadherin antibody; PI-PLC, phosphoinositol-specific phospholipase C.

Gonzalez et al., 1991). Thus, the binding of ligands to the GalNAcPTase appears to initiate a signal transduction pathway that ultimately has a global effect on many adhesion systems.

This effect appears to be mediated by a change in the association of adhesion molecules with the cytoskeleton. The cytoplasmic domain of cadherins is associated with the actin-containing network through its interaction with the catenins, an association essential to cadherin function (see Magee and Buxton, 1991; Takeichi et al., 1992; Tsukita et al., 1992; Kemler, 1993; Gumbiner, 1993 for reviews). Binding of adhesion-inhibitory anti-GalNAcPTase antibodies to cells results in disruption of this essential linkage between cadherin and the cytoskeleton (Balsamo et al., 1991).

Our working hypothesis has been that the anti-adhesive effect of anti-GalNAcPTase antibodies mimics the activity of an endogenous ligand for the GalNAcPTase and that the interaction of the GalNAcPTase/ligand pair in situ would have the potential to control morphogenetic processes. Because several proteoglycans inhibit adhesion in vitro, proteoglycans are candidate ligands for the GalNAcPTase. Both keratan sulfate and chondroitin sulfate proteoglycans have been reported to be inhibitors of neuronal outgrowth in vitro (Cole and McCabe, 1991; Oohira et al., 1991; Guo et al., 1993) and to be present at barriers to neuronal outgrowth in situ (Oakley and Tosney, 1991; Pinzola et al., 1993; Geisert and Bidanset, 1994; see also Schwab et al., 1993; Margolis and Margolis, 1993 for reviews). Furthermore, it has been suggested that proteoglycans act as "anti-adhesion" molecules by binding to cell surface receptors and thereby initiating transmembrane signals that alter the structure of the cytoskeleton, resulting in the inhibition of cell spreading and migration in vitro (Hoffman et al., 1994).

It is controversial whether the anti-adhesive effects of proteoglycans are due to nonspecific interference with cell apposition, resulting in lack of appropriate contact between adhesion molecules on adjacent cells (Morris, 1993; Vleminckx et al., 1994), or result from the specific interactions of proteoglycans with cell surface receptors. Recently, chondroitin sulfate proteolycans have been observed to bind to cell surface receptors and to inhibit adhesion by two distinct mechanisms: (a) Homophilic adhesion mediated by N-CAM or Ng-CAM is inhibited by the direct binding of neurocan (a rat brain-derived proteoglycan) to N-CAM or Ng-CAM (Grumet et al., 1993). (b) Cell-extracellular matrix adhesion mediated by integrins is inhibited by an indirect mechanism involving the binding of chicken brain proteoglycans with 400- and 250-kD core proteins to cell surface receptors other than the integrin subunits themselves (H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, manuscript submitted for publication).

The ability of the 400- and 250-kD proteoglycans of H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, (manuscript submitted for publication) to initiate a signal which affects adhesion involving other ligands is similar to the effects produced by select anti-GalNacPTase antibodies following binding to the GalNAcPTase (Balsamo et al., 1991). Therefore, we tested whether these proteoglycans might be endogenous ligands for the GalNAcPTase. We found that the proteoglycan with the 250-kD core protein (250-kD PG) binds directly to the GalNAcPTase at the cell surface, resulting in inhibition of homophilic cadherin-mediated adhesion, as well as neurite outgrowth. In contrast, the proteoglycan with the 400-kD core protein (400-kD PG) has little or no effect. We further show that incubating cells with the 250-kD PG, but not the 400-kD PG, alters the tyrosine phosphorylation state of β-catenin and the association of cadherin with actin, providing a reasonable mechanism for the inhibition of cadherin-mediated adhesion by the GalNAcPTase/250-kD PG interactive pair. This interactive pair may play an important role in modulating morphogenetic movements by regulating the function of cadherins and potentially other adhesion molecules.

## Materials and Methods

### Antibodies

Anti-GalNAcPTase antibodies 1B11 and 7A2 are mouse monoclonal IgMs prepared as described (Scott et al., 1990; Balsamo et al., 1991). Cells producing anti–N-cadherin antibody NCD-2 (a gift from M. Takeichi) were cultured in our laboratory and the antibody prepared as described (Balsamo et al., 1991). The anti–β-catenin antibody was prepared in rabbits from the following synthetic 15–amino acid peptide deduced from the published cDNA sequence (Butz et al., 1992): CPGDSNQLAWFDTDL. The anti-phosphotyrosine antibody clone PY20 was from Transduction Laboratories (Lexington, KY). Anti-actin antibody was a monoclonal IgM purchased from Amersham Corp. (Arlington Heights, IL). As controls, commercial mouse IgM or rabbit IgG were purchased from Sigma Immunochemicals (St. Louis, MO).

To prepare anti-proteoglycan antibodies, total chicken brain proteoglycan monomers were isolated as described below and were used to immunize rabbits. This avoids making antibodies against the highly antigenic and cross-reactive stubs of chondroitin sulfate that remain on chondroitin ABC lyase-treated core proteins. Antibodies specific for the 250 and 400-kD core proteins were then affinity purified. Core proteins were covalently linked to CNBr-activated Sepharose 4B (Pharmacia LKB Nuclear, Gaithersburg, MD) following the manufacturer's instructions using 50 μg of protein per ml of beads. 10 mg of anti-proteoglycan IgG (isolated from serum as described by Brackenbury et al., 1977) was dissolved in PBS and run over a column containing 1 ml of immobilized 250-kD core protein. After extensive washing with PBS, bound IgG was eluted with 50 mM diethylamine. The diethylamine eluate was neutralized with 1/10 vol of 1 M sodium phosphate (pH 6.3), then run over a column containing 1 ml of immobilized 400-kD core protein. Both the unbound fraction and the bound and eluted fraction from this second column were saved and characterized in terms of their reactivity on Western transfers of embryonic brain extracts. As expected, IgG retained on the immobilized 250-kD core protein, but not retained on the immobilized 400-kD core protein, recognized the 250-kD core protein but not the 400-kD core protein and is referred to as anti-250. IgG bound and eluted from the 250-kD core protein, then bound and eluted from the 400-kD core protein recognized the 400-kD core protein, but not the 250-kD core protein, and is therefore referred to as anti-400. The fact that the anti-400 antibody was retained by the immobilized 250-kD core protein appears to result from the presence of partially degraded 400-kD core proteins in the 250-kD core protein preparation. This is supported by data from NH₂-terminal sequence analyses of CNBr fragments derived from the 250-kD core protein preparation (see Discussion). The patterns of reactivity of the anti-250 and anti-400 antibodies on brain and retina extracts are shown in Fig. 8.

### Preparation of Proteoglycan Fractions

Proteoglycan fractions were prepared as recently described (H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, manuscript submitted for publication). Briefly, 14-d-old embryonic chicken brains were homogenized and the supernatant was fractionated on CsCl density gradients under nondenaturing conditions. The densest material from the CsCl gradients, when fractionated by HPLC gel filtration under nondenaturing

conditions, yielded a higher molecular weight fraction containing proteoglycan aggregates and a lower molecular weight fraction containing proteoglycan monomers. In addition, each of these fractions contains the glycoprotein restriction. We refer to the lower molecular weight fraction as native monomers because it was never exposed to hyaluronidase. Approximately 75% of the proteoglycan monomers with core proteins of 400 and 250 kD are isolated in the native monomer fraction, while only 25% are isolated in the aggregate fraction (Ernst et al., manuscript submitted for publication), suggesting that proteoglycans are present both in aggregates and as monomers in vivo. To purify the major proteoglycan monomers and core proteins individually, the dense fraction from the CsCl gradients was digested with *Streptomyces* hyaluronidase. Restrictin was separated from the proteoglycan monomers by rate-zonal sedimentation on sucrose gradients. Proteoglycan monomers containing 400-kD core proteins and proteoglycan monomers containing 250-kD core proteins were then separated by HPLC gel filtration in the presence of 4 M guanidine-HCl. To isolate core proteins, the proteoglycan monomers from the sucrose gradient were treated with chondroitin ABC lyase, and the 400- and 250-kD core proteins resolved by HPLC gel filtration in the presence of 4 M guanidine-HCl. These preparations are referred to as 400-kD monomer, 250-kD monomer, 400-kD core protein, and 250-kD core protein. When describing the form of one of these proteoglycan preparations present in vivo or when referring to both the monomer and core protein of a proteoglycan, these proteoglycans will be referred to as 400-kD PG and 250-kD PG.

## Preparation of Single Cells and Adhesion Assays

For adhesion assays, E8 neural retina tissue was incubated overnight at 37°C in leucine-free F12 medium (GIBCO BRL, Gaithersburg, MD) containing 10 μCi/ml of [$^3$H]Leucine (53 Ci/mM; DuPont NEN, Boston MA). Single cells were prepared by trypsinization of tissues in the presence of 1 mM Ca$^{2+}$ (Grunwald et al., 1980; Brackenbury et al., 1981; Magnani et al., 1981). The radiolabeled cells were resuspended in HBSGKCa (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM glucose, 3 mM KCl, and 1 mM CaCl$_2$) at a concentration of 1× 10$^6$/ml. Cells were incubated with the appropriate additives in HBSGKCa in a total volume of 200 μl for 15 min at 4°C; 50-μl aliquots were then added to each well of a 96-well plate (previously coated with purified N-cadherin [~0.1 μg/well] or anti–N-cadherin antibody NCD-2 [5 μg/well]) and incubated for 30 min at 37°C (Balsamo et al., 1991). The wells were washed 4× with HBSGKCa containing 1 mg/ml BSA (HBSGKCaBSA), the attached cells solubilized in 0.1 N NaOH, and radioactivity determined by liquid scintillation counting. To determine the effect on adhesion of treating cells with phospholipase $^3$H-labeled cells were incubated with 5 u/ml of phosphoinositol-specific phospholipase C (PI-PLC) for 30 min at 37°C before assay, as described previously (Balsamo and Lilien, 1993).

## Assay for Neurite Outgrowth

These assays were performed essentially as described (Gaya-Gonzalez et al., 1991). N-cadherin was prepared from E10 chick neural retinas by affinity purification on anti–N-cadherin antibody (NCD-2) conjugated to CNBr-Sepharose as described by Bixby and Zhang (1990). Single cells prepared by trypsinization of E7 neural retinas were plated in each well of 24-well plates (previously coated with affinity-purified N-cadherin [100 μl of a solution of 1 μg/ml]) and allowed to adhere for a period of 2 h. Aliquots of the appropriate additives were then added and the cells were cultured for another 20 h. After this time, the cells were observed using an Olympus inverted microscope. Cells bearing extensions shorter than one cell diameter were considered negative for neurite extension. 50–70 cells were counted for each treatment in three different experiments. To test for the effect of treatment with phospholipase, cells were treated with PI-PLC as described above before plating onto the N-cadherin–coated wells.

## Binding of Radiolabeled Proteoglycan Core Proteins to Cells

$^{125}$I-labeled proteoglycan core proteins were prepared using the chloramine T method according to Friedlander et al. (1988). For binding assays, single cells were resuspended in HBSGKCaBSA and aliquots containing 1 × 10$^5$ cells were plated into individual wells of 96-well plates precoated with poly-L-lysine (100 μl of a solution of 50 μg/ml). The wells were then washed with poly-L-glutamic acid to block nonspecific binding to the poly-L-lysine substrate (blocking of nonspecific binding was also reduced by the

presence of BSA in the cell suspension during formation of the cell layer and during incubation with iodinated proteoglycan). The wells were incubated with increasing concentrations of radiolabeled 250- or 400-kD core proteins in a total volume of 100 μl of HBSGKCaBSA for 30 min at 4°C. Unbound material was removed and the wells were washed 4× with 200 μl of HBSGKCaBSA. The cell layers were solubilized in 0.1 N NaOH and bound radioactivity was determined by liquid scintillation counting.

## Binding of the GalNAcPTase to Immobilized Proteoglycan Core

Immobilized core proteins were prepared as described above for the affinity purification of antibodies. GalNAcPTase and other GPI-anchored cell surface proteins were released from crude cell membranes from 20 E10 retinas using 10 U/ml PI-PLC as previously described (Balsamo and Lilien, 1993). Following centrifugation at 150,000 g the supernatant (200 μl in 20 mM Hepes [pH 7.4], 0.15 M NaCl, 1 mM PMSF, 1.5 μg antipain, 25 μg DNAase) was incubated with sulfo-NHS-Biotin (1 mg/ml; Pierce Chem. Co., Rockford, IL) for 30 min at room temperature. Unreacted and inactivated biotin was removed by microconcentration (Centricon 10; Millipore Corp., Bedford, MA). 250-μl aliquots containing the material released from 10 retinas were incubated with 50 μl of packed beads for 1 h at 4°C. Unbound material was collected and concentrated for analysis by SDS-PAGE. The beads were washed 3× with 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 0.1% Triton X-100 and 0.1% BSA, and bound material eluted with SDS sample buffer (0.125 M Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, and 10% glycerol). The total bound and unbound material were fractionated by SDS-PAGE and transferred to Immobilon. To detect the GalNAcPTase, the transfers were reacted with anti-GalNAcPTase antibody followed by HRP-conjugated goat anti–mouse IgM and developed using the enhanced chemiluminescence (ECL) substrate (Amersham). Biotinylated species in the bound and unbound fractions were detected with alkaline phosphatase conjugated avidin.

GalNAcPTase bound to beads was also measured by assaying GalNAcPTase activity. Material released from 20 E10 retinas by PI-PLC treatment was incubated with 100 μl of packed beads bearing either the 250- or the 400-kD core proteins. Bound GalNAcPTase was assayed by measuring the incorporation of [$^3$H]-GalNAc from UDP[$^3$H]-GalNAc into asialo-agalactofetuin as described previously (Balsamo and Lilien, 1982).

## Effect of the 250-kD Core Protein on the Association of N-cadherin with Actin

The effect of the 250- or 400-kD core proteins on the association between N-cadherin and actin was determined as previously described (Balsamo et al., 1991). 1 × 10$^8$ single cells were incubated in 2 ml of HBSGKCa containing 10 μg/ml of the 250- or 400-kD core proteins for 30 min, at 37°C and 120 rpm. The cells were pelleted, washed in HBSGKCa and homogenized in homogenization buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM PMSF, 5 μg/ml each of antipain, leupeptin, and chymostatin, 1 mM NaF, 4 mM o-vanadate, 1 mM EDTA, and 100 μg/ml DNAase). After 30 min on ice, the homogenates were centrifuged at 14,000 g and the supernatants layered onto 1 ml of 20% sucrose on top of 100 μl of 50% sucrose each made in 1% Triton X-100, 50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, and centrifuged at 150,000 g for 1 h. The 50% layer and the 20/50% interface were collected (referred to as the rapidly sedimenting fraction; see Balsamo et al., 1986), diluted with an equal volume of homogenization buffer, and incubated with anti-actin antibody for 4 h at 4°C with gentle rocking. The samples were then incubated with goat anti–mouse IgG conjugated to magnetic beads (Advanced Magnetics Inc., Cambridge, MA) for another hour, the beads collected using magnetic stands, washed 3× in homogenization buffer containing 0.1% SDS and 1% BSA, then 3× in homogenization buffer and once in H$_2$O. The immunoprecipitated material was eluted in SDS sample buffer, fractionated by SDS-PAGE, transferred to Immobilon, reacted sequentially with anti–N-cadherin monoclonal antibody NCD-2 and HRP-conjugated goat anti–rat antibody, and detected using the ECL procedure.

## Effect of GalNAcPTase Ligands on β-catenin Phosphorylation

To examine the effects of core proteins or anti-GalNAcPTase mAbs on the phosphorylation of β-catenin, single cells (~1 × 10$^8$ cells in 2 ml) were incubated in HBSGKCa for 30 min at 37°C and 120 rpm in the presence of

the 250- or 400-kD core proteins (15 μg/ml) or mAbs 1B11 or 7A2 (15 μg/ml). The cells were collected and homogenized in homogenization buffer and the rapidly sedimenting fraction isolated. This fraction was made 1% in SDS, boiled to dissociate the complex, diluted to 0.1% SDS with homogenization buffer, and incubated with anti–β-catenin antibody for 4 h at 4°C. The samples were then incubated with goat anti–rabbit antibody conjugated to magnetic beads and the immunoprecipitates collected as described above, fractionated by SDS-PAGE, transferred to Immobilon, reacted sequentially with anti-phosphotyrosine antibody and HRP-conjugated goat anti–mouse IgG, and developed using the ECL system. The same transfers were stripped and probed with anti–β-catenin antibodies and developed with alkaline-phosphatase conjugated goat anti–rabbit IgG.

## Preparation of Tissue Extracts for Western Blots with Anti-250 and Anti-400

E9 brain or retina tissue was homogenized in 10 vol of 0.1 M Tris-HCl (pH 8.0) containing protease inhibitors (10 mM N-ethylmaleimide, 5 mM benzamidine, 50 μg/ml leupeptin, 1 μg/ml aprotinin, 5 μg/ml pepstatin A, and 2 mM PMSF and the homogenates centrifuged at 14,000 rpm for 5 min. A portion of the resulting supernatant was then treated with chondroitin ABC lyase (0.5 u/ml) for 30 min at 37°C. The protein concentration in the extracts was determined using the BCA assay (Pierce). 25-μg aliquots of each sample were fractionated by SDS-PAGE, transferred to nitrocellulose, reacted sequentially with anti-250 or anti-400 and HRP-conjugated goat anti–rabbit antibodies, and detected using the ECL substrate.

## Coprecipitation of the GalNAcPTase, the 250-kD Core Protein and N-cadherin from Tissue

The rapidly sedimenting fraction isolated from 20 retinas (1 ml), prepared as described above, was treated with 0.5 U chondroitin ABC lyase (Seikagaku Corp., Tokyo, Japan) for 30 min at 37°C. Equal volume aliquots were incubated with anti-GalNAcPTase mAb 1B11, anti-250, or control nonimmune mouse IgM or rabbit IgG for 4 h at 4°C. Immunoprecipitates were collected using the appropriate second antibodies as described above and washed 3× in 10 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 1% Triton X-100, and 1% BSA, then 3× in the same buffer but containing only 0.1% Triton X-100 and 0.1% BSA. The antigen was eluted from the magnetic beads by boiling in SDS sample buffer, fractionated by SDS-PAGE, transferred to Immobilon (Millipore), reacted sequentially with the antibodies indicated in Fig. 9 and appropriate HRP-conjugated second antibodies, and detected using the ECL procedure.

## Results

In these studies we examine the ability of proteoglycan aggregates, monomers, and core proteins to inhibit cadherin-mediated adhesion and neurite outgrowth of embryonic chick neural retina cells when added to the culture medium. The purification scheme and the composition of the proteoglycan fractions tested are as recently described (H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, manuscript submitted for publication). These methods and the nomenclature used in this paper are summarized in the Materials and Methods.

We have used both purified N-cadherin and anti–N-cadherin mAb NCD-2 as the substrate for cell adhesion and neurite outgrowth; they are both highly active in their ability to mediate these processes (Balsamo et al., 1991; Gaya-Gonzales et al., 1991). All of the assays reported here have been performed using both substrates with identical results.

### Inhibition of N-cadherin-mediated Adhesion by Proteoglycans

We first compared the ability of proteoglycan aggregates and native monomers to affect cadherin-mediated adhe-

sion. Native monomers inhibit cadherin mediated adhesion to either purified N-cadherin or to the anti–N-cadherin antibody NCD-2 (Fig. 1 A). Furthermore, inhibition of cadherin mediated adhesion by the native proteoglycan
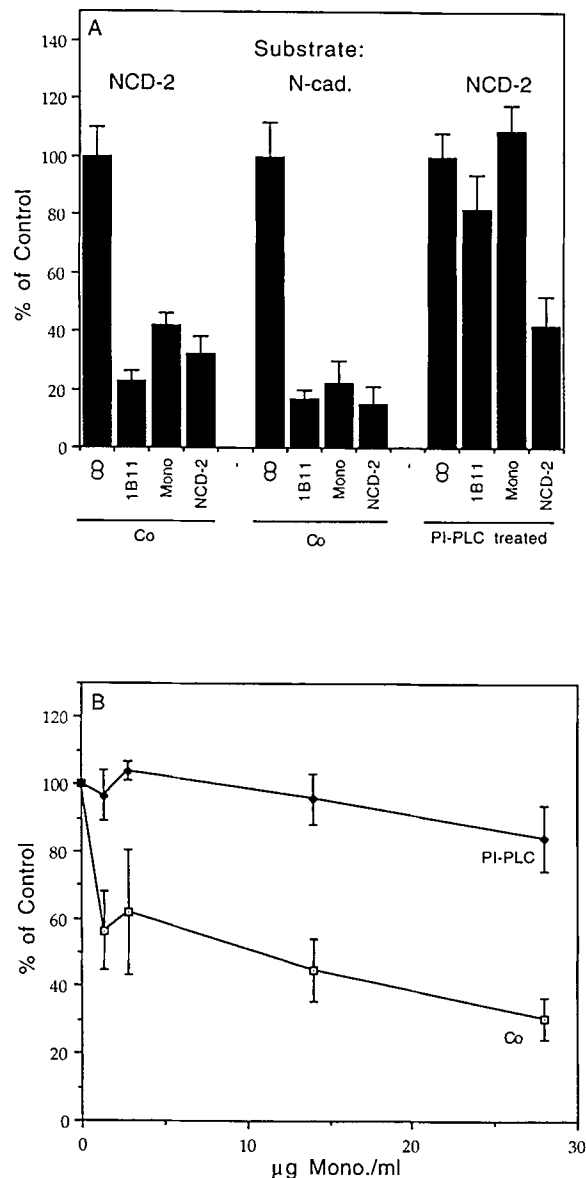




Figure 1. Inhibition of N-cadherin–mediated adhesion by native chick brain proteoglycan monomers. The adhesion of ³H-labeled cells to purified N-cadherin or anti–N-cadherin antibody-coated 96-well plates was evaluated in the presence of the indicated additives in the culture fluid. Adhesion is represented as the percent of the adhesion obtained with no additives. Up to 70% of the added cells attach under control conditions. The data are representative of several experiments, each point is the average of three measurements. Error bars indicate the standard error of the mean. (A) Adhesion before or after treatment of cells with PI-PLC. CO, control, no additives; 1B11, anti-GalNAcPTase mAb 1B11 (10 μg/ml); Mono, native proteoglycan monomer mixture (15 μg/ml); NCD-2, anti–N-cadherin mAb (10 μg/ml). (B) Adhesion to anti–N-cadherin antibody in the presence of increasing concentrations of the native proteoglycan monomer mixture. □, control cells (Co); ◆, cells treated with PI-PLC (PL).
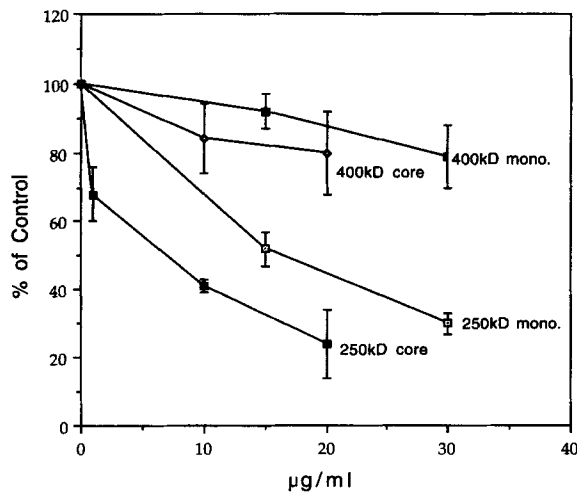
*Figure 2.* Effect of purified proteoglycan monomers and core proteins on N-cadherin–mediated adhesion. The adhesion of ³H-labeled cells to anti–N-cadherin–coated 96-well plates was evaluated in the presence of increasing concentrations of the indicated purified chick brain proteoglycan monomers (*250 kD mono.* and *400 kD mono.*) or core proteins (*250 kD core* and *400 kD core*) in the culture fluid. Adhesion is represented as the percent of the adhesion obtained in wells with no additives. Each point represents the average of three measurements. Error bars indicate the standard error of the mean.

monomers is dose dependent (Fig. 1 *B*). In contrast, the hyaluronic acid-containing aggregates are inactive, suggesting that the moieties responsible for triggering the inhibition of cadherin-mediated adhesion are masked in the aggregate (not shown). Because PI-PLC removes the GalNAcPTase from the cell surface and abolishes the ability of anti-GalNAcPTase mAb 1B11 to inhibit cadherin-me-

diated adhesion (Balsamo and Lilien, 1993), we assayed the effect of native monomers on cadherin-mediated adhesion using PI–PLC–treated cells. With these cells the native monomers no longer inhibit cadherin-mediated adhesion; however, anti–N-cadherin mAb NCD-2 is still inhibitory (Fig. 1 *A*). These data suggest that a component of the native monomer preparation interacts with a cell surface polypeptide containing a glycophosphatidylinositol (GPI) tail, possibly the GalNAcPTase.

To determine which component of the native monomer preparation is active in inhibiting cadherin-mediated adhesion, we evaluated the activity of the 400- and 250-kD monomer and core protein preparations. Only the 250-kD monomer and core protein preparations show significant inhibition of N-cadherin–mediated adhesion (Fig. 2).

## The 250-kD Core Protein Inhibits Neurite Extension on N-cadherin

We have previously shown that E7 chick neural retina cells extend neurites when plated on purified N-cadherin or anti–N-cadherin antibody NCD-2 and that addition of anti-GalNAcPTase mAb 1B11 inhibits this neurite extension by ~70% (Gaya-Gonzalez, 1991). The 250-kD core protein is as effective as anti-GalNAcPTase mAb 1B11 in inhibiting neurite extension. Only 18% (±4%) of cells bear neurites in the presence of the 250-kD core protein, as opposed to 77% (±3%) in the absence of additives. The percent of cells with neurites in the presence of the 400-kD core protein is similar to the controls (67 ± 9%).

Representative fields of cells cultured on purified N-cadherin with or without the addition of the 250-kD core protein or anti-GalNAcPTase mAb 1B11 are shown in Fig. 3. As described above for cell adhesion, the inhibitory effect of the 250-kD core protein and anti-GalNAcPTase mAb 1B11 are eliminated by treating the cells with PI-PLC (Fig.
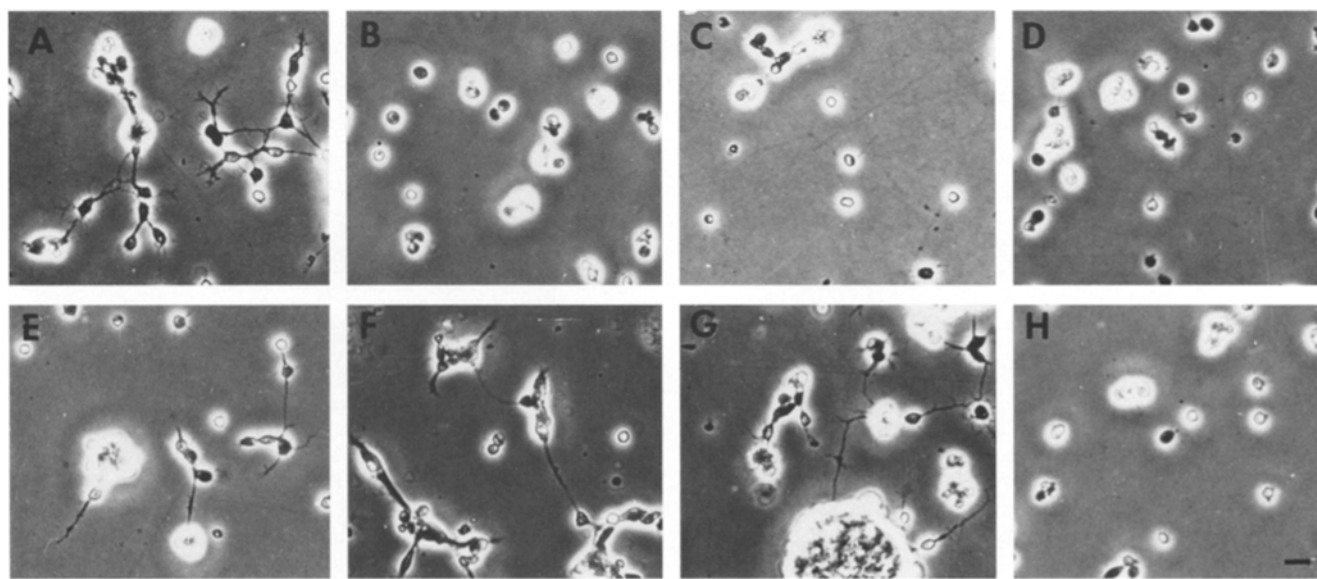


*Figure 3.* Effect of the 250-kD core protein on neurite extension. E7 chick neural retina cells (*A–D*, control cells; *E–H*, PI-PLC–treated cells) were plated on 24-well plates coated with affinity purified N-cadherin and allowed to adhere for 2 h. Culture medium containing the indicated components was added and the cells incubated for 20 h. (*A* and *E*) No additives; (*B* and *F*) 250-kD core protein (15 μg/ml); (*C* and *G*) anti-GalNAcPTase mAb 1B11 (10 μg/ml); (*D* and *H*) anti–N-cadherin mAb NCD-2 (10 μg/ml). Bar, 50 μm.

3, compare *B* with *F*, and *C* with *G*). In contrast anti–N-cadherin mAb NCD-2 inhibits neurite outgrowth before and after PI-PLC treatment of cells (Fig. 3, compare *D* with *H*). As in the case of adhesion, the 400-kD core protein does not inhibit neurite outgrowth (data not shown). These results further highlight that the interaction of the 250-kD core protein (and mAb 1B11) with the cell surface involves a GPI-anchored receptor.

## The 250-kD Proteoglycan Core Protein Binds to Cells

The results described above are consistent with the idea that the 250-kD core protein binds to a cell surface receptor, possibly the GalNAcPTase. To demonstrate that this is indeed the case, we assayed the binding of $^{125}$I-labeled 250- and 400-kD core proteins to cells. Binding of the 250- and 400-kD core proteins increases with concentration; however, a much higher level of binding is observed with
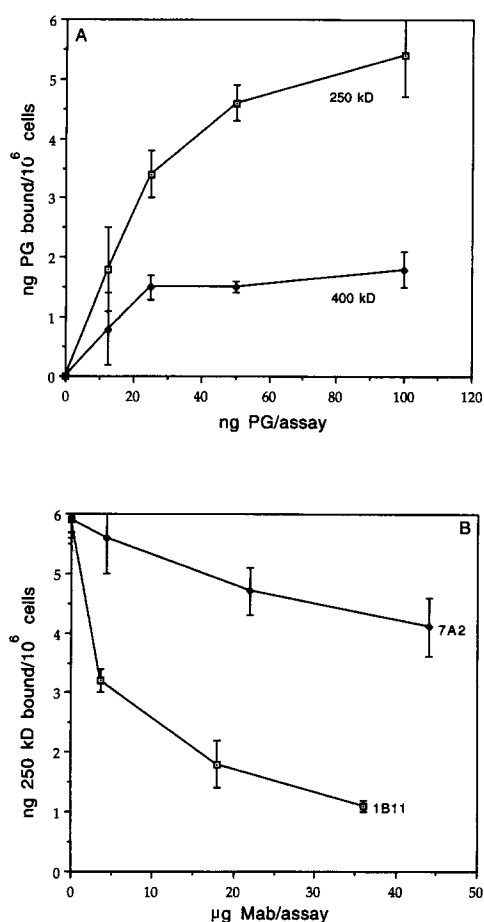


*Figure 4.* Binding of the 250-kD core protein to cells and inhibition by the anti-GalNAcPTase mAb 1B11. Cells attached to a poly-L-lysine coated substrate were incubated with radiolabeled core proteins (170,000 cpm/µg protein), washed, and the bound radioactivity determined. Each point is the average of three measurements. Error bars represent the standard error of the mean. (*A*) 250-kD core protein (*open squares*); 400-kD core protein (*closed circles*). (*B*) increasing concentrations of anti-Gal-NAcPTase mAbs 1B11 (*open squares*) or 7A2 (*filled circles*) were added to cells followed by the addition of 30 ng of radiolabeled 250-kD core protein.

the 250-kD core protein (Fig. 4 *A*). Based on the amount of label bound to cells incubated with 100 ng/ml of the 250-kD core protein (at which concentration the binding curve is approaching an asymptote), there appear to be ∼1 × 10⁵ receptors for the 250-kD core protein per cell. These results are consistent with the presence of a specific receptor for the 250-kD core protein at the cell surface.

Binding of the 250-kD monomer or core protein to cells has the same effect on cadherin-mediated adhesion and neurite outgrowth as binding of anti-GalNAcPTase mAb 1B11 (Balsamo et al., 1991; Gaya-Gonzalez, 1991). This observation suggests that the 250-kD core protein and mAb 1B11 might bind to the same critical site on the GalNAcPTase. To test this possibility, we measured the binding of a subsaturating amount of $^{125}$I-labeled 250-kD core protein to cells in the presence of increasing concentrations of mAb 1B11. As a control, we replaced mAb 1B11 with mAb 7A2, which also binds to the cell surface GalNAcPTase but does not inhibit cadherin-mediated adhesion (Balsamo et al., 1991). Binding of 250-kD core protein is inhibited by mAb 1B11, while mAb 7A2 has little effect (Fig. 4 *B*), strongly suggesting that the 250-kD core protein and mAb 1B11 bind to the same, or an overlapping, site on the GalNAcPTase.

## The 250-kD Core Protein Binds to the GalNAcPTase

The results presented thus far, that the binding of the 250-kD core protein to cells is inhibited by mAb 1B11 and eliminated by treatment of cells with PI-PLC, are consistent with the GalNAcPTase being a cell surface receptor for the 250-kD core protein. To verify that the two molecules interact directly, we tested the ability of the 250-kD core protein covalently attached to Sepharose beads to bind soluble GalNacPTase released from E10 neural retina membranes by treatment with PI-PLC (Balsamo and Lilien, 1993). As a control for nonspecific adsorption,
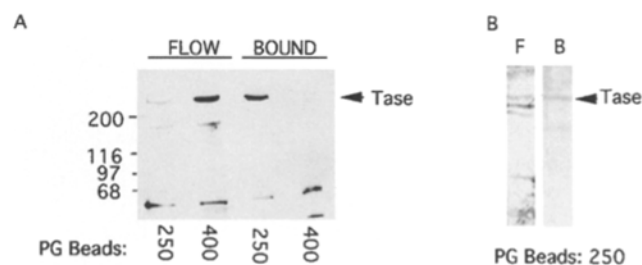


*Figure 5.* Binding of the GalNAcPTase to immobilized core proteins. The GalNAcPTase, along with other GPI-linked proteins, was released by PI-PLC treatment of crude membranes from E10 retina tissue and was labeled with biotin. This released material was then incubated with immobilized 250- or 400-kD core proteins. Unbound (*Flow* or *F*) and bound and eluted (*Bound* or *B*) material was fractionated by SDS PAGE, transferred to Immobilon, and immunoblotted with: (*A*) anti-GalNacPTase mAb 1B11, or (*B*) alkaline phosphatase-conjugated avidin. Arrowheads labeled Tase indicate the migration of the GalNAcPTase. The 180-kD polypeptide recognized by anti-GalNAcPTase antibody is a fragment of the 220-kD GalNAcPTase (J. Balsamo and J. Lilien, unpublished). The migration of standard proteins for both panels is indicated at the left of *A* by their molecular mass.

Sepharose beads conjugated with the 400-kD core protein were used. A comparison of bound and unbound material by Western blotting with anti-GalNAcPTase antibody reveals that the GalNAcPTase is bound by the immobilized 250-kD core protein, but not by the immobilized 400-kD core protein (Fig. 5 *A*). Similarly, the GalNAcPTase is removed from solution by the immobilized 250-kD core protein, but not by the immobilized 400-kD core protein (Fig. 5 *A*). Moreover, the GalNAcPTase appears to be the predominant polypeptide bound by the immobilized 250-kD core protein (Fig. 5 *B*). Whereas a polypeptide with the migration of the GalNAcPTase is one of four major polypeptides released by PI-PLC, it is the predominant polypeptide bound by the 250-kD core protein.

The differential binding of the GalNAcPTase to the 250- and 400-kD core proteins was also measured by assaying the amount of GalNAcPTase activity associated with the beads. Consistent with the Western blot data, no GalNAcPTase activity above background is associated with the immobilized 400-kD core protein (<3.5 pmol GalNAc incorporated per 100 μl of packed beads), while the GalNAcPTase associated with the immobilized 250-kD core protein catalyzed the incorporation into asialo-agalactofetuin of 13 pmol of labeled GalNAc per 100 μl of packed beads.

## Binding of 250-kD Core Protein to Cells Alters the Association of N-cadherin with Actin

We have previously shown that the inhibition of cadherin-mediated adhesion by binding of anti-GalNAcPTase mAb 1B11 to cells is accompanied by uncoupling of N- and E-cadherin from their association with the actin-containing cytoskeleton (Balsamo et al., 1991; Bauer et al., 1992). This association is critical to cadherin function (see Magee and Buxton, 1991; Takeichi et al., 1992; Tsukita et al., 1992; Kemler, 1993; Gumbiner, 1993 for reviews). To determine if binding of the 250-kD core protein also uncouples the association of N-cadherin with the actin-containing cytoskeleton, cells were incubated with the 250- or 450-kD core proteins, homogenates prepared, and fractionated on sucrose gradients. In unperturbed cells, N-cadherin can be precipitated from the rapidly sedimenting fraction by anti-actin antibodies, suggesting that a complex containing actin, N-cadherin, and the GalNAcPTase is found in this fraction (Balsamo et al., 1991). Similarly, in cells incubated with the 400-kD core protein, N-cadherin is precipitated from the rapidly sedimenting sucrose gradi-
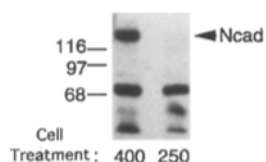
ent fraction by anti-actin antibodies (Fig. 6). However, in the rapidly sedimenting fraction from cells incubated with the 250-kD core protein, no detectable N-cadherin coprecipitates with actin (Fig. 6). The amount of actin precipitated is similar in all cases (not shown). These data strongly suggest that binding of the 250-kD core protein to the GalNAcPTase uncouples N-cadherin from its association with the actin-containing cytoskeleton.

## Binding of GalNAcPTase Ligands to Neural Retina Cells Results in Increased Levels of Phosphorylated Tyrosine in β-catenin

Phosphorylation of β-catenin has previously been correlated with the loss of cadherin function (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994). Because the binding of GalNAcPTase ligands to cells inhibits cadherin function, we also examined their effect on the tyrosine phosphorylation of β-catenin. E10 cells were incubated with the 250-kD core protein or the anti-GalNAcPTase mAb 1B11. As controls, the cells were incubated with the 400-kD core protein and the anti-GalNAcPTase mAb that binds but does not inhibit adhesion (mAb 7A2). The rapidly sedimenting fractions from each of the cell homogenates were incubated with anti–N-cadherin mAb NCD-2 to precipitate N-cadherin and the associated catenins. Under these conditions no tyrosine phosphorylated proteins are detected in material from cells treated with the 400-kD core protein or mAb 7A2 and only one polypeptide at ∼93 kD, the molecular mass of β-catenin, is seen in material from cells treated with the 250-kD core protein or mAb 1B11 (not shown). To unequivocally identify this tyrosine-phosphorylated polypeptide as β-catenin, the same experiment was performed using anti–β-catenin as the immunoprecipitating antibody. Little or no phosphorylated β-catenin is seen in the absence of treatment (not shown) or following treatment of the cells with the 400-kD core protein or mAb 7A2; however, following incubation with the 250-kD core protein or anti-GalNAcPTase mAb 1B11, high levels of tyrosine-phosphorylated β-catenin are detected (Fig. 7,

*Figure 6.* Effect of the 250-kD core protein on the association of N-cadherin with actin. Cells incubated with soluble 250- or 400-kD core proteins were homogenized. The rapidly sedimenting fraction was isolated and incubated with anti-actin antibody. The resulting immunoprecipitates were fractionated by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti-N-cadherin mAb NCD-2. The arrowhead labeled N-cad indicates the position of N-cadherin. The migration of standard proteins is indicated by their molecular mass. The lower molecular mass bands seen in both lanes are due to recognition of the precipitating antibody by the secondary antibody conjugate.

*Figure 7.* Effect of anti-GalNAcPTase mAb 1B11 and the 250-kD core protein on the level of phosphorylated tyrosine in β-catenin. Single cells were incubated with anti-GalNAcPTase mAbs 7A2 or 1B11 or the 250- or 400-kD core proteins. The cells were then homogenized, the rapidly sedimenting fraction isolated, treated with SDS, and incubated with anti–β-catenin antibody. The anti–β-catenin immunoprecipitates from the indicated cell preparations were fractionated by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti-phosphotyrosine antibody (*top*) or anti–β-catenin antibody (*bottom*). The arrowheads labeled β cat. indicate the migration of β-catenin. The migration of standard proteins is indicated on the left by their molecular mass. The lower molecular mass bands seen in all lanes of the upper panel are due to recognition of the precipitating antibody by the secondary antibody conjugate.
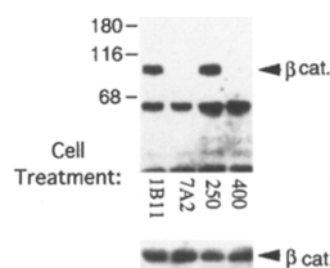
*upper panel*). This difference does not reflect a difference in the amount of precipitated β-catenin as evidenced by the fact that similar levels of β-catenin protein are detected in all four immunoprecipitates using anti-β-catenin as the detecting antibody on Western blots (Fig. 7, *lower panel*). An increase in phosphorylated tyrosine residues on β-catenin is also seen following incubation of cells with the 250-kD monomer (not shown).

### The 250-kD Monomer Is Associated with the GalNAcPTase in Tissue

We have shown that the isolated chick brain-derived 250-kD monomer and core protein bind to the cell surface GalNAcPTase in vitro, initiating a signal that results in increased levels of phosphorylated tyrosine in β-catenin, uncoupling of N-cadherin from its association with actin, and loss of N-cadherin function. If the 250-kD monomer is indeed an endogenous ligand for the GalNAcPTase, it should be present in vivo in retina, possibly associated with the GalNAcPTase. To confirm that the 250-kD (and 400-kD) monomers are present in retina, affinity-purified antibodies specific for the 250- and 400-kD core proteins were prepared and used in Western blotting experiments with E9 brain and retina extracts. The anti-250 antibody recognizes a 250-kD core protein in retina and 250- and 140-kD core proteins in brain (Fig. 8). Even after exposure for an extended period, only one major band is seen in retina (Fig. 8, second set of retina lanes). As expected, no immunologically crossreactive material is seen to enter the gels prior to chondroitin ABC lyase treatment. The anti-400-kD antibody recognizes a major constituent at 400 kD, seen only after chondroitin ABC lyase treatment of retina and brain, and additional components in the retina at ~300 and 200 kD both before and after treatment with chondroitin ABC lyase.

To determine if the 250-kD monomer and the GalNAcPTase are associated in vivo, E10 retina tissue was

homogenized in neutral detergent and the rapidly sedimenting material containing cadherin/GalNAcPTase/actin complexes incubated with anti-GalNAcPTase antibody. The resulting immunoprecipitates were examined for the presence of the 250-kD core protein and N-cadherin. Similarly, anti–250-kD immunoprecipitates were examined for the presence of the GalNAcPTase and N-cadherin. Immunoprecipitates prepared using anti-GalNAcPTase antibody 1B11 contain the 250-kD core protein and N-cadherin (Fig. 9). The polypeptide at ~140 kD detected by the anti–250-kD antibody is not N-cadherin as anti–250-kD antibody does not recognize purified N-cadherin (not shown). Immunoprecipitates prepared using the anti–250-kD core protein antibody contain the GalNAcPTase and N-cadherin (Fig. 9). None of these molecules are precipitated by control antibodies (Fig. 9). Similar results are obtained whether the samples are treated with chondroitin ABC lyase before or after immunoprecipitation.

## Discussion

We have shown that the embryonic chick retina cell surface GalNAcPTase interacts specifically with an endogenous proteoglycan with a 250-kD core protein but not with a distinct proteoglycan with a 400-kD core protein. The native 250-kD monomer and core protein generated by chondroitin ABC lyase treatment are equally active; however, we have used the 250-kD core protein for the majority of our analyses, as it can be isolated in larger quantities (H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, manuscript submitted for publication). Binding of the 250-kD PG to the GalNAcPTase on cells initiates a signal cascade which culminates in the inhibition of homophilic cadherin-mediated cell–cell adhesion and neurite outgrowth. Treatment of cells with PI-PLC, which releases the GalNAcPTase from the cell surface, abolishes both of these effects although the cells continue to express N-cadherin and exhibit N-cadherin–mediated adhesion. Radiolabeled
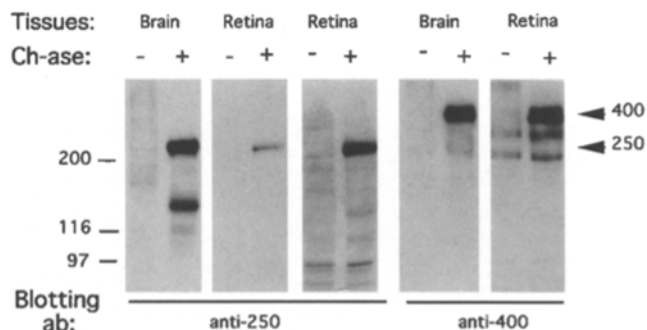


*Figure 8.* Presence of the 250- and 400-kD PG's in E9 chick retina and brain. Tissue extracts were prepared and, where indicated, treated with chondroitin ABC lyase (*Ch-ase*). Samples were then fractionated by SDS-PAGE and immunoblotted with the indicated anti-proteoglycan core protein antibodies. All lanes were loaded with 25 μg of extract. The second set of retina lanes blotted with anti-250 was exposed for 5× longer to match the intensity of the signal in the brain. The migration of standard proteins is indicated on the left by their molecular mass. The migration of the 400-kD component of laminin (not shown) was also used as a standard in estimating the molecular mass of the core proteins larger than 200 kD.
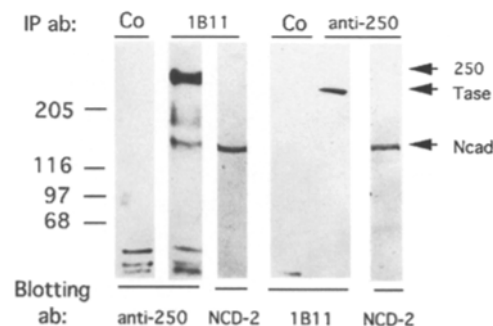


*Figure 9.* Coprecipitation of the GalNAcPTase, the 250-kD core protein, and N-cadherin from tissue. The rapidly sedimenting fraction from neutral detergent extracts of E10 neural retina was treated with chondroitin ABC lyase and incubated with anti-GalNAcPTase mAb 1B11, nonimmune mouse IgM (*Co*), anti–250-kD core protein antibody, or nonimmune rabbit IgG (*Co*). The resulting immunoprecipitates were fractionated by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti–250-kD core protein antibody, anti-GalNAcPTase mAb 1B11 and anti–N-cadherin mAb NCD-2. The migration of standard proteins is indicated on the left by their molecular mass.

250-kD core protein binds to cells; this binding is inhibited by anti-GalNAcPTase mAb 1B11, but not by anti-Gal-NAcPTase mAb 7A2. This observation, and the fact that the binding of mAb 1B11, but not 7A2, to the GalNAcPTase also inhibits cadherin mediated adhesion (Balsamo et al., 1991), strongly suggests that both the 250-kD PG and mAb 1B11 bind to an overlapping epitope on the GalNAcPTase. Binding between the 250-kD core protein and the GalNAcPTase can also be demonstrated directly; GalNAcPTase liberated from cells by PI-PLC treatment binds to immobilized 250-kD core protein but not to immobilized 400-kD core protein. Finally, our data indicate that binding of the 250-kD core protein or mAb 1B11 to the GalNAcPTase leads to enhanced tyrosine phosphorylation of β-catenin. This, in turn, inhibits the association of N-cadherin with the actin-containing cytoskeleton leading to the observed inhibition of cadherin-mediated adhesion and neurite outgrowth.

This work describes a new regulatory mechanism potentially important in controlling cadherin-dependent morphogenetic processes; binding of the 250-kD PG to the GalNAcPTase resulting in loss of cadherin function. Our working hypothesis is that the temporal and spatial regulation of the expression of the GalNAcPTase and of the 250-kD PG plays a role in the control of morphogenesis by inhibiting cadherin function at certain select times and places during development. Consistent with this idea, the GalNAcPTase and the 250-kD PG are developmentally regulated. The GalNAcPTase is initially found throughout the chick eye but gradually becomes localized to the fiber layer and outer segments (Balsamo et al., 1986) and is found in the outer segments of all vertebrates tested (Sweatt et al., 1991). It is also present at the mature neuro-muscular junction, where its localization is controlled by innervation (Scott et al., 1990). During early development, the GalNAcPTase is localized to the dorsal neural tube and notochord, suggesting an important function in neuronal induction and development (J. Balsamo and J. Lilien, unpublished observation).

The distribution of the 250- and 400-kD PG's are also regulated in time and space. In the developing retina, anti–250- and anti–400-kD stain fiber layers beginning at E10 (A. Sweat, S. Hoffman, J. Balsamo, and J. Lilien, unpublished observation). In the 4-d-old embryo several sites have been identified where the 250- and the 400-kD PG's appear to have mutually exclusive distributions (M. K. B. Zanin, H. Ernst and S. Hoffman, unpublished observation). For example, in the heart, anti-250 stains the endocardial surface of the myocardium; in contrast, anti-400 stains the endocardial cushion tissue, particularly the portion of loosely packed cells nearest the myocardium.

Our data also suggest an additional mode through which the inhibitory function of the 250-kD PG may be regulated. The active form of the proteoglycan is the monomeric form; macromolecular complexes of proteoglycan monomers bound non-covalently to hyaluronic acid (i.e., proteoglycan aggregates) are inactive. While the monomer is the most abundant form of the 250-kD PG in E14 brain, we have no information on the relative abundance of aggregates and monomers at other stages of development and in other tissues. These observations raise the possibility that the interaction of the 250-kD PG with hyaluronic acid may interfere with its ability to bind to the Gal-NAcPTase. If so, the regulated expression of hyaluronic acid or hyaluronidase in vivo may, in turn, regulate the interaction of the 250-kD PG and the GalNAcPTase and thus cadherin-mediated adhesion.

Regulation of adhesion may also occur through the interaction of the GalNAcPTase with multiple ligands at different times and places during development. It is currently unknown what structural features of the 250-kD PG are required for interaction with the GalNAcPTase. While it is clear that the bulk of the chondroitin sulfate is not required, we do not know whether the remaining carbohydrate in the 250-kD core protein or the polypeptide portion is responsible for its specific interaction with the GalNAcPTase. If the carbohydrate portion of the 250-kD core protein is involved in binding to the GalNAcPTase, the possibility must be considered that similar oligosaccharides are present on additional proteoglycans or glycoproteins. Furthermore, the distribution of oligosaccharides able to interact with the GalNAcPTase could further be altered by the patterned expression of other glycosyltransferases and glycosidases. Alteration of oligosaccharides on ECM molecules affecting their biological activity is consistent with observed differences in cell spreading on laminin, before and after glycosylation of the laminin by galactosyltransferase (Runyan et al., 1988), and with the observed inhibition of the ability of mesenchymal cells to migrate through a three-dimensional collagen gel if similar cells have already passed through and thereby "conditioned" the gel (Markwald et al., 1984). The 250- and 400-kD core proteins used in this study bind cytotactin (H. Ernst, M. K. B. Zanin, and S. Hoffman; unpublished). The original cytotactin-binding (CTB) proteoglycan (Hoffman and Edelman, 1987) was reported to have a 280-kD core; using laminin as a standard, we now estimate the molecular mass of this core protein to be 400 kD. Recently, the proteoglycans neurocan and phosphacan (Grumet et al., 1994) have been reported to bind to cytotactin (tenascin); it has also been suggested that versican may be a "CTB proteoglycan" (Perides et al., 1993). While it is noteworthy that the 250-kD core protein binds to cytotactin, it is unlikely that this activity is involved in its ability to bind to the GalNAcPTase because the 400-kD core protein also binds to cytotactin but not to the GalNAcPTase. The glycoprotein restrictin, which is structurally related to cytotactin (Nörenberg et al., 1992), is associated with our proteoglycan aggregates and native monomer preparations. Our recent results suggest that this association occurs because restrictin also binds proteoglycans (H. Ernst, M. K. B. Zanin, and S. Hoffman, unpublished observation). Restrictin also does not appear to be involved in the inhibition of cadherin-mediated adhesion; the 250-kD monomer and the 250-kD core protein preparations that are fully active in inhibiting cadherin-mediated adhesion are free of restrictin.

Recently we have succeeded in determining the NH2-terminal sequences of at least 13 CNBr fragments derived from our 250- and 400-kD core protein preparations (H. Ernst, M. K. B. Zanin and S. Hoffman; unpublished observations). Although both the 250- and the 400-kD core proteins appear as well-resolved sharp bands on Coomassie blue-stained gels, comparison of these data to the se-

quences of chicken aggrecan (Chandrasekaran and Tanzer, 1992) and chicken versican (Shinomura et al., 1993) and to the sequences of rat neurocan (Rauch et al., 1992) and phosphacan (Maurel et al., 1994) suggest that both the 250- and the 400-kD core proteins are mixtures of known proteoglycans. The 250-kD core protein preparation appears to contain neurocan, phosphacan, and aggrecan. This size is consistent with the known molecular mass of the neurocan core protein and suggests that partial degradation of phosphacan and aggrecan may occur creating fragments in this size range. The 400-kD core protein preparation appears to contain phosphacan, versican, and aggrecan which is consistent with the reported molecular weights for these proteoglycans. All of the $NH_2$-terminal sequences that we obtained could be accounted for by one of these four proteoglycans, suggesting that there are no novel proteoglycans in our preparations. As neurocan is the only proteoglycan found exclusively in the 250-kD core protein preparation, and only this core protein preparation is active in our assays, it is likely that neurocan is the proteoglycan that binds to the GalNAcPTase leading to the inhibition of cadherin-mediated adhesion.

These data are totally consistent with the specificity of the anti-250 and anti-400 antibodies. The anti-250 antibody was isolated on the basis of its ability to bind to the immobilized 250-kD core protein and not the 400-kD core protein. Because neurocan is unique to the 250-kD core protein preparation, an antibody prepared in this manner should be an anti-neurocan antibody. Like anti-neurocan antibodies (Rauch et al., 1992), the anti-250 recognizes a brain proteoglycan with a 140-kD core protein in addition to the full-length 250-kD core protein. The fact that the anti-250 has the specificity appropriate for an anti-neurocan antibody, and also immunoprecipitates from retinal tissue extracts a 250-kD proteoglycan along with the GalNAcPTase, is further evidence that neurocan is likely to be the proteoglycan that interacts with the GalNAcPTase.

It is interesting to note that, if our speculation that the 250-kD PG is neurocan is correct, neurocan has the potential to interact with multiple molecules at different times and places during development. The 250-kD PG interacts with cytotactin, restrictin, hyaluronic acid, and the GalNAcPTase, while neurocan (and phosphacan) has been reported to bind directly to N-CAM and Ng-CAM, inhibiting N-CAM-to-N-CAM and Ng-CAM-to-Ng-CAM–mediated adhesion (Grumet et al., 1993; Friedlander et al., 1994). This mechanism of inhibition of adhesion is, however, quite distinct from that demonstrated here. The 250-kD core protein cannot be functioning by binding to N-CAM or Ng-CAM in our experiments because the procedure used to prepare single cells, trypsinization in the presence of calcium, strips the cell surface of N-CAM and Ng-CAM and leaves the cells unable to adhere through a calcium-independent mechanism (Grunwald et al., 1980; Brackenbury et al., 1981; Magnani et al., 1981). However, cells prepared in this manner have intact and functional N-cadherin and GalNAcPTase at their surface (Balsamo and Lilien, 1982, 1990).

The most plausible explanation for the inhibition of cadherin function on binding of the 250-kD PG to the GalNAcPTase is that a signaling pathway is activated which culminates in the dissociation of cadherin from the actin-

containing cytoskeleton. As with the binding of anti-GalNAcPTase mAb 1B11 (Balsamo et al., 1991), we have found that the association of cadherin with the cytoskeleton is uncoupled following binding of the 250-kD PG to the GalNAcPTase. The association of cadherins with the cytoskeleton has been shown to be essential to cadherin function by several techniques, most convincingly by deletion of the cytoplasmic domain of cadherin (Nagafuchi and Takeichi, 1988, 1989; Kintner, 1992) and through the transfection into cells of a truncated, dominant negative construct containing only the cytoplasmic domain of cadherin (Fujimori and Takeichi, 1993). In both of these cases, cadherin continues to be expressed at the cell surface, but function is lost. Interestingly, the interaction of the 250-kD PG (and the 400-kD PG) with its receptor(s) on fibroblasts also initiates a signal cascade affecting adhesion molecule function through alterations at the level of the cytoskeleton, resulting in the failure to spread on fibronectin and vitronectin, and the failure to phosphorylate the focal adhesion kinase (H. Ernst, M. K. B. Zanin, D. Everman, and S. Hoffman, manuscript submitted for publication).

Cadherin interaction with actin is mediated by $\alpha$-, $\beta$- and $\gamma$-catenin (Ozawa et al., 1989). $\alpha$-catenin is related to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) and $\beta$- and $\gamma$-catenins are related to the product of the Drosophila segment polarity gene *armadillo* (McCrea et al., 1991; Peifer et al., 1992) and to plakoglobin (Butz et al., 1992; Knudsen and Wheelock, 1992). Aberrant or missing $\alpha$-catenin (Shimoyama et al., 1992; Oda et al., 1993) and phosphorylation of $\beta$-catenin (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994) have been correlated with loss of cadherin function. Consistent with these observations, we have found that binding of the 250-kD PG or anti-GalNAcPTase mAb 1B11 to cells results in increased levels of phosphorylated tyrosine in $\beta$-catenin. This further establishes the link between the presence of phosphorylated tyrosine residues on $\beta$-catenin and the loss of cadherin function. Thus, cadherin-dependent morphogenetic processes may be controlled by several distinct physiological signals, one of which is the interaction of the 250-kD PG with the GalNAcPTase, and all of which initiate signal cascades that regulate the level of phosphorylated tyrosine residues in $\beta$-catenin and thereby cadherin function.

*References*

Balsamo, J., and J. Lilien. 1982. An N-Acetylgalactosaminyltransferase and its acceptor in embryonic chick neural retina exist in interconvertible particulate forms depending on their cellular location. *J. Biol. Chem.* 257:349–354.

Balsamo, J., and J. Lilien. 1990. N-Cadherin is stably associated with and is an acceptor for a cell surface N-Acetylgalactosaminylphosphotransferase. *J. Biol. Chem.* 265:2923–2928.

Balsamo, J., and J. Lilien. 1993. The retina cell surface N-acetylgalactosaminylphosphotransferase is anchored by a glycophosphatidylinositol. *Biochemistry.* 32:8246–8250.

Balsamo, J., R. S. Pratt, M. R. Emmerling, G. B. Grunwald, and J. Lilien. 1986. Identification of the chick neural retina cell surface N-Acetylgalactosaminyltransferase using monoclonal antibodies. *J. Cell. Biochem.* 32:125–141.

Balsamo, J., R.Thiboldeaux, N. Swaminathan, and J. Lilien. 1991. Antibodies to

the retina N-Acetylglactosaminylphosphotransferase modulate N-cadherin-mediated adhesion and uncouple the N-cadherin transferase complex from the actin-containing cytoskeleton. *J. Cell Biol.* 113:429–436.

Bauer, G. E., J. Balsamo, and J. Lilien. 1992. Cadherin-mediated adhesion in pancreatic islet cells is modulated by a cell surface N-acetylgalactosaminylphosphotransferase. *J. Cell Sci.* 103:1235–1241.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M. M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* 120:757–766.

Bixby, J. L., and R. Zhang. 1990. Purified N-Cadherin is a potent substrate for the rapid induction of neurite outgrowth. *J. Cell Biol.* 110:1253–1260.

Brackenbury, R., J.-P. Thiery, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell–cell binding. *J. Cell Biol.* 252:6835–6840.

Brackenbury, R., U. Rutishauser, and G. M. Edelman. 1981. Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells. *Proc. Natl. Acad. Sci. USA.* 78:387–391.

Butz, S., J. Stappert, H. Weissig, and R. Kemler. 1992. Plakoglobin and β-catenin: distinct but closely related. *Science (Wash. DC).* 257:1142–1144.

Chandrasekaran, L., and M. L. Tanzer. 1992. Molecular cloning of chicken aggrecan. *Biochem. J.* 288:903–910.

Cole, G. J., and C. F. McCabe. 1991. Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. *Neuron.* 7:1007–1018.

Friedlander, D. R., S. Hoffman, and G. M. Edelman. 1988. Functional mapping of cytotactin: proteolytic fragments active in cell substrate adhesion. *J. Cell Biol.* 107:2329–2340.

Friedlander, D. R., P. Milev, L. Karthikeyan, R. K. Margolis, R. U. Margolis, and M. Grumet. 1994. The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM and inhibits neuronal adhesion and neurite outgrowth. *J. Cell Biol.* 125:669–680.

Fujimori, T., and M. Takeichi. 1993. Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. *Mol. Biol. Cell.* 4:37–47.

Gaya-Gonzalez, L., J. Balsamo, N. Swaminathan, and J. Lilien. 1991. Antibodies to the retina N-Acetylgalactosaminylphosphotransferase inhibit neurite outgrowth. *J. Neurosci. Res.* 29:474–480.

Geisert, E. E., Jr., and D. J. Bidanset. 1993. A central nervous system keratan sulfate proteoglycan: localization to boundaries in the neonatal rat brain. *Dev. Brain Res.* 75:163–173.

Grumet, M., A. Flaccus, and R. U. Margolis. 1993. Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules. *J. Cell Biol.* 120:815–824.

Grumet, M., P. Milev, T. Sakurai, L. Karthikeyan, M. Bourdon, R. K. Margolis, and R. U. Margolis. 1994. Interactions with tenascin and differential effects on cell adhesion of neurocan and phosphacan, two major chondroitin sulfate proteoglycans of nervous tissue. *J. Biol. Chem.* 269:12142–12146.

Grunwald, G. B., R. L. Geller, and J. Lilien. 1980. Enzymatic dissection of embryonic cell adhesive mechanisms. *J. Cell Biol.* 85:766–776.

Gumbiner, B. M. 1993. Proteins associated with the cytoplasmic surface of adhesion molecules. *Neuron.* 11:551–564.

Guo, M., K. E. Dow, R. Kisilevsky, and R. J. Riopelle. 1993. Novel neurite growth-inhibitory properties of an astrocyte proteoglycan. *J. Chem. Neuroanat.* 6:239–245.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60ᵛ⁻ˢʳᶜ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307–314.

Herrenknecht, K., M. Ozawa, C. Eckerskorn, F. Lottspeich, M. Lenter, and R. Kemler. 1991. The uvomorulin-anchorage protein α catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. USA.* 88:9156–9160.

Hoffman, S., and G. M. Edelman. 1987. A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. *Proc. Natl. Acad. Sci. USA.* 84:2523–2527.

Hoffman, S., S. L. Dutton, H. Ernst, M. K. Boackle, D. Everman, A. Tourkin, and J. D. Loike. 1994. Functional characterization of anti-adhesion molecules. *Perspect. Dev. Neurobiol.* 2:101–110.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.

Kintner, C. 1992. Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. *Cell.* 69:225–236.

Knudsen, K. A., and M. J. Wheelock. 1992. Plakoglobin, or an 83-kD homologue distinct from β-catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol.* 118:671–679.

Magee, A. I., and R. S. Buxton. 1991. Transmembrane molecular assemblies regulated by the greater cadherin family. *Curr. Opin. Cell Biol.* 3:854–861.

Magnani, J. L., W. A. Thomas, and M. S. Steinberg. 1981. Two distinct adhesion mechanisms in embryonic chick neural retina cells. I. A kinetic analysis. *Dev. Biol.* 81:96–105.

Margolis, R. K., and R. U. Margolis. 1993. Nervous tissue proteoglycans. *Experientia (Basel).* 49:429–446.

Markwald, R. R., R. B. Runyan, G. T. Kitten, F. M. Funderberg, D. H. Bernanke, and P. R. Brauer. 1984. Use of collagen gel cultures to study heart development: protein and glycoprotein interactions during the formation of

endocardial cushion tissue. *In* The Role of the Extracellular Matrix in Development. Alan R. Liss, Inc., New York. 323–350.

Matsuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell–cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703–714.

Maurel, P., U. Rauch, M. Flad, R. K. Margolis, and R. U. Margolis. 1994. Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 91:2512–2516.

McCrea, P. D., C. W. Turck, and B. Gumbiner. 1991. A homolog of the *Armadillo* protein in Drosophila (Plakoglobin) associated with E-cadherin. *Science (Wash. DC).* 254:1359–1361.

Morris, J. E. 1993. Proteoglycans and the modulation of cell adhesion by steric exclusion. *Dev. Dyn.* 196:246–251.

Nagafuchi, A., and M. Takeichi. 1988. Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3679–3684.

Nagafuchi, A., and M. Takeichi. 1989. Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. *Cell Regul.* 1:37–44.

Nagafuchi, A., M. Takeichi, and S. Tsukita. 1991. The 102 kD cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell.* 65:849–857.

Nörenberg, U., H. Wille, J. M. Wolff, R. Frank, and F. G. Rathjen. 1992. The chicken neural extracellular matrix molecule restrictin: similarity with the EGF-, fibronectin type III- and fibrinogen-like motifs. *Neuron.* 8:849–863.

Oakley, R. A., and K. W. Tosney. 1991. Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. *Dev. Biol.* 147:187–206.

Oda, T., Y. Kanai, Y. Shimoyama, A. Nagafuchi, S. Tsukita, and S. Hirohashi. 1993. Cloning of the human α-catenin cDNA and its aberrant mRNA in a human cancer cell line. *(Biochem. Biophys. Res. Commun.)* 193:897–904.

Oohira, A., F. Matsui, and R. Katoh-Semba. 1991. Inhibitory effects of brain chondroitin sulfate proteoglycans on neurite outgrowth from PC12D cells. *J. Neurosci.* 11:822–827.

Ozawa, M., H. Baribault, and R. Kemler. 1989. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1711–1717.

Peifer, M., P. D. McCrea, K. J. Green, E. Wieschaus, and B. M. Gumbiner. 1992. The vertebrate adhesive junction proteins β-catenin and plakoglobin and the *Drosophila* segment polarity gene *armadillo* form a multigene family with similar properties. *J. Cell Biol.* 118:681–691.

Perides, G., H. P. Erickson, F. Rahemtulla, and A. Bignami. 1993. Colocalization of tenascin with versican, a hyaluronate-binding chondroitin sulfate proteoglycan. *Anat. Embryol.* 188:467–479.

Pindzola, R. R., C. Doller, and J. Silver. 1993. Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Dev. Biol.* 156:34–48.

Rauch, U., L. Karthikeyan, P. Maurel, R. U. Margolis, and R. K. Margolis. 1992. Cloning and primary structure of neurocan, a developmentally regulated, aggregating chondroitin sulfate proteoglycan of brain. *J. Biol. Chem.* 267:19536–19547.

Runyan, R. B., J. Versalovic, and B. D. Shur. 1988. Functionally distinct laminin receptors mediate cell adhesion and spreading: the requirement for surface galactosyltransferase in cell spreading. *J. Cell Biol.* 107:1863–1871.

Schwab, M. E., J. P. Kapfhammer, and C. E. Bandtlow. 1993. Inhibitors of neurite growth. *Neuroscience.* 16:565–595.

Scott, J. C. L., J. Balsamo, J. R. Sanes, and J. Lilien. 1990. Synaptic localization and neural regulation of an N-acetylgalactosaminyl transferase in skeletal muscle. *J. Neurosci.* 10:346–350.

Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, N. Oko, K. Miyazawa, N. Kitamura, M. Takeichi, and F. Ito. 1994. Tyrosine phosphorylation of β catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. *Cell Adhes. Commun.* 1:295–305.

Shimoyama, Y., A. Nagafuchi, S. Fujita, M. Gotoh, M. Takechi, S. Tsukita, and S. Hirohashi. 1992. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. *Cancer Res.* 52:5770–5774.

Shinomura, T., Y. Nishida, K. Ito, and K. Kimata. 1993. cDNA cloning of PG-M, a large chondroitin sulfate proteoglycan expressed during chondrogenesis in chick limb buds. Alternative spliced multiforms of PG-M and their relationship to versican. *J. Biol. Chem.* 268:14461–14469.

Sweatt, A., J. Balsamo, and J. Lilien. 1991. Immunolocalization of N-acetylgalactosaminyl-phosphotransferase in the adult retina and subretinal space. *Exp. Eye Res.* 53:479–487.

Takeichi, M., S. Hirano, N. Matsuyoshi, and T. Fujimori. 1992. Cytoplasmic control of cadherin-mediated cell-cell adhesion. *Cold Spring Harbor Symp. Quant. Biol.* 57:327–334.

Tsukita, S., S. Tsukita, A. Nagafuchi, and S. Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Curr. Opin. Cell Biol.* 4:834–839.

Vleminckx, K. L., J. J. Deman, E. A. Bruyneel, G. M. R. Vandenbossche, A. A. Keirsebilck, M. M. Mareel, and F. M. van Roy. 1994. Enlarged cell-associated proteoglycans abolish E-cadherin functionality in invasive tumor cells. *Cancer Res.* 54:873–877.