

Regulated Binding of a PTP1B-like Phosphatase to N-Cadherin: Control of Cadherin-mediated Adhesion by Dephosphorylation of β -Catenin

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Abstract. Cadherins are a family of cell–cell adhesion molecules which play a central role in controlling morphogenetic movements during development. Cadherin function is regulated by its association with the actin containing cytoskeleton, an association mediated by a complex of cytoplasmic proteins, the catenins: α , β , and γ . Phosphorylated tyrosine residues on β -catenin are correlated with loss of cadherin function. Consistent with this, we find that only nontyrosine phosphorylated β -catenin is associated with N-cadherin in E10 chick retina tissue. Moreover, we demonstrate that a PTP1B-like tyrosine phosphatase associates with N-cadherin and may function as a regulatory switch controlling cadherin function by dephosphorylating β -catenin, thereby maintaining cells in an adhesion-competent state.

The PTP1B-like phosphatase is itself tyrosine phosphorylated. Moreover, both direct binding experiments performed with phosphorylated and dephosphorylated molecules, and treatment of cells with tyrosine kinase inhibitors indicate that the interaction of the PTP1B-like phosphatase with N-cadherin depends on its tyrosine phosphorylation. Concomitant with the tyrosine kinase inhibitor-induced loss of the PTP1B-like phosphatase from its association with N-cadherin, phosphorylated tyrosine residues are retained on β -catenin, the association of N-cadherin with the actin containing cytoskeleton is lost and N-cadherin-mediated cell adhesion is prevented. Tyrosine phosphatase inhibitors also result in the accumulation of phosphorylated tyrosine residues on β -catenin, loss of the association of N-cadherin with the actin-containing cytoskeleton, and pre-

vented N-cadherin mediated adhesion, presumably by directly blocking the function of the PTP1B-like phosphatase.

We previously showed that the binding of two ligands to the cell surface N-acetylgalactosaminylphosphotransferase (GalNAcPTase), the monoclonal antibody 1B11 and a proteoglycan with a 250-kD core protein, results in the accumulation of phosphorylated tyrosine residues on β -catenin, uncoupling of N-cadherin from its association with the actin containing cytoskeleton, and loss of N-cadherin function. We now report that binding of these ligands to the GalNAcPTase results in the absence of the PTP1B-like phosphatase from its association with N-cadherin as well as the loss of the tyrosine kinase and tyrosine phosphatase activities that otherwise co-precipitate with N-cadherin. Control antibodies and proteoglycans have no such effect. This effect is similar to that observed with tyrosine kinase inhibitors, suggesting that the GalNAcPTase/proteoglycan interaction inhibits a tyrosine kinase, thereby preventing the phosphorylation of the PTP1B-like phosphatase, and its association with N-cadherin.

Taken together these data indicate that a PTP1B-like tyrosine phosphatase can regulate N-cadherin function through its ability to dephosphorylate β -catenin and that the association of the phosphatase with N-cadherin is regulated via the interaction of the GalNAcPTase with its proteoglycan ligand. In this manner the GalNAcPTase–proteoglycan interaction may play a major role in morphogenetic cell and tissue interactions during development.

THE cadherin family of calcium-dependent adhesion molecules mediate homophilic cell–cell adhesion (reviewed in Grunwald, 1993). During normal de-

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velopment they are suggested to play an important causal role in the onset and cessation of cell migration (Akitaya and Bronner-Fraser, 1992; reviewed in Vallés et al., 1991) and in the separation of tissues (reviewed in Takeichi, 1991). Three lines of evidence have been marshaled in support of this critical role for cadherins in development:

first, ectopic or altered expression leads to altered development (Detrick et al., 1990; Dufour et al., 1994; Levine et al., 1994; Heasman et al., 1994a,b) and failure of implantation (Larue et al., 1994; Riethmacher et al., 1995). Second, in vitro analysis of function reveals that N-cadherin plays an important role in the outgrowth of nerve fibers (Bixby and Zhang, 1990; Gaya Gonzalez et al., 1991; Matsunaga et al., 1988; Doherty et al., 1991) and E-cadherin plays a crucial role in the formation of epithelia (reviewed in Eaton and Simons, 1995). Third, an extensive body of data demonstrates that cadherins are temporally and spatially regulated in a manner that correlates with key developmental events (reviewed in Takeichi, 1991; Ranscht, 1991; Grunwald, 1993; Dalseg et al., 1994).

The function of cadherins depends on their association with the actin containing cytoskeleton. Deletion or truncation of the cytoplasmic domain of cadherin results in loss of function, in spite of continued expression at the cell surface (Nagafuchi and Takeichi, 1988, 1989; Kintner, 1992; Fugimori and Takeichi, 1993). The interaction of cadherins with the actin containing cytoskeleton is mediated by α , β , and γ catenins (reviewed in Magee and Buxton, 1991; Kemler, 1993; Gumbiner, 1993). Consistent with a role for the catenins in linking cadherins to the cytoskeleton, loss of cadherin function has been correlated with alterations in catenins. Loss of α -catenin has been correlated with loss of cadherin function in cell lines (Shimoyama et al., 1992; Breen et al., 1994; Ochiai et al., 1994a), which in one case may be due to a deletion in the α -catenin gene (Oda et al., 1993). Mutation of β -catenin (Kawanishi et al., 1995), as well as the presence of phosphorylated tyrosine residues on β -catenin are also correlated with a loss of cadherin-mediated adhesion. We have shown that the interaction of the chick retina N-acetylgalactosaminylphosphotransferase (GalNAcPTase) with its proteoglycan ligand results in the accumulation of phosphorylated tyrosine residues on β -catenin concomitant with loss of cadherin-mediated adhesion and uncoupling of cadherin from its association with the cytoskeleton (Balsamo et al., 1995). Additionally, treatment of human cancer cells with growth factors (Shibamoto et al., 1994) or transformation of cells with *v-src* (Behrens et al., 1993; Hamaguchi et al., 1993) or Rous sarcoma virus (Matsuyoshi et al., 1992) results in the accumulation of phosphorylated tyrosine residues on β -catenin concomitant with loss of cadherin-mediated adhesion.

Taken together these data suggest that the presence or absence of phosphorylated tyrosine residues on β -catenin appears to be a critical parameter in controlling cadherin function, possibly by regulating the association of cadherins with the cytoskeleton. In this manuscript we have examined the regulation of the tyrosine phosphorylation/dephosphorylation of β -catenin. In E10 chick retina tissue only β -catenin molecules lacking phosphorylated tyrosine residues are associated with N-cadherin. Moreover, we show that a novel PTP1B-like tyrosine phosphatase (PTP1B-LP)¹ is associated with N-cadherin and that the accumulation of phosphorylated tyrosine residues on β -cate-

nin, uncoupling of cadherin from its association with the cytoskeleton, and loss of cadherin-mediated adhesion are correlated with inhibition of the function of this phosphatase, or loss of its association with N-cadherin. Furthermore, the association of the PTP1B-LP with N-cadherin requires its tyrosine phosphorylation as demonstrated by both direct binding experiments and by experiments in which cells were treated with tyrosine kinase inhibitors. We also show that the binding to cells of GalNAcPTase ligands results in the loss of tyrosine kinase and tyrosine phosphatase activity that co-precipitates with N-cadherin and the failure of the PTP1B-LP to associate with N-cadherin. Based on these observations we propose that the PTP1B-LP acts as a regulatory switch modulating cadherin-mediated cell-cell adhesion and that GalNAcPTase-proteoglycan interactions can alter the state of this switch by inhibiting a tyrosine kinase whose activity is necessary to keep the phosphatase bound to N-cadherin.

Materials and Methods

Antibodies

The anti-N-cadherin hybridoma cell line NCD-2 is a rat IgG and was generously provided by M. Takeichi (Kyoto University, Kyoto, Japan). The anti- β -catenin antibody used in immunoprecipitation experiments is a rabbit polyclonal, prepared from a 15-amino acid synthetic peptide derived from the published sequence (Butz et al., 1992). In experiments where immunoblotting was also necessary, a mouse monoclonal IgG anti- β -catenin antibody from Transduction Labs (Lexington, KY) was used. Anti-phosphotyrosine antibody PY20, horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20H, biotin conjugated anti-phosphotyrosine antibody RC20B, and anti-PTP1B antibody, prepared from a 166-amino acid fragment corresponding to the carboxy terminus of the enzyme, were also mouse IgG's from Transduction Labs. Streptavidin-conjugated magnetic beads were from Promega Biotech (Madison, WI). The anti-actin antibody was a monoclonal mouse IgG from Chemicon Inc. (Temecula, CA). HRP-conjugated goat anti-rat, -rabbit, or -mouse IgG or IgM were from Cappel (Organon Teknika Corp., Durham, NC). HRP-conjugated Streptavidin was from Amersham Corp. (Chicago, IL).

The monoclonal anti-GalNAcPTase antibodies, 7A2 and 1B11, are mouse IgMs prepared in our laboratory and partially purified from culture medium as described (Scott et al., 1990; Balsamo et al., 1991). Both antibodies bind equally to the cell surface GalNAcPTase, but only 1B11 triggers the pathway resulting in inhibition of N-cadherin-mediated adhesion (Balsamo et al., 1991).

Other Reagents

o-Vanadate, genistein, and protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). Phenylarsine oxide was from Eastman Kodak and herbimycin from Calbiochem-Behring Corp. (San Diego, CA). Chicken brain-derived chondroitin sulfate proteoglycans (PG) with core molecular masses of 250 kD (250-kD PG) and 400 kD (400-kD PG) were purified and core proteins prepared according to Ernst et al. (1995). We have previously demonstrated that the 250-kD core protein, but not the 400-kD core protein, binds directly to the GalNAcPTase resulting in inhibition of N-cadherin-mediated adhesion (Balsamo et al., 1995).

Preparation of a Rapidly Sedimenting Fraction from Triton X-100 Tissue Extracts

This procedure has been described in previous publications (Balsamo et al., 1991, 1995). Briefly, neural retina tissue or cell suspensions were homogenized in Triton buffer (five retinas/ml; 1% Triton X-100 in 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl) containing protease and phosphatase inhibitors and DNase (5 μ g/ml each leupeptin and antipain, 1 mM PMSF, 10 mM NaF, 4 mM *o*-vanadate, 100 μ g/ml DNAase). After 30 min on ice, the homogenates were clarified by centrifugation at 14,000 *g*. To prepare

1. *Abbreviations used in this paper:* B, bound; F, flowthrough; GalNAcPTase, N-acetylgalactosaminylphosphotransferase; PTP1B-LP, PTP1B-like tyrosine phosphatase; PG, proteoglycan.

the rapidly sedimenting fraction the 14,000 g supernatant solution was layered on sucrose (100 μ l of 50% and 1 ml of 20% sucrose in Triton buffer) and centrifuged for 30 min at 150,000 g. The fraction from the 20/50% interface down constitutes the rapidly sedimenting fraction, referred to as the H fraction, and includes \sim 80% of the total N-cadherin present. This distribution remains unchanged by treatment of cells with phosphatase or kinase inhibitors as described below.

Affinity Purification of N-Cadherin and Analysis of Associated Proteins

IgG prepared from NCD-2 culture media was coupled to CnBr-activated Sepharose (Sigma Chemical Co.) according to the manufacturer's instructions. The H fraction from 20 retinas was diluted with an equal volume of Triton buffer and applied to the column. The column was washed with 10 volumes of Triton buffer and eluted with two column volumes of 50 mM diethylamine. The eluate was neutralized with 1 M Tris-HCl, pH 8, before further use.

To determine the distribution of tyrosine phosphorylated β -catenin in retina tissue, the bound and flowthrough fractions from the NCD-2 affinity column were concentrated using a Centricon 30 (Amicon Corp., Danvers, MA), made 1% in SDS to disrupt protein-protein interactions, diluted with Triton buffer to a final concentration of 0.1% SDS, and then immunoprecipitated with anti- β -catenin. The immunoprecipitates were fractionated by SDS-PAGE, transferred to PVDF membranes and probed with anti-phosphotyrosine or anti- β -catenin antibodies.

To evaluate whether a PTP1B-like phosphatase was associated with N-cadherin and whether it contained phosphorylated tyrosine residues, the bound and flow-through fractions from an NCD-2 affinity column were treated as above and immunoprecipitated with anti-PTP1B or PY20. The immunoprecipitates were fractionated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody RC20H or anti-PTP1B.

Immunoprecipitations were carried out for 4 h at 4°C. 100 μ g of magnetic beads (Advanced Magnetics, Cambridge, MA) conjugated with the appropriate second antibody were then added and the mixture incubated for 1 h at 4°C with end to end rotation. The immunoprecipitates were collected using a magnetic stand, the beads washed five times with Triton buffer, and precipitated material eluted with SDS-PAGE sample buffer.

Immunoblotting

SDS-polyacrylamide gels were transferred to PVDF membranes using a semi-dry transfer unit (Bio-Rad Laboratories, Cambridge, MA) for 36 min at 15V. The membranes were blocked for 1 h at 37°C in 5% dry milk solids in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8) or in 3% BSA-1% gelatin in TBST when reacting with anti-phosphotyrosine antibodies. The membranes were incubated with the primary antibody for 1 to 2 h, washed for 30 min in three changes of TBST, and incubated with HRP-conjugated secondary antibody (1:1,000 in TBST) for 45 min. After washing the membranes were developed using the ECL system (Amersham Corp., Arlington Heights, IL). When necessary the membranes were stripped of bound antibody by incubating stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) for 30 min at 50°C, washed in TBST, blocked, and reused as above.

Adhesion Assays

Single cells were prepared from 3 H-labeled E9 chick retina by trypsin dissociation in the presence of calcium (Grunwald et al., 1980; Brackenbury et al., 1981; Magnani et al., 1981). Such cells have an intact and functional cadherin adhesion system. Labeling of tissues and adhesion of labeled cells to immobilized N-cadherin or the anti-N-cadherin antibody NCD-2 was assayed as described previously (Balsamo et al., 1991).

To 3 H-label cells during a prolonged treatment with tyrosine kinase inhibitors, single cells were incubated in F12 medium containing 10 μ Ci 3 H-leucine, 50 μ g/ml gentamycin, and 1 μ g/ml herbimycin A, on 30 mm Petri dishes ($\approx 1 \times 10^7$ cells/2 ml), overnight, at 37°C, under an atmosphere of 10% CO₂. Just before assaying, cells were removed from the Petri plate by gentle flushing with a Pasteur pipette, collected by centrifugation, washed and resuspended in HBSGKCa (20 mM Hepes, pH 7.2, 150 mM NaCl, 2 mM glucose, 3 mM KCl, and 1 mM Ca).

To correlate the effects of tyrosine kinase and tyrosine phosphatase inhibitors and GalNAcPTase ligands on adhesion with the phosphorylated tyrosine content of β -catenin and with the association of the PTP1B-LP with N-cadherin, single cells were incubated with or without the desired

inhibitor in HBSGKCa for 45 min at 37°C and 70 rpm ($\approx 10^8$ cells/3 ml). The cells were collected, H fractions were prepared and the phosphorylated tyrosine content of β -catenin or the association of the PTP1B-LP with N-cadherin analyzed as described above.

Immunofluorescence

E9 neural retina cells prepared as above were plated on coverslips coated with poly-L-lysine (50 μ g/ml in PBS) and incubated overnight in DME containing 10% FCS and 1% ITS (insulin, transferrin, selenium; GIBCO BRL, Gaithersburg, MD). The cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were then incubated for 1 h, at room temperature with the desired antibody diluted in DME containing 5% goat serum. The cells were washed four times with DME/goat serum and incubated for another hour with rhodamine-conjugated second antibody. After washing another four times in the same buffer, the coverslips were mounted and examined under epifluorescence.

Preparation of Biotinylated N-Cadherin and Overlay Assays

Purified N-cadherin was prepared according to the procedure described by Bixby and Zhang (1990) and biotinylated using the ECL protein biotinylation kit (Amersham, Corp.). The purity of the preparation was assessed by SDS-PAGE followed by transfer to PVDF membrane and probing with HRP-streptavidin or anti-N-cadherin antibody followed by HRP goat anti-rat IgG (see Fig. 8).

To assay direct binding between N-cadherin and the PTP1B-LP, H preparations were immunoprecipitated with anti-PTP1B antibody, the precipitates fractionated by SDS-PAGE and transferred to PVDF membranes. The membranes were rinsed in 50 mM Tris buffer, pH 9, containing 5 mM MgCl₂, 2 mM CaCl₂ and 1 mM PMSF. Parallel lanes were incubated in the same buffer with 10 U/ml alkaline phosphatase (Promega Biotec, Madison, WI) with or without 4 mM o-vanadate for 1 h, at 37°C to inhibit phosphatase activity. The membranes were blocked for 30 min in 3% BSA in 50 mM Tris (pH 8.0) with 150 mM NaCl and incubated overnight with biotinylated N-cadherin in the Tris-NaCl buffer with 2 mM CaCl₂. After extensive washing, the membranes were incubated with HRP-streptavidin and developed using enhanced chemiluminescence.

Determination of Protein Tyrosine Kinase and Protein Tyrosine Phosphatase Activity

Single cells were incubated with the desired perturbing agent in HBSGKCa for 45 min at 37°C and 70 rpm ($\approx 10^8$ cells/3 ml). The N-cadherin bound fraction was prepared from an H fraction as above. Aliquots were tested for kinase or phosphatase activity using the respective non radioactive enzyme assay kits (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Tyrosine phosphatase activity was measured using a stable tyrosine phosphorylated peptide which is biotinylated at the amino terminus. This substrate is bound to microtiter wells coated with streptavidin and reacted with the source of tyrosine phosphatase. Phosphotyrosine residues are determined immunochemically with a specific anti-phosphotyrosine antibody covalently bound to peroxidase. Absorbance at 405 nm reflects the amount of phosphotyrosine residues remaining: the higher the absorbance, the lower the phosphatase activity.

The protein tyrosine kinase assay measures addition of a phosphate to tyrosine residues in a synthetic peptide substrate labeled with biotin. The peptide is incubated with the kinase source in suspension, the reaction stopped by the specific inhibitor piceatannol and the biotinylated peptide attached to streptavidin coated microtiter wells. As in the phosphatase assay, phosphotyrosine residues are determined by a specific anti-phosphotyrosine antibody, and activity determined as absorbance at 405 nm. In this case, higher absorbance values correspond to higher kinase activity.

Results

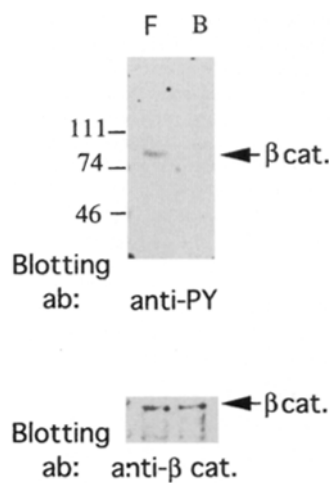
Only β -Catenin Lacking Phosphorylated Tyrosine Residues Is Associated with N-Cadherin

There is a correlation between the presence of phosphorylated tyrosine residues on β -catenin and loss of cadherin

function (Matsuyoshi et al., 1992; Behrens, et al., 1993; Hamaguchi et al., 1993; Shibamoto et al., 1994; Balsamo et al., 1995). One possible interpretation of this correlation is that the presence of phosphorylated tyrosine residues on β -catenin prevents its association with N-cadherin and therefore cadherin is unable to form its critical connection to the actin containing cytoskeleton. To further test this idea, we examined the association of nontyrosine-phosphorylated and tyrosine-phosphorylated β -catenin with N-cadherin. A 14,000-g supernatant from E10 retina tissue homogenized in Triton X-100 was applied to an anti-N-cadherin affinity column and separated into bound (B) and unbound or flowthrough (F) fractions. Greater than 90% of the N-cadherin in the extract was recovered in the bound fraction (not shown). The bound and unbound fractions were then treated with SDS to eliminate protein-protein interactions and immunoprecipitated with anti- β -catenin antibody. These precipitates were immunoblotted with anti- β -catenin and anti-phosphotyrosine antibodies. Although similar amounts of β -catenin were present in the B and F fractions, β -catenin that had coprecipitated with N-cadherin (B fraction) was not phosphorylated on tyrosine residues whereas β -catenin that did not co-precipitate with N-cadherin (F fraction) contained phosphorylated tyrosine residues (Fig. 1).

Inhibition of Either Tyrosine Kinases or Tyrosine Phosphatases Results in β -Catenin Containing Phosphorylated Tyrosine Residues, Uncoupling of N-Cadherin from Its Association with Actin and Inhibition of Cadherin-mediated Adhesion

To determine the roles of tyrosine kinases and tyrosine phosphatases in regulating the level of phosphorylated tyrosine residues on β -catenin, E10 retina cells were assayed for cadherin-mediated adhesion and for phosphorylated tyrosine residues on β -catenin in the presence or absence of specific tyrosine kinase and phosphatase inhibitors. Genistein (Akiyama et al., 1987) or herbimycin A (Uehara and Fukazawa, 1991) were used to inhibit tyrosine kinase; per-



anti- β -catenin antibody. The arrowhead at the right labeled β cat. indicates the position of β -catenin. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

Figure 1. β -Catenin phosphorylated on tyrosine residues is not associated with N-cadherin. (Top) Anti-N-cadherin bound (B) and flowthrough (F) fractions from the 14,000 g supernatant of the Triton X-100 homogenate were made 1% in SDS to disrupt protein-protein interactions and immunoprecipitated with anti- β -catenin antibody. The precipitates were fractionated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody PY20. (Bottom) The same transfer was stripped and reprobed with

vanadate (Gordon, 1991; Volberg et al., 1991) or phenylarsine oxide (Levenson and Blackshear, 1989) were used to inhibit tyrosine phosphatase. Both sets of inhibitors result in the inhibition of N-cadherin-mediated adhesion (Fig. 2A). Fig. 2A represents adhesion to anti-N-cadherin antibody NCD-2, however, identical results were obtained

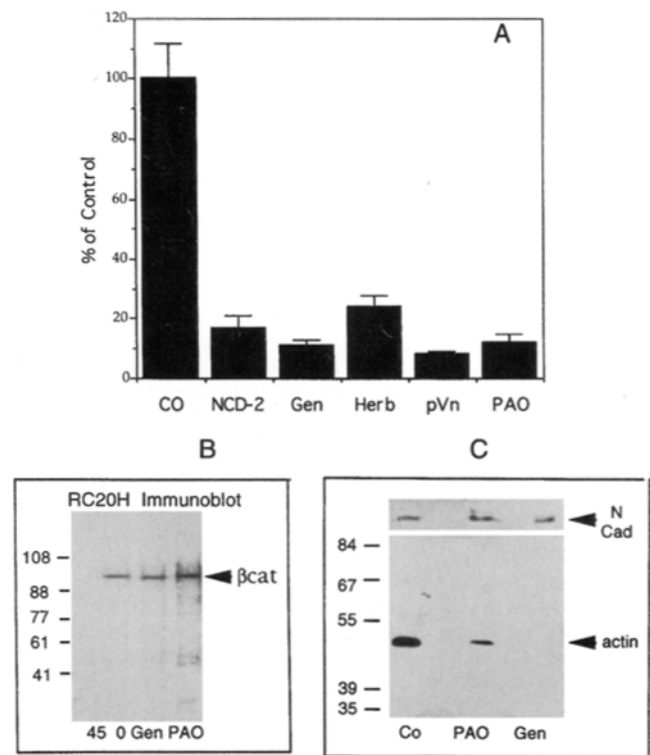


Figure 2. The effect of tyrosine kinase and tyrosine phosphatase inhibitors on N-cadherin-mediated adhesion (A), β -catenin phosphorylation (B), and the association of N-cadherin with actin (C). (A) Adhesion. 3 H-labeled single cells were preincubated with the indicated additive for 45 min and added to microtiter wells coated with anti-cadherin antibody NCD-2. Adhesion was calculated as percent of control value. The data represent the results of three independent experiments and each point is the average of three measurements. Co, no additives; Gen, 150 μ M genistein; Herb., 1 μ g/ml herbimycin; pVn, 2 mM per-vanadate; PAO, 10 μ g/ml phenylarsine oxide. (B) Phosphorylated tyrosine residues on β -catenin. Single cells were either immediately processed for analysis (0) or incubated for 45 min in the presence of: 45, no additives; Gen, 150 μ M genistein; PAO, 10 μ g/ml phenylarsine oxide. H fractions were prepared, immunoprecipitated with anti- β -catenin antibody, the immunoprecipitates fractionated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody PY20. The arrow labeled β cat indicates the position of β -catenin. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$). (C) Association of N-cadherin with actin. Single cells were treated as in B, the H fraction isolated and immunoprecipitated with anti-N-cadherin antibody NCD-2. The immunoprecipitates were fractionated by SDS-PAGE on a 10% gel and immunoblotted with an anti-actin antibody. As a control, the same gel was stripped and immunoblotted with anti-N-cadherin antibody NCD-2 (top). Co, no additions; PAO, 10 μ g/ml phenylarsine oxide; Gen, 150 μ M genistein. The arrowheads labeled N-Cad and actin indicate the position of N-cadherin and actin, respectively. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

using purified N-cadherin as the adhesive substrate (data not shown). Fig. 2 B shows the results of kinase and phosphatase inhibitors on phosphorylation of β -catenin. As seen in intact tissue (Fig. 1), immediately following dissociation of tissue into single cells there is a population of tyrosine phosphorylated β -catenin. However, after 45 min of culture under conditions allowing adhesion, phosphorylated tyrosine residues are no longer detected on β -catenin. Consistent with their inhibitory effect on adhesion, the presence of tyrosine kinase or tyrosine phosphatase inhibitors prevent the removal of phosphate residues from β -catenin (Fig. 2 B). The amount of total β -catenin in each lane is similar (not shown), indicating that the differences represent changes in the degree of tyrosine phosphorylation. Thus adhesion competent cells incubated in suspension for 45 min have a lower level of phosphorylated tyrosine residues on β -catenin than cells in vivo, possibly because during cell preparation endogenous inhibitors (such as the 250-kD PG; Balsamo et al., 1995) are removed, allowing the removal of tyrosine phosphate from β -catenin.

To determine if these drugs also affect the association of N-cadherin with actin, we determined if actin remains associated with the N-cadherin-catenin complex in the rapidly sedimenting fraction (H) isolated from the 14,000 g supernatant from Triton X-100 homogenates of E10 retina cells. We have previously shown that the H fraction contains cadherin- β -catenin-actin complexes in adhesion competent cells, but not in cells rendered adhesion incompetent by treatment with monoclonal anti-GalNAcPTase antibody 1B11 or the GalNAcPTase binding proteoglycan with a 250-kD core protein (Balsamo et al., 1991, 1995; Bauer et al., 1992). Cells were incubated with tyrosine kinase and tyrosine phosphatase inhibitors; the H fraction prepared, immunoprecipitated with anti-N-cadherin antibody NCD-2 and the precipitates analyzed by immunoblot with anti-actin antibody (Fig. 2 C). Indeed, both sets of drugs result in a reduction in the amount of actin associated with N-cadherin.

A PTP1B-like Phosphatase Is Associated with N-Cadherin and Is Itself Tyrosine Phosphorylated

The fact that incubation in the presence of tyrosine phosphatase inhibitors increases the level of phosphate on β -catenin suggests that tyrosine phosphatases are involved in the removal of phosphate from β -catenin. To directly test for the association of protein tyrosine phosphatases with N-cadherin-catenin complexes, the H fraction was passed over an anti-N-cadherin affinity column and the bound and flowthrough fractions analyzed for the presence of tyrosine phosphatases using commercially available antibodies. Strikingly, a \approx 37-kD polypeptide cross-reactive with anti-PTP1B antibody is consistently found associated with the N-cadherin bound fraction, but not in the flowthrough fraction (Fig. 3).

Short term incubations in the presence of tyrosine kinase inhibitors prevent the dephosphorylation of β -catenin seen following preparation of single cells (Fig. 2 B). This suggests that a tyrosine kinase is involved in one or more steps in the removal of phosphate from tyrosine resi-

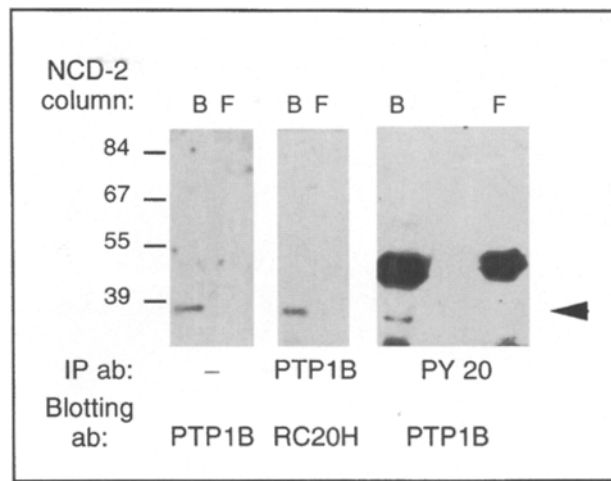


Figure 3. Association of a tyrosine phosphatase with the N-cadherin-catenin complex. Flowthrough (F) and bound (B) fractions from an H preparation fractionated on an anti-N-cadherin affinity column were made 1% in SDS to dissociate protein-protein interactions. After dilution of the SDS to 0.1%, the F and B fractions were immunoprecipitated with the indicated antibody (IP), the immunoprecipitates fractionated by SDS-PAGE on a 7.5% gel and immunoblotted as indicated. PY20 is an anti-phosphotyrosine antibody. The heavily stained bands \approx 55-kD correspond to IgG heavy chain and are absent from the second panel as RC20H is an anti-phosphotyrosine antibody conjugated with HRP and this serves as primary and secondary antibody. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

dues on β -catenin, possibly by modulating the effect of the phosphatase. To test this possibility we determined if the PTP1B-LP itself was tyrosine phosphorylated. The bound and flowthrough fractions from the N-cadherin affinity column were treated with SDS to dissociate protein complexes and immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitated material was fractionated by SDS-PAGE and immunoblotted with anti-PTP1B antibody. The results of this experiment reveal that cadherin-bound PTP1B-LP is indeed tyrosine phosphorylated (Fig. 3). When these antibodies are used in the reverse order (NCD-2 bound and flowthrough fractions precipitated with anti-PTP1B antibody then blotted with anti-phosphotyrosine antibody), similar results are obtained, further indicating that the PTP1B-LP is tyrosine phosphorylated (Fig. 3).

To further reinforce our conclusion that the PTP1B-LP is localized to cadherin/catenin complexes we examined the distribution of the immunoreactive species immunohistochemically. The PTP1B-LP is distributed at the cell periphery (Fig. 4, A and B) and on neurites (Fig. 4 A). This pattern is similar to that seen for β -catenin (Fig. 4 C) and N-cadherin (Fig. 4 D). However, the anti-PTP1B antibody also reveals some cytoplasmic staining consistent with prior reports (Frangioni et al., 1992).

Inhibition of the Tyrosine Phosphorylation of the PTP1B-LP Blocks Its Association with N-Cadherin

To further clarify the significance of tyrosine phosphoryla-

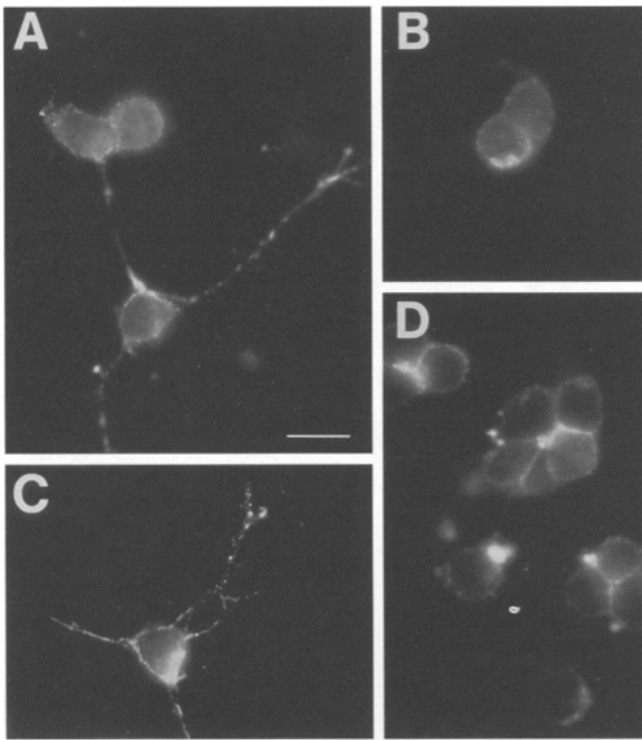


Figure 4. Immunofluorescent localization of the PTP1B-LP, β -catenin and N-cadherin. Single cells cultured on coverslips coated with poly-L-lysine (50 $\mu\text{g}/\text{ml}$ in PBS) were fixed, permeabilized, and reacted with the indicated primary antibody followed by rhodamine-labeled second antibody. Each panel represents a different field. (A and B) Anti-PTP1B antibody; (C) anti- β -catenin antibody; (D) Anti-N-cadherin antibody. Bar, 10 μm .

tion of the PTP1B-LP, we assayed the effect of the tyrosine kinase inhibitor genistein and the tyrosine phosphatase inhibitor phenylarsine oxide on the association of the PTP1B-LP with N-cadherin. Single cells were incubated with or without these inhibitors prior to homogenization and preparation of the H fraction. The N-cadherin-associated fraction was then prepared and analyzed by immunoblot for the presence of the PTP1B-LP (Fig. 5). The PTP1B-LP is co-immunoprecipitated with N-cadherin from control (untreated) cells and cells treated with phenylarsine oxide, but does not co-immunoprecipitate with N-cadherin from the cells treated with genistein (see Table I). Furthermore, when present the PTP-LP is tyrosine phosphorylated (Fig. 5 A). This suggests that the tyrosine phosphorylation of the phosphatase is essential for its association with the cadherin-catenin complex.

We further assayed for tyrosine kinase and phosphatase activities that co-immunoprecipitate with N-cadherin. Cells were treated with genistein or phenylarsine oxide, H fractions prepared, and fractionated on anti-N-cadherin affinity columns. The bound fraction was then assayed for these activities. The tyrosine kinase inhibitor genistein results in the loss of both the kinase and phosphatase activities associated with N-cadherin. In contrast, the tyrosine phosphatase inhibitor phenylarsine oxide results in the loss of only phosphatase activity (Fig. 6). Genistein has no effect on phosphatase activity when added during the as-

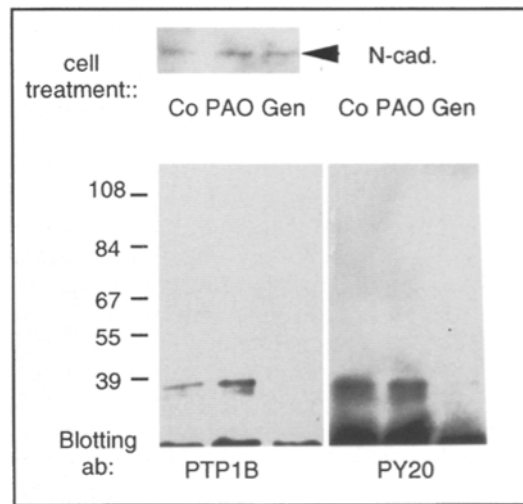


Figure 5. The effect of inhibitors of tyrosine kinase and tyrosine phosphatase on the association of the PTP1B-LP with N-cadherin and phosphorylation of the PTP1B-LP. Single cells were incubated with the indicated additives, H fractions prepared and immunoprecipitated with Sepharose-conjugated NCD-2. The immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with anti-PTP1B antibody or anti-phosphotyrosine antibody PY20. As a control, the same transfer membrane was immunoblotted with NCD-2 (top). Co, control, no additives; PAO, 10 $\mu\text{g}/\text{ml}$ phenylarsine oxide; Gen, 150 μM genistein. The arrow labeled N-cad indicates the position of N-cadherin. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

say (not shown). These results are consistent with the idea that the phosphorylation of the PTP1B-LP (which is inhibited by genistein) is essential for its association with the N-cadherin-catenin complex.

The fact that phosphorylation of the PTP1B-LP is required for binding to N-cadherin and that genistein leads to loss of cadherin-bound PTP1B-LP, suggests that at least a component of the soluble or non cadherin-bound PTP1B-LP should lack phosphorylated tyrosine residues. To determine if this is the case, a cadherin free fraction was prepared to insure that no cadherin-bound PTP1B-LP was present. To accomplish this, the 14,000 g supernatant was fractionated as usual. However, in this case, the material above the $^{20}/_{50}\%$ interface, (i.e., the fraction lacking the vast majority of N-cadherin) was collected and further depleted of N-cadherin by incubation with anti-N-cadherin antibody. The unbound fraction was then immunoprecipitated with anti-phosphotyrosine antibody (RC20B) and the bound and unbound fractions separated by SDS-PAGE and immunoblotted with anti-PTP1B antibody. After this fractionation, PTP1B-LP was detected only in the RC20B unbound fraction (Fig. 7). The RC20B-bound fraction, but not the unbound fraction, does contain several other tyrosine-phosphorylated components indicating that the separation of tyrosine-phosphorylated and -nonphosphorylated components by RC20B is effective (not shown). Thus, the PTP1B-LP not associated with N-cadherin is not tyrosine phosphorylated. Treatment with genistein, which releases the PTP1B-LP from its association with cadherin,

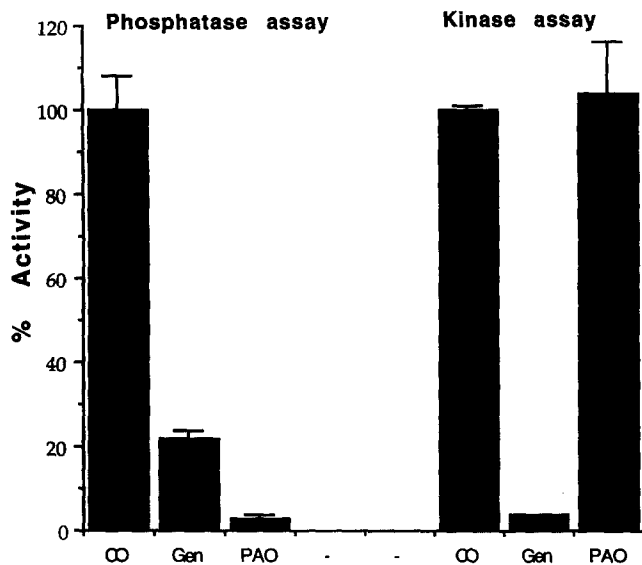


Figure 6. Inhibition of protein tyrosine kinase and tyrosine phosphatase activities in N-cadherin-catenin complexes. Single cells were incubated with the indicated additive, H fractions prepared, fractionated on immobilized anti-N-cadherin antibody NCD-2, and enzyme activity assayed in the bound fraction. The results are presented as percent activity based on 100% for control cells with no additives and 0% for assays carried out in the presence of the appropriate inhibitor (4 mM vanadate for the phosphatase assay and 60 μ M piceatannol for the kinase assay). Each point represents the mean of duplicate measurements and bars represent deviations from the mean. *Gen*, 150 μ M genistein; *PAO*, 10 μ g/ml phenylarsine oxide. (Left) Tyrosine phosphatase activity. (Right) Tyrosine kinase activity.

does cause an apparent increase in the amount of nontyrosine-phosphorylated PTP1B-LP; however, this increase is small which may reflect the fact that the nontyrosine-phosphorylated pool of PTP1B-LP is far larger than the tyrosine-phosphorylated, cadherin-bound pool.

N-Cadherin Binds to Tyrosine-phosphorylated PTP1B-LP

The association of the PTP1B-LP with N-cadherin suggests a direct interaction between the two molecules. To determine if the PTP1B-LP interacts directly with N-cadherin, N-cadherin was purified and labeled with biotin to use as a probe (Fig. 8, top). The rapidly sedimenting fraction (H) was immunoprecipitated with anti-PTP1B-LP, the precipitate fractionated by SDS-PAGE and Western transfers prepared as the target. Biotin-labeled N-cadherin binds specifically to the same polypeptide recognized by the anti-PTP1B antibody (Fig. 8, bottom). Furthermore, cadherin fails to bind to PTP1B-LP which has been dephosphorylated. Whether the PTP1B-LP sample is treated with alkaline phosphatase after separation by SDS-PAGE and transfer (Fig. 8, bottom left) or before separation by SDS-PAGE (not shown), N-cadherin fails to bind to immobilized PTP1B-LP. Taken together these data demonstrate that N-cadherin binds directly to the PTP1B-LP, but only when the PTP1B-LP is tyrosine phosphorylated. Table I summarizes the data linking phosphorylation of the

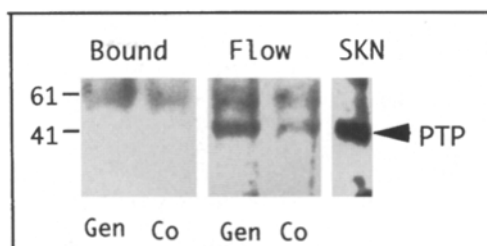


Figure 7. PTP1B-LP that is not associated with N-cadherin is not tyrosine phosphorylated. Single cells were incubated in the presence (*Gen*) or absence (*Co*) of genistein, the 14,000 g supernatant prepared and fractionated as described for the preparation of the H fraction. The material above the 20/50% sucrose interface was incubated with NCD-2 and the precipitates harvested using goat anti-rat IgG conjugated to magnetic beads. The supernatant from the NCD-2 fractionation was further incubated with anti-phosphotyrosine antibody conjugated to biotin (RC20B) and the bound fractions collected using streptavidin conjugated magnetic beads. The bound and unbound (*Flow*) fractions were then resolved by SDS-PAGE and immunoblotted with anti-PTP1B antibody. SKN refers to the cell lysate provided by the vendor as a PTP1B standard. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$). The identity of the crossreactive band present in each lane at approximately 60-kD is unknown.

PTP1B-LP, its association with N-cadherin and N-cadherin function.

Binding of GalNAcPTase Ligands to Cells Results in Inhibition of Tyrosine Kinase and Absence of PTP1B-LP from the Cadherin/Catenin Complex

We have previously demonstrated that interaction of the retina cell surface GalNAcPTase with a proteoglycan with a 250-kD core protein (apparently neurocan) triggers a signaling pathway which results in tyrosine-phosphorylated β -catenin, uncoupling of cadherin from its association with actin, and loss of cadherin function (Balsamo et al., 1995). This effect is similar to that shown above for tyrosine kinase and phosphatase inhibitors and suggests that inhibition of cadherin-mediated adhesion after interaction of the GalNAcPTase with the 250-kD PG ligand might involve alteration of the activity of one or both of these enzymes. To test this hypothesis, cells were incubated with GalNAcPTase ligands, H fractions prepared, and purified on an N-cadherin affinity column. Bound material was assayed for tyrosine kinase and phosphatase activities. Binding of the 250-kD core protein as well as anti-GalNAcPTase antibody 1B11 to cells bearing the GalNAcPTase results in inhibition of protein tyrosine phosphatase activity (Fig. 9, top) and protein tyrosine kinase activity (Fig. 9, bottom). In contrast, anti-GalNAcPTase antibody 7A2, which binds equally well to the cell surface GalNAcPTase, is without effect, as is a mixture of 400-kD proteoglycan core proteins including versican, aggrecan, and phosphacan.

This is identical to the effects observed with tyrosine kinase inhibitors, suggesting that the binding of the 250-kD PG to the GalNAcPTase results in inhibition of a tyrosine kinase which prevents phosphorylation of the PTP1B-LP

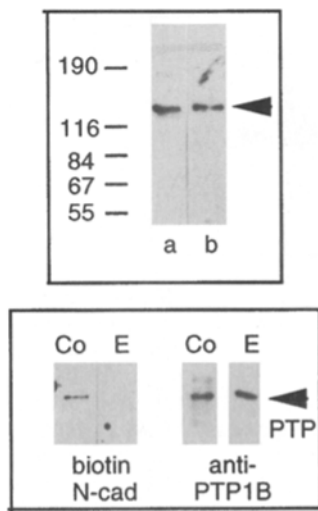


Figure 8. Binding of purified N-cadherin to immobilized PTP1B-LP. (Top) Biotin labeled purified N-cadherin was fractionated by SDS-PAGE, transferred to PVDF membrane and probed with HRP-streptavidin (a) or NCD-2 (b). The arrow indicates the position of N-cadherin. (Bottom) The PTP1B-LP was immunoprecipitated from an H preparation with anti-PTP1B antibody, fractionated by SDS-PAGE and transferred to PVDF. The membranes were incubated with 10 U/ml alkaline phosphatase in the presence (Co) or absence of (E) the phosphatase inhibitor 4 mM *o*-vanadate (Co). The membranes were then reacted with purified, biotin-labeled N-cadherin (biotin/N-cad), followed by HRP-streptavidin. The same membranes were stripped and reacted with anti-PTP1B antibody (anti-PTP1B). The arrow labeled PTP indicates the position of the PTP1B-LP. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

and therefore prevents association of the PTP1B-LP with N-cadherin. Indeed, in cells treated with anti-GalNAcPTase antibody 1B11 or the 250-kD core protein preparation, but not anti-GalNAcPTase antibody 7A2 or the 400-kD core protein, the PTP1B-LP is not associated with N-cadherin (Fig. 10).

Blocking the Tyrosine Phosphorylation of β -Catenin Eliminates the Ability of GalNAcPTase Ligands and Phosphatase Inhibitors to Inhibit Cadherin-mediated Adhesion

Taken together the results described above indicate that binding of the 250-kD PG to the GalNAcPTase inhibits

Table 1. Cadherin-mediated Adhesion Requires Bound, Functional PTP1B-LP*

Cell Treatment	Cadherin mediated adhesion	PTP1B-LP associated with N-cadherin	PTP1B-LP is tyrosine phosphorylated	Phosphatase associated with N-cadherin
None (control) [‡]	Yes	Yes	Yes	Yes
Phenylarsine oxide [§]	No	Yes	Yes	No
Genistein [¶]	No	No	No	No

*For a full explanation of the results summarized here see the data in Fig. 5, 6, and 7.
[‡] Among control cells cadherin is fully functional. The PTP1B-LP is tyrosine phosphorylated and bound to the cytoplasmic domain of N-cadherin.

[§] Treatment of cells with the phosphatase inhibitor, phenylarsine oxide, inactivates cadherin. The drug directly inhibits N-cadherin-associated phosphatase activity, but has no effect on the tyrosine phosphorylation of the PTP1B-LP or its association with N-cadherin.

[¶] Treatment of cells with the tyrosine kinase inhibitor, genistein, also inactivates cadherin. This drug results in a loss of N-cadherin-associated phosphatase activity as a consequence of the fact that it inhibits the tyrosine phosphorylation of the PTP1B-LP, and thus its association with N-cadherin.

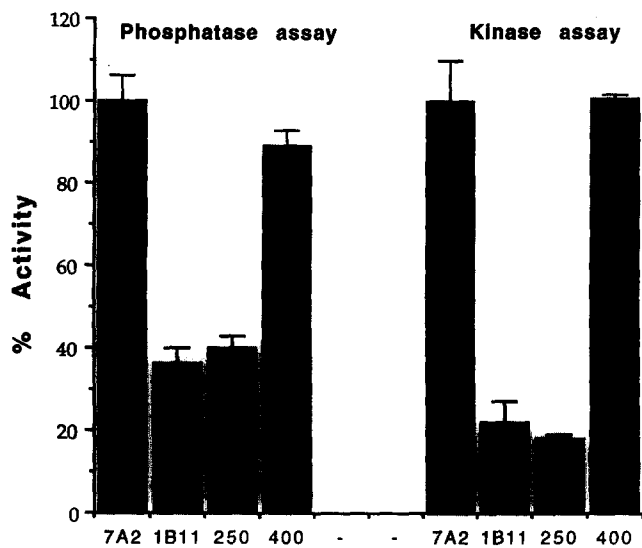


Figure 9. Inhibition of protein tyrosine kinase and tyrosine phosphatase activities in N-cadherin-catenin complexes in response to binding of GalNAcPTase ligands. Single cells were incubated with the indicated additive, H fractions prepared, fractionated on immobilized anti-N-cadherin antibody NCD-2, and enzyme activity assayed in bound fraction. The results are presented as percent activity based on 100% for control cells with no additives and 0% for assays carried out in the presence of the appropriate inhibitor (4 mM vanadate for the phosphatase assay and 60 μ M piceatannol for the kinase assay). Each point represents the mean of duplicate measurements and bars represent deviations from the mean. 7A2, 10 μ g/ml anti-GalNAcPTase mAb 7A2; 1B11, 10 μ g/ml anti-GalNAcPTase mAb 1B11; 250, 10 μ g/ml 250-kD core proteoglycan; 400, 10 μ g/ml 400-kD core proteoglycan. (Left) Tyrosine phosphatase activity; (right) tyrosine kinase activity.

cadherin-mediated adhesion by inhibiting a tyrosine kinase. This results in lack of phosphorylation of the PTP1B-LP, preventing its association with N-cadherin and its ability to dephosphorylate β -catenin. This hypothesis suggests that cells lacking phosphorylated β -catenin will form cadherin-mediated adhesions in the presence of GalNAcPTase ligands or inhibitors of tyrosine phosphatase. To test this hypothesis we prepared cells with reduced levels of phosphotyrosine residues by overnight incubation in culture medium containing herbimycin A. The cells were then assayed for the presence of phosphorylated β -catenin and N-cadherin-mediated adhesion in the presence or absence of GalNAcPTase ligands or tyrosine phosphatase inhibitors. To ensure that the preincubation resulted in cells lacking phosphorylated β -catenin, the rapidly sedimenting fraction (H) was immunoprecipitated with anti- β -catenin antibody and analyzed by immunoblot with anti-phosphotyrosine antibody. While the amount of β -catenin is unaffected by prolonged herbimycin treatment (Fig. 11, top left), the herbimycin-treated cells have a significantly lower level of phosphorylated tyrosine residues on β -catenin than do control cells (Fig. 11, top right).

While phosphorylated tyrosine residues are presumably absent from many cellular proteins following herbimycin treatment, the ability of cells to adhere via an N-cadherin-mediated mechanism is not compromised as evidenced by the ability of anti-N-cadherin to inhibit adhesion. Further-

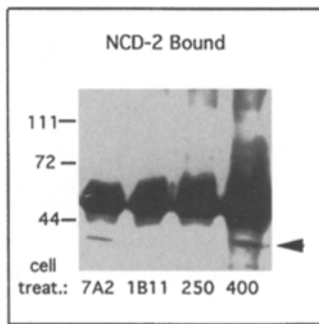


Figure 10. Effect of GalNAcPTase ligands on the association of the PTP1B-LP with N-cadherin. Single cells were incubated for 45 min at 37°C in the presence of the GalNAcPTase mAbs 7A2 or 1B11, or in the presence of the 250- or 400-kD proteoglycans (see Fig. 6 for concentrations). H fractions were prepared and fractionated using anti-N-cadherin antibody. The immunoprecipitated material was fractionated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-PTP1B antibody. The arrow indicates the position of the PTP1B-LP. The heavy band at ~55 kD is immunoglobulin heavy chain. The migration of standard proteins is indicated at the left by their molecular mass ($\times 10^{-3}$).

more, ~80% of the cells are competent to form N-cadherin-mediated adhesions after incubation in the presence or absence of herbimycin. However, after prolonged herbimycin treatment, cadherin-mediated adhesion is no longer inhibited by the 250-kD PG (not shown) or anti-GalNAcPTase antibody 1B11 (Fig. 11, *bottom*). Furthermore, the tyrosine phosphatase inhibitor phenylarsine oxide is also without effect (Fig. 11, *bottom*). These observations suggest that prolonged herbimycin treatment blocks the constitutive phosphorylation of β -catenin and thereby eliminates the need for an N-cadherin-associated tyrosine phosphatase to dephosphorylate β -catenin in order to maintain the adhesion competence of cells.

Discussion

Our data indicate that in embryonic chick retina tissue and cells the presence of phosphorylated tyrosine residues on β -catenin is inversely related to the association of N-cadherin with the actin containing cytoskeleton and the ability of cells to adhere via N-cadherin. Furthermore, the level of phosphorylated tyrosine residues on β -catenin depends on the function of a PTP1B-like phosphatase associated with N-cadherin, an association dependent on tyrosine phosphorylation of the PTP1B-LP. Agents which inhibit the activity of the PTP1B-LP (i.e., tyrosine phosphatase inhibitors) or its binding to N-cadherin (i.e., tyrosine kinase inhibitors) or binding of the 250-kD PG to the cell surface GalNAcPTase; Balsamo et al., 1995), result in the accumulation of phosphorylated tyrosine residues on β -catenin, uncoupling of cadherin from its association with the cytoskeleton and loss of N-cadherin function.

Phosphorylated Tyrosine Residues on β -Catenin Are an Indicator of the Integrity of the Cadherin/Actin Linkage

The correlation between the presence of phosphorylated tyrosine residues on β -catenin and loss of cadherin-mediated adhesion is consistent with reports from other laboratories. Transformation of cells bearing E-cadherin with *v-src* leads to an invasive phenotype, with a concomitant loss of cadherin function and the presence of phosphorylated ty-

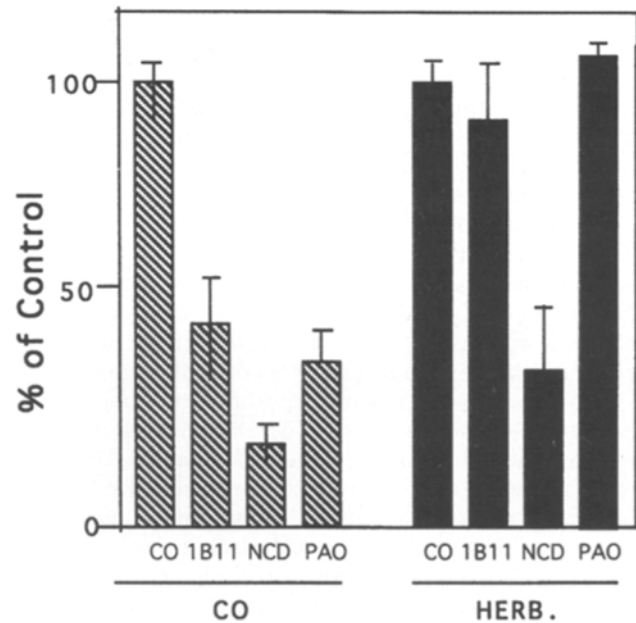
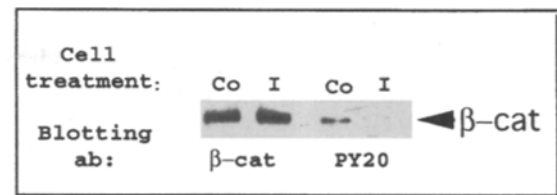


Figure 11. Effect of long term treatment of cells with herbimycin A on phosphorylated tyrosine residues on β -catenin and on N-cadherin-mediated adhesion. Single cells were incubated overnight in medium with or without 1 μ g/ml herbimycin A and assayed for phosphorylated tyrosine residues on β -catenin and cadherin-mediated adhesion. (*Top*) Phosphorylated tyrosine residues on β -catenin. The H fraction was incubated with anti- β -catenin antibody and the immunoprecipitated material was fractionated by SDS-PAGE and immunoblotted with anti- β -catenin antibody or anti-phosphotyrosine antibody. Co, no additives; I, cells incubated overnight with herbimycin A. The arrowhead labeled β -cat marks the position of β -catenin. (*Bottom*) N-cadherin-mediated adhesion. Single cells were allowed to adhere to microtiter wells coated with purified N-cadherin in the presence of the indicated additives. CO, control, no additives; 1B11, 10 μ g/ml anti-GalNAcPTase mAb 1B11; NCD, 10 μ g/ml NCD-2; PAO, 10 μ g/ml phenylarsine oxide. Results are presented as percent of control values and represent the average of three measurements. Error bars indicate the standard deviation.

rosine residues on β -catenin (Matsuyoshi et al., 1992; Behrens et al., 1993). Similarly, suppression of N-cadherin-mediated adhesion after transformation of chick embryo fibroblasts with Rous sarcoma virus correlates with the presence of phosphorylated tyrosine residues on β -catenin (and α -catenin) (Hamaguchi et al., 1993). Treatment of human carcinoma cells with hepatocyte growth factor or epidermal growth factor enhances dispersal of cells, concomitant with the appearance of phosphorylated tyrosine residues on β -catenin (Shibamoto et al., 1994).

In contrast to other reports (Matsuyoshi et al., 1992; Behrens et al., 1993, Hamaguchi et al., 1993, Shibamoto et al., 1994) we do not find tyrosine phosphorylated β -catenin to

be associated with cadherin, even though the 14,000 g supernatant analyzed here includes the Triton X-100 high speed supernatant analyzed in these other studies. These observations may be reconciled if the presence of phosphorylated tyrosine residues on β -catenin does not completely eliminate the ability of β -catenin to interact with cadherin, but rather reduces its affinity for cadherin. Thus there may be a small amount of tyrosine-phosphorylated β -catenin that remains associated with cadherin, but this comprises a very small proportion when compared to tyrosine-phosphorylated β -catenin not associated with N-cadherin. Furthermore, the ability to detect this residual population of tyrosine-phosphorylated β -catenin associated with cadherin may depend on the cells being examined and the specific techniques being used.

We have previously shown that loss of cadherin function is correlated with loss of the association of cadherin with actin in both epithelial (Bauer et al., 1992) and neural cells (Balsamo et al., 1991, 1995). In this manuscript we extend these observations, showing that tyrosine kinase and phosphatase inhibitors also result in loss of cadherin-mediated adhesion and loss of the integrity of the cadherin-actin connection. A recent study of adherens junctions also suggests that the behavior of a rapidly sedimenting fraction, similar to our H fraction, reflects the function of E-cadherin. After ras transformation, the integrity of the adherens junction and the function of E-cadherin are compromised. This is correlated with displacement of β -catenin from a rapidly sedimenting pool into a soluble pool and the detection of tyrosine-phosphorylated β -catenin primarily in the soluble pool. Furthermore, after herbimycin treatment of ras-transformed cells, which decreases phosphorylation of many proteins and restores the integrity of the adherens junction, β -catenin is found in a rapidly sedimenting fraction in association with E-cadherin (Kinch et al., 1995).

A PTP1B-LP Is Associated with N-Cadherin

Our data strongly suggest that the dephosphorylation of tyrosine residues in β -catenin is essential for cells to form cadherin-mediated adhesions and that this depends on a PTP1B-LP associated with N-cadherin and the N-cadherin-catenin complex. Conditions which prevent association of the PTP1B-LP with N-cadherin (inhibition of tyrosine kinase activity), or which result in loss of PTP1B-LP activity (inhibition of tyrosine phosphatase activity), invariably lead to the accumulation of phosphorylated tyrosine residues on β -catenin and loss of cadherin-mediated adhesion.

The association of the PTP1B-LP with N-cadherin is evidenced by their co-isolation. The significance of this interaction is most effectively illustrated by the lack of association of the PTP1B-LP with cadherin under conditions which result in loss of cadherin function (the presence of tyrosine kinase inhibitors or GalNAcPTase ligands). Moreover we have shown that purified N-cadherin interacts directly with the PTP1B-LP on Western transfers.

The association of the PTP1B-LP with cadherin depends on the phosphorylation state of tyrosine residues on the PTP1B-LP; only tyrosine phosphorylated PTP1B-LP is associated with cadherin. This association is blocked by

drugs that prevent tyrosine phosphorylation. Furthermore, removal of phosphate from the PTP1B-LP prevents its association with N-cadherin as assayed by Western overlays. The transmembrane phosphatase PTP μ has also been reported to associate with the intracellular domain of cadherin (Brady-Kalnay et al., 1995); however, it is not yet possible to determine if the two phosphatases interact through the same or an overlapping domain. A key difference may be the requirement for tyrosine phosphorylation of the PTP1B-LP in order for it to interact with N-cadherin.

The requirement for tyrosine phosphorylation of the PTP1B-LP to bind to N-cadherin implicates a tyrosine kinase as a critical component of the signaling pathway controlling the level of phosphorylated tyrosine residues on β -catenin and therefore cadherin function. Whether this kinase is the same one that is required for tyrosine phosphorylation of β -catenin (see discussion below) remains to be determined.

Analysis of recombinant PTP's has revealed several distinct sequences which target them to specific cellular locations (Mauro and Dixon, 1994). However, more relevant to our studies, tyrosine phosphorylation of PTP's has been demonstrated to produce a consensus site for interaction with SH2 domains (Sun and Tonks, 1994). We searched the carboxy terminal cytoplasmic domain of N-cadherin for SH2 (see Margolis, 1992) domains and as well as PID domains which also are recognized by peptides containing phosphorylated tyrosine residues (Kavanaugh and Williams, 1994). We were unable to identify unequivocally any such sequence motifs in N-cadherin. This suggests that tyrosine phosphorylated PTP1B-LP interacts with a sequence motif present in N-cadherin not (yet) recognized as a site for interaction with phosphorylated tyrosine residues or that phosphorylated tyrosine residues are not directly involved in this interaction.

We have referred to the PTP1B immunoreactive species as a PTP1B-like phosphatase because of its immunological cross reactivity with characterized antibodies and the similarity of its apparent molecular mass to the human placental enzyme (Tonks et al., 1988). The monoclonal antibody used in these studies was prepared against a protein fragment corresponding to amino acids 269 through 435 of the human enzyme. This sequence encompasses a portion of the conserved phosphatase domain and the COOH-terminal 35-amino acid targeting domain and could well cross-react with other related PTP's. PTP1B has been reported to be primarily localized to the endoplasmic reticulum by virtue of the COOH-terminal targeting domain (Frangioni et al., 1992). However, it has been stated that PTP1B is also present at focal adhesions (Mauro and Dixon, 1994), a locale more in keeping with the localization of the tyrosine phosphatase identified in this study. While these similarities suggest that the tyrosine phosphatase identified here is closely related to PTP1B, definitive identification will have to await molecular characterization.

Phosphorylation/Dephosphorylation of β -Catenin: a Dynamic Equilibrium

Our data suggest the hypothesis that there is an equilibrium between phosphorylation and dephosphorylation of

β -catenin which controls the integrity of the cadherin cytoskeletal linkage (Fig. 12). This equilibrium may be perturbed by a variety of transmembrane signals altering cell behavior during development. For example, binding of a 250-kD PG to the GalNAcPTase results in inhibition of cadherin-mediated adhesion concomitant with the accumulation of phosphorylated tyrosine residues on β -catenin and uncoupling of cadherin from its association with the actin containing cytoskeleton (Balsamo et al., 1995).

Here we show that the increased levels of phosphorylated tyrosine residues on β -catenin after binding of the 250-kD PG appear to be due to inhibition of a tyrosine kinase activity essential for the phosphorylation of the PTP1B-LP, binding of the PTP1B-LP to N-cadherin and dephosphorylation of β -catenin. Lack of PTP1B-LP bound to N-cadherin is correlated with the accumulation of phosphorylated tyrosine residues on β -catenin. As one test of this proposed chain of events, we incubated cells overnight in the presence of a protein tyrosine kinase inhibitor in order to prepare cells which lack phosphorylated tyrosine residues on β -catenin. Cadherin-mediated adhesion among these cells occurs at a normal level; however, this adhesion is no longer inhibited by the 250-kD PG or by inhibitors of tyrosine kinase or phosphatase. Thus, inhibition of cadherin-mediated adhesion through the interaction of the 250-kD PG with the GalNAcPTase or by inhibitors of tyrosine kinase and phosphatase requires that cells are capable of phosphorylating β -catenin and further reinforces our conclusion that regulated removal of phosphate from β -catenin is a critical event controlling cadherin function. Together these data suggest that, under normal conditions, there is a dynamic equilibrium between phosphorylation and dephosphorylation of tyrosine residues on β -catenin (see Fig. 12).

Binding of ligand to two transmembrane tyrosine kinase receptors, the epidermal growth factor and the scatter factor/hepatocyte growth factor receptors, has also been shown to result in accumulation of phosphorylated tyrosine residues on β -catenin and the loss of cadherin-mediated adhesion (Shibamoto, 1995). The cytoplasmic domain of the EGF receptor tyrosine kinase has been reported to associate directly with β -catenin and to phosphorylate β -catenin on activation by EGF (Hoschuetzky et al., 1994). The closely related transmembrane tyrosine

kinase *c-erbB-2* (Yamamoto et al., 1986) has also been reported to associate directly with β -catenin (Ochiai et al., 1994b; Kanai et al., 1995) and is thus positioned appropriately to directly phosphorylate β -catenin and therefore downregulate cadherin function. Consistent with this possibility, amplification or overexpression of *c-erbB-2* has been correlated with highly malignant tumors and poor prognosis (Slamon et al., 1987). The SF/HGF-receptor, *c-met*, may act similarly. As the name implies, interaction of SF/HGF with its receptor does appear to be one trigger for cells to assume a migratory phenotype (i.e., lose their characteristic cell-cell adhesion). Indeed, the interaction of SF/HGF with *c-met* is essential for the migration of myogenic precursors into the limb (Bladt et al., 1995). In each case, the increased tyrosine phosphorylation of β -catenin may overwhelm the ability of the PTP1B-LP or other phosphatases to dephosphorylate β -catenin, resulting in loss of cadherin-mediated adhesion.

While the 250-kD PG/GalNAcPTase interaction, as well as interaction of transmembrane receptor tyrosine kinases with their receptors, have the potential to downregulate cadherin function, the transmembrane phosphatase PTP μ has the potential to upregulate cadherin function in much the same manner as we are proposing for the PTP1B-LP. PTP μ interacts directly with N-cadherin (Brady-Kalnay et al., 1995) and may be specifically activated through homophilic interaction (Brady-Kalnay et al., 1993) creating opportunities for temporal or spatial control of cadherin function. We have not yet determined if the PTP1B-LP and the PTP μ are both present in the same complex. The presence of two distinct phosphatases in the same complex might add to the potential for precise control of N-cadherin function through the interaction of other cell surface proteins with their ligands. One set of signals may cause inactivation or loss of the PTP1B-LP, increasing the accumulation of phosphorylated tyrosine residues on β -catenin; another set of signals may activate the transmembrane phosphatase, thereby reducing the level of phosphorylated tyrosine residues on β -catenin.

Our hypothesis is also consistent with the effect of kinase and phosphatase inhibitors on the integrity of adherens junctions which depend on E-cadherin. We propose that junctional integrity is lost after treatment with phosphatase inhibitors due to the inability of cells to dephos-

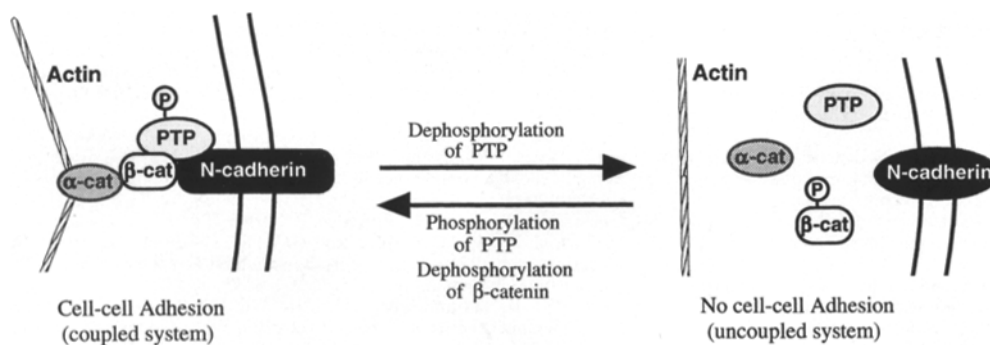


Figure 12. Diagram depicting the effect of the phosphorylation or dephosphorylation of the PTP1B-LP on its association with N-cadherin and the concomitant alteration in the state of tyrosine phosphorylation of β -catenin and the integrity of the N-cadherin-actin connection. Our data suggest that tyrosine phosphorylation of the PTP1B-LP results in its association with N-cadherin,

dephosphorylation of β -catenin, and association of N-cadherin with the actin containing cytoskeleton (via α - and β -catenin), resulting in adhesion competent cells. In contrast, dephosphorylation of the PTP1B-LP results in its inability to associate with N-cadherin, accumulation of phosphorylated tyrosine residues on β -catenin, dissociation of the cadherin-actin connection, and loss of adhesive competence. The illustrated changes in intermolecular association involving α -catenin are speculation.

phorylate β -catenin. Furthermore, restoration of junctional integrity by tyrosine kinase inhibitors, after transformation of cells with *src*, may be due to a reduction in the rate or level of tyrosine phosphorylation of β -catenin (Volberg et al., 1992; see also Kinch et al., 1995). It should be emphasized that these effects are seen following long term incubations and are analogous to the results seen in our system after long term incubation with tyrosine kinase inhibitors. While *src* is present at adherens junctions (Tsukita et al., 1991) and could directly phosphorylate β -catenin, it is not yet clear whether β -catenin is in fact phosphorylated directly by *src* or by a more complex pathway involving other kinases.

In summary, β -catenin appears to play a central role in the translation of extracellular information into altered cadherin function. One class of external ligands: e.g., the 250-kD PG, hepatic growth factor and epidermal growth factor, bind to specific cell surface receptors, decreasing the stability of cadherin-mediated adhesion by maintaining a high level of phosphorylated tyrosine residues on β -catenin. At least two distinct pathways are possible: loss of the ability to remove phosphorylated tyrosine residues from β -catenin due to loss of the PTP1B-LP from the cadherin-catenin complex, as demonstrated for binding of the 250-kD PG; and enhanced phosphorylation of β -catenin, through direct activation of associated tyrosine kinases, the likely explanation for the effects of EGF and HGF. A second class of ligands, e.g., the *Wnt* gene product, also probably bind to specific cell surface receptors, resulting in an increase in the amount of β -catenin associated with cadherin and increasing the stability of cadherin-mediated adhesion (Hinck et al., 1994a; Bradely et al., 1994). Thus, cadherin function may be controlled through the activation of at least two distinct types of signaling pathways, both ramifying on the stability and/or extent of linkage of cadherins with the cytoskeleton (see also Hinck et al., 1994b). We are just beginning to appreciate the diversity of molecular interactions impinging on control of cadherin function. It is already clear that the temporal and spatial control of cadherin function through ligand receptor interactions, which themselves are temporally and spatially controlled, has the potential to profoundly affect the course of development.

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