The Nonreceptor Protein Tyrosine Phosphatase PTP1B Binds to the Cytoplasmic Domain of N-Cadherin and Regulates the Cadherin–Actin Linkage

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Abstract. Cadherin-mediated adhesion depends on the association of its cytoplasmic domain with the actin-containing cytoskeleton. This interaction is mediated by a group of cytoplasmic proteins: α-and β- or γ- catenin. Phosphorylation of β-catenin on tyrosine residues plays a role in controlling this association and, therefore, cadherin function. Previous work from our laboratory suggested that a nonreceptor protein tyrosine phosphatase, bound to the cytoplasmic domain of N-cadherin, is responsible for removing tyrosine-bound phosphate residues from β-catenin, thus maintaining the cadherin–actin connection (Balsamo et al., 1996). Here we report the molecular cloning of the cadherin-associated tyrosine phosphatase and identify it as PTP1B. To definitively establish a causal relationship between the function of cadherin-bound PTP1B and cadherin-mediated adhesion, we tested the effect of expressing a catalytically inactive form of PTP1B in L cells constitutively expressing N-cadherin. We find that expression of the catalytically inactive PTP1B results in reduced cadherin-mediated adhesion. Furthermore, cadherin is uncoupled from its association with actin, and β-catenin shows increased phosphorylation on tyrosine residues when compared with parental cells or cells transfected with the wild-type PTP1B. Both the transfected wild-type and the mutant PTP1B are found associated with N-cadherin, and recombinant mutant PTP1B binds to N-cadherin in vitro, indicating that the catalytically inactive form acts as a dominant negative, displacing endogenous PTP1B, and rendering cadherin nonfunctional. Our results demonstrate a role for PTP1B in regulating cadherin-mediated cell adhesion.

Key words: β-catenin • cadherin • protein tyrosine phosphatase • cell–cell adhesion

C ELL–CELL adhesion mediated by the cadherin family of calcium-dependent cell adhesion molecules is crucial for normal embryonic development. For example, the neural cadherin, N-cadherin, plays an essential role in heart development (Radice et al., 1997) and in the outgrowth of nerve fibers (Bixby et al., 1987; Matsunaga et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988; Doherty et al., 1991) and the epithelial cadherin, E-cadherin, is essential for normal implantation (Larue et al., 1994; Riethmacher et al., 1995) and the formation of normal epithelial morphology (Nelson, 1994; Tepass et al., 1996; Uemura et al., 1996). It is now well established that cadherin function depends on its association with the actin-containing cytoskeleton (Nagafuchi and Takeichi, 1988; Kintner, 1992; Fujimori and Takeichi, 1993). This association is mediated by the cytoplasmic proteins, α- and β- or γ-catenin. β-catenin is bound to the cytoplasmic domain of cadherin and interacts with α-catenin, which in turn binds to actin either directly, or through α-actinin (Knudsen et al., 1995; Rimm et al., 1995).

Phosphorylation of β-catenin on tyrosine residues appears to be an important mechanism for regulating cadherin function (for review see Lilien et al., 1997). Our work in embryonic chick neural retina cells demonstrated that only the population of β-catenin not associated with N-cadherin is phosphorylated on tyrosine residues (Balsamo et al., 1995). The level of tyrosine phosphorylation on β-catenin appears to be controlled by a protein tyrosine phosphatase that interacts directly with the cytoplasmic domain of N-cadherin (Balsamo et al., 1996). The phosphatase itself must be tyrosine phosphorylated in order to associate with N-cadherin. Agents that inhibit activity of the phosphatase or its tyrosine phosphorylation result in accumulation of tyrosine-phosphate on β-catenin. The end

result is the uncoupling of N-cadherin from the cytoskeleton, and concomitant loss of cadherin function. In this study we demonstrate that the N-cadherin–associated phosphatase is the nonreceptor protein tyrosine phosphatase PTP1B. Using L cells constitutively expressing N-cadherin and transfected with a catalytically inactive form of PTP1B, we present evidence that decreasing or eliminating cadherin-bound phosphatase activity disrupts cadherin function, increases the level of tyrosine phosphorylation on β-catenin, and uncouples N-cadherin from its association with the cytoskeleton. Thus PTP1B acts as a switch, modulating cadherin function by dephosphorylating β-catenin and maintaining an intact cadherin–cytoskeleton interaction.

Materials and Methods

Cloning of PTP1B and Preparation of Expression Vectors

Chick PTP1B cDNA clones were isolated from a 10-d-old embryonic chick neural retina cDNA ZapII library. A human PTP1B cDNA (provided by J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA) containing the COOH-terminal region was used to screen the cDNA library. Several clones were isolated, including a 1.9-kb clone containing the complete open reading frame (ORF)[1] and both 5′ and 3′ untranslated regions. A sequence of 27 nucleotides corresponding to a human hemagglutinin sequence (HA) was added in frame at the 5′ end of the complete PTP1B ORF. Catalytically inactive PTP1B, in which cysteine 215 (within the consensus catalytic domain) was changed to serine, was obtained by site-directed mutagenesis using recombinant PCR (Guan and Dixon, 1990). HA-tagged wild-type (wt) and mutant (mut) chkPTP1B were cloned into the eukaryotic expression vector pcDNA3.1/zeo (Invitrogen, Carlsbad, CA) for introduction into L cells. Wt and mut chkPTP1B were also cloned into the pEGFP-C3 vector (Clontech Laboratories, Palo Alto, CA), for eukaryotic expression of GFP fusion protein.

Wt and mut chkPTP1B containing a c-myc tag at the 5′ end were cloned in pGEX-KG and expressed as glutathione-S-transferase (GST) fusion proteins in TKB1 bacteria (Stratagene, La Jolla, CA). This bacterial strain carries a tyrosine kinase gene and is able to phosphorylate a wide variety of proteins. Recombinant PTP1B was purified from bacterial cell lysates on glutathione–Sepharose 4B (Pharmacia Biotech, Piscataway, NJ) and phosphatase activity was determined using a Boehringer Mannheim Biochemicals (Indianapolis, IN) phosphatase assay kit. For the in vitro binding assays to N-cadherin, the GST fusion protein was cleaved with thrombin after binding to the glutathione affinity column (Guan and Dixon, 1991) and then pure recombinant PTP1B was eluted. All constructs were verified by sequencing.

Antibodies

Chicken-specific polyclonal anti-PTP1B (chkPTP) was prepared in rabbits using a synthetic peptide specific to the chick PTP1B (amino acids 357 to 367; see Fig. 1 A) as immunogen (Bio-Synthesis Inc., Lewisville, TX). The antiserum was affinity purified on immobilized peptide. This antibody shows no cross reactivity with species present in L cells. Two other anti-PTP1B antibodies were used: TLPTP (monoclonal mouse IgG from Transduction Laboratories [TL], Lexington, KY) recognizes chicken, as well as many other species but not mouse PTP1B; and UBIPTP (polyclonal rabbit IgG from Upstate Biotechnology Inc., Lake Placid, NY) recognizes both species. The anti–N-cadherin antibody NCD-2 (Hatta and Takeichi, 1986) was produced as described (Balsamo et al., 1991). Anti–β-catenin antibody is a polyclonal rabbit IgG prepared from a 15-amino

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1. *Abbreviations used in this paper*: GFP, green fluorescent protein; GST, glutathione-S-transferase; HA, hemagglutinin; mut, mutant; ORF, open reading frame; PVDF, polyvinylene difluoride; RT, reverse transcription; TL, Transduction Laboratories; wt, wild-type.

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acid synthetic peptide derived from the published sequence (Butz et al., 1992). The antiphosphotyrosine antibody PY20 and the HRP-conjugated RC20H are mouse monoclonal IgGs obtained from TL. Anti-actin is a mouse IgG monoclonal from Chemicon Inc. (Temecula, CA). Anti-HA and anti-GFP are rabbit polyclonal antibodies purchased from Berkeley Antibody Co. (Berkeley, CA) and Clontech Laboratories, respectively. Anti–c-myc is a mouse monoclonal IgG from Santa Cruz Biotechnology (Santa Cruz, CA). Enzyme (AP or HRP)-conjugated anti-mouse, -rat, or -rabbit IgG were from Cappel Laboratories (ICN Pharmaceuticals, Costa Mesa, CA). The secondary antibodies conjugated to magnetic beads used in immunoprecipitations were obtained from PerSeptive Diagnostics (Cambridge, MA).

Other Materials

N-cadherin, used as a substrate for adhesion assays and for in vitro binding assays, was purified from embryonic chick brains as described previously (Bixby and Zhang, 1990; Balsamo et al., 1991). These preparations are routinely analyzed for contaminating proteins after biotinylation, fractionation by SDS-PAGE, and reaction with HRP-streptavidin and anti–N-cadherin antibody followed by HRP goat anti–rat IgG (Balsamo et al., 1996).

Cell Fractionation

Retina homogenates were fractionated into a plasma membrane-enriched and ER-enriched fractions, as described by Frangioni et al. (1992) with minor modifications. In brief, 12 neural retinas dissected from day 9 embryos were homogenized in 50 mM Tris, pH 7.5, containing 0.25 M sucrose, 5 mM EGTA, 3 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM o-vanadate, 5 μg/ml leupeptin, 5 μg/ml DNase, and 5 μg/ml calpeptin. The postnuclear supernatant was centrifuged at 150,000 g, and the pellet was resuspended in 1 ml of the same buffer and layered on a step gradient consisting of 1 ml of 2 M sucrose and 1 ml of 1.2 M sucrose prepared in homogenization buffer. The sample was centrifuged at 100,000 g for 2.5 h at 4°C. The interfaces between 0.25 and 1.2 M sucrose (plasma membrane) and 1.2 and 2 M sucrose (ER) were collected and assayed for protein content using the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were fractionated on SDS-polyacrylamide gels and immunoblotted with anti-chkPTP.

Transfection of L Cells

L cells constitutively expressing N-cadherin were obtained by transfection with the mammalian expression vector pHβApr-1/neo (provided by L. Kedes, University of Southern California, Los Angeles, CA), containing the full length N-cadherin cDNA. This vector uses the β-actin gene promoter to control the expression of the inserted DNA and the neomycin resistance gene as the selection system. Stable lines were selected and maintained in the presence of G418 (GIBCO BRL, Grand Island, NY). Clones of cells expressing high levels of N-cadherin, as determined by immunoblotting with anti–N-cadherin antibody and by their ability to adhere to an N-cadherin–coated substrate (Balsamo et al., 1991), were expanded and used for transfection with PTP1B. pcDNA3.1/zeo vectors containing wild-type or mutant full-length or truncated PTP1B were transfected into LN cells using Lipofectin (GIBCO BRL). Stable cells lines were selected with 1mg/ml Zeocin (Invitrogen) and 400 μg/ml G418. Vector without insert was used as a control. Several clonally derived cell lines, expressing both N-cadherin and the wild-type or mutant PTP1B, were obtained. These cell lines were maintained for ~2 wk before freezing for use at a future time. Results of representative lines are shown.

LN cells were also transfected with wt and mut chkPTP1B cloned into the pEGFP-C3 vector (Clontech Laboratories) and used for analysis 72 h later.

Expression of Transfected cDNAs

To determine protein expression, confluent cell layers in 100-mm dishes were rinsed free of serum and solubilized in MLB buffer (1% NP-40, 0.15 M NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 50 mM NaF, 0.2 mM Na vanadate, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml calpeptin, and 100 μg/ml DNase). The lysates were centrifuged at 15,000 g and aliquots of the supernatant containing equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylene difluoride (PVDF) membranes. The transfers were immunoblotted with the antibodies indicated in the figures as described previously (Balsamo et al., 1995).

Adhesion Assays

Cell layers were washed free of serum and harvested using 0.1% trypsin (GIBCO BRL) in buffer containing 1 mM Ca^{2+}. Single cells were aliquoted to 96-well plates previously coated with purified N-cadherin, anti–N-cadherin antibody NCD-2, or with monolayers of LN cells as described previously (Balsamo et al., 1991). The number of cells adhering to the substrate was estimated after 45 min at 37°C by measuring the amount of staining with crystal violet in a microplate reader (Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitations

To determine the association of N-cadherin with PTP1B and actin, transfected L cells grown to confluency were washed in serum-free medium and lysed in MLB buffer. After centrifugation at 14,000 g, equivalent amounts of supernatant protein were immunoprecipitated with the indicated antibody (5 μg IgG/ml of cell lysate) for 4 h at 4°C. The precipitate was collected with the appropriate IgG-conjugated magnetic beads, washed several times with MLB, separated by SDS-PAGE, and immunoblotted as described in the figure legends. To determine the association of β-catenin with N-cadherin, transfected cells were lysed and immunoprecipitated as described above. The N-cadherin immunoprecipitated fraction was separated by SDS-PAGE and immunoblotted with anti–β-catenin antibody. The N-cadherin–free fraction was made 1% in SDS, boiled for 2 min, diluted with MLB to 0.1% SDS, and then immunoprecipitated with anti–β-catenin antibody. The immunoprecipitate was fractionated by SDS-PAGE and blotted with the antiphosphotyrosine antibody PY20. The membranes were stripped according to the Bio-Rad Laboratories protocol and reprobed with the anti–β-catenin antibody.

Immunofluorescence

LN cells were transfected with either the pEGFP-C3 vector alone or with vector containing wt or mut chkPTP1B. After 48 h, the cells were plated on acid-washed, round coverslips precoated with serum. After an overnight incubation, the cells were fixed in 3% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and then blocked with 3% BSA in PBS for 1 h at room temperature. The cells were then incubated with anti-GFP or anti–β-catenin antibody for 1 h at 37°C, washed three times in PBS, and then incubated with fluorescein- or rhodamine-conjugated goat anti–rabbit IgG for 1 h at 37°C. After extensive washes in PBS, the cells were mounted in 0.1 M Tris, pH 8.5/glycerol (1:1, vol/vol), containing 1 mg/ml p-phenylenediamine, and observed with a Zeiss laser confocal microscope (model LSM 310; Carl Zeiss Inc., Thornwood, NY).

Results

Isolation and Analysis of Chick PTP1B cDNA Clones

The chicken N-cadherin–associated PTP is recognized by an anti-PTP1B antibody, from TL, raised to a peptide encompassing the entire COOH terminus (amino acids 269–435) in the human PTP1B sequence (Balsamo et al., 1996). We used a fragment of the human PTP1B cDNA (Chernoff et al., 1990) encompassing this sequence to screen a 10-d-old embryonic chick retina cDNA library. Several
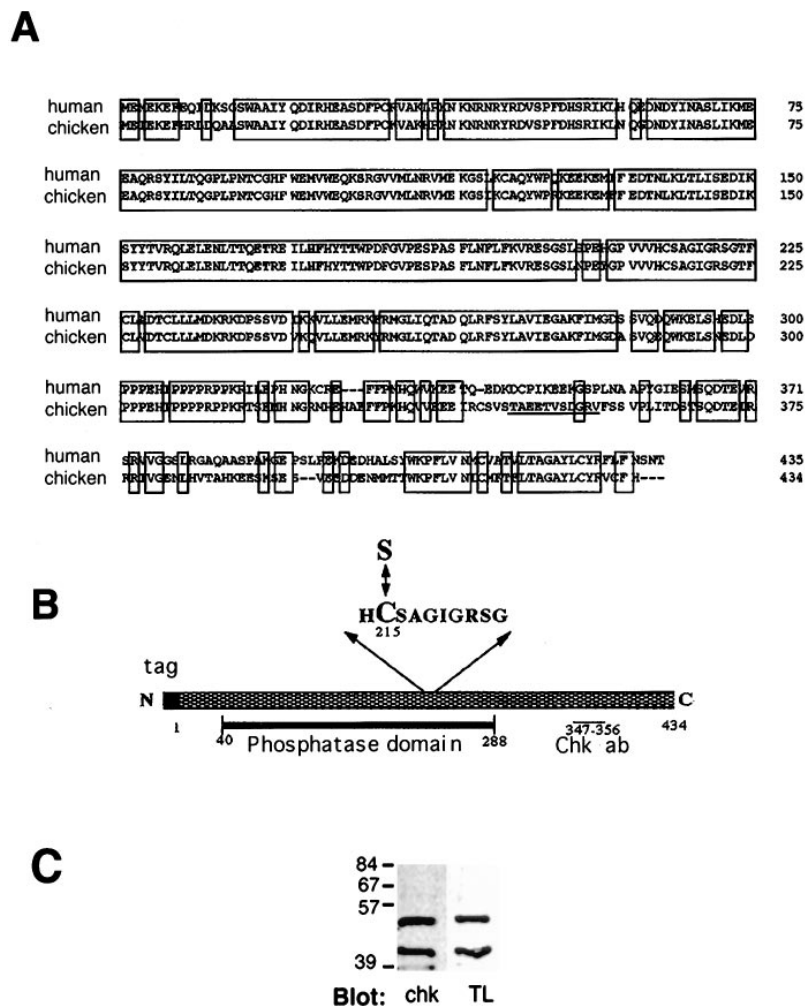
Figure 1. Chick PTP1B cDNA and protein. (*A*) Comparison of the amino acid sequence of chick PTP1B (GenBank/EMBL/DDBJ accession number U86410) and its human homologue PTP1B (GenBank/EMBL/DDBJ accession number M31724). Alignments were done with Geneworks software (Intelligenetics, Campbell, CA). *Boxes*, identical residues; *underline*, unique chicken sequence used to raise a polyclonal antibody. (*B*) Recombinant chick PTP1B was constructed with a c-myc or HA tag in frame at the NH2 terminus of the full-length PTP1B sequence. *Thick underline*, consensus PTP domain. The position of the chicken specific sequence is indicated (amino acids 347–356). Catalytically inactive PTP1B was constructed by site-directed mutagenesis in which cysteine 215, within the consensus PTP catalytic domain, was changed to serine. (*C*) A Triton X-100 homogenate of embryonic chick neural retinas was fractionated by SDS-PAGE, transferred to a PVDF membrane, and then immunoblotted with the chicken-specific antibody (*chk*) or the anti-PTP1B antibody from TL. *Left*, numbers indicate the migration of the molecular mass standards.

overlapping clones were isolated, one of which was 1.9 kb and contained the complete ORF, including 3′ and 5′ untranslated regions. The predicted amino acid sequence of this cDNA insert shows 75% identity with human PTP1B (Fig. 1 *A*) and codes for a polypeptide of 431 amino acids, corresponding to a molecular mass of 49 kD. We conclude that this clone represents the chicken homologue of human PTP1B (chkPTP1B).

To verify that this clone codes for the cadherin-associated PTP1B, we used an amino acid sequence specific to chkPTP1B in order to generate a rabbit polyclonal antibody (anti-chkPTP1B) and used this antibody to examine the distribution of chkPTP1B and its association with N-cadherin. This sequence is underlined in Fig. 1, *A* and *B* (amino acids 347–356). The antibody recognizes the GST fusion protein (data not shown) and predominant bands at ∼50 and ∼40 kD in chick retina homogenates separated by SDS-PAGE (Fig. 1 *C*, *chk*). This is similar to the pattern seen in retina homogenates fractionated by SDS-PAGE and immunoblotted with the anti-human PTP1B antibody we used previously (TL) (Fig. 1 *C*) (Balsamo et al., 1996).

To demonstrate that the chick PTP1B localizes, at least in part, to the plasma membrane, we separated a crude membrane fraction from chick retina into low and high density fractions, corresponding to the plasma membrane and endoplasmic reticulum, respectively. Aliquots of each were separated by SDS-PAGE and transfers immunoblotted with the anti-chkPTP1B antibody. PTP1B is present in both membrane fractions, however, the ER-enriched fraction (Fig. 2 *A*, *M1*) contains both the ∼50- and ∼40-kD forms, whereas the plasma membrane fraction (Fig. 2 *A*, *M2*) is enriched for a ∼40-kD form. To further demonstrate that chkPTP1B does indeed associate with N-cadherin, retina cell homogenates were immunoprecipitated with the anti–N-cadherin monoclonal antibody NCD-2, the precipitates fractionated by SDS-PAGE and immuno-

Figure 2. Distribution of chick PTP1B in neural retina and association with N-cadherin in retina and N-cadherin–expressing L cells. (*A*) Homogenates of embryonic chick neural retina were fractionated into ER-enriched (*M1*) and plasma membrane-enriched (*M2*) fractions. Each were separated by SDS-PAGE, transferred to PVDF, and then immunoblotted with the anti-chkPTP1B antibody. (*B*) Chick neural retina or N-cadherin–expressing L cells were homogenized in neutral detergent, immunoprecipitated with the anti–N-cadherin antibody NCD-2 (*NCD*) or control rat IgG (*Co*). The precipitates were separated by SDS-PAGE, transferred to PVDF, and then immunoblotted with anti-chkPTP1B antibody. *Left*, numbers indicate the migration of the molecular mass standards.

blotted with anti-chkPTP1B antibody (Fig. 2 *B*). The predominant form of chkPTP1B associated with N-cadherin migrates at ∼40 kD.

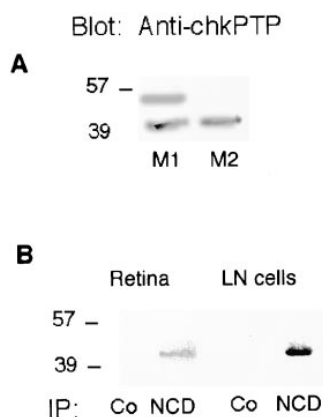## Establishment of Stable Cell Lines Expressing both N-cadherin and PTP1B

To examine the role of PTP1B in N-cadherin–mediated adhesion, we first generated a cell line stably transfected with chick N-cadherin cDNA using the neomycin resistance gene as the selection system (LN cells). These cells express N-cadherin constitutively and show N-cadherin–mediated adhesion and the altered phenotype reported previously (Hatta et al., 1988) (see Fig. 4 *B*). L cells constitutively expressing cadherin have been shown to have α- and β-catenin associated with cadherin (Butz and Kemler, 1994). Based on our data with retina cells we further analyzed cadherin complexes immunoprecipitated from LN cells for the presence of PTP1B. Immunoblots of homogenates separated by SDS-PAGE do indeed show cadherin-associated PTP1B (Fig. 2 *B*).

To analyze the role of PTP1B in N-cadherin–mediated cell–cell adhesion, we created a dominant-negative form of the chkPTP1B lacking phosphatase activity by introducing a single amino acid substitution within the catalytic domain, changing cysteine 215 to serine (C215S; Guan and Dixon, 1990) (Fig. 1 *B*). We hypothesized that the mutant form of the enzyme would compete with endogenous PTP1B for association with N-cadherin and thus block N-cadherin function. Both the wild-type and the C215S chkPTP1B were expressed as GST fusion proteins and assayed for tyrosine phosphatase activity in vitro. As expected, the mutant construct shows no phosphatase activity, whereas the wild-type enzyme is fully active (data not shown).
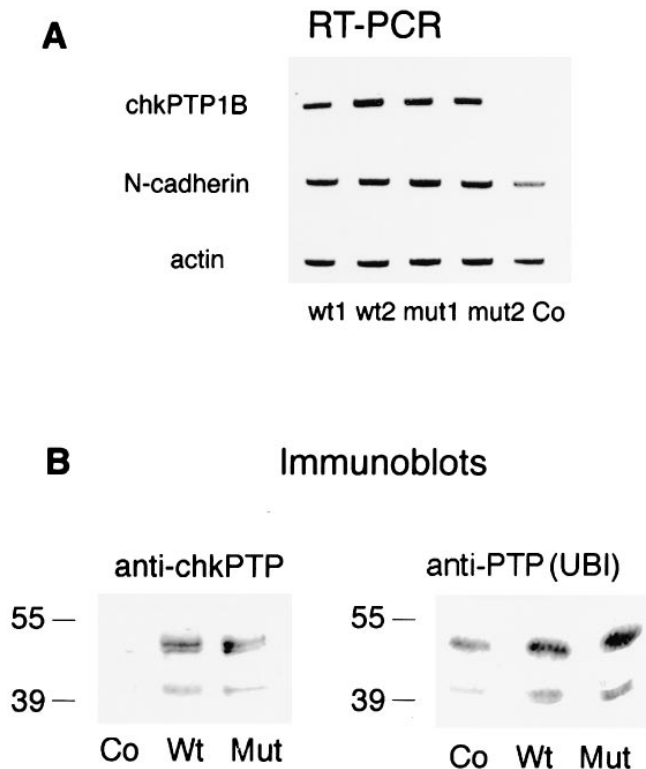
Both the wt and C215S mut chkPTP1B were cloned into an eukaryotic expression vector and transfected into LN cells. Cells were also transfected with vector lacking the insert as controls (Fig. 2 *B*, *Co*). To easily differentiate between endogenous and transfected chkPTP1B, we incorporated the human HA nonapeptide in frame at the 5′ end of the chkPTP1B (Fig. 1 *B*). Stable cell lines expressing both N-cadherin and wt or mut chkPTP1B or vector alone were selected using neomycin and zeocin. We confirmed that the transfected cells were indeed expressing chkPTP1B mRNA by reverse transcription (RT)-PCR, using primers corresponding to the tag sequence and to a unique chick PTP1B sequence and by immunoblot using anti-chkPTP1B. Both procedures enabled us to distinguish endogenous mouse PTP1B from transfected chicken PTP1B.

RT-PCR reveals the presence of chick-specific transcripts (Fig. 3 *A*) and the anti-chkPTP1B antibody recognizes a major band at ∼50 kD and another at ∼40 kD in homogenates of cells transfected with wild-type or mutant chkPTP1B, but not in homogenates of control transfected LN cells (Fig. 3 *B*, *anti-chkPTP*). To determine the relative increase in total PTP1B expression after transfection of cells, we immunoblotted SDS-PAGE of lysates of wild-type, mutant, and control transfected cells with a pan-specific anti-PTP1B antibody (UBI) (Fig. 3 *B*, *anti-PTP UBI*). Densitometric analysis of the band at ∼50 kD in wild-type and mutant transfectants shows an approximately twofold

A RT-PCR

chkPTP1B

N-cadherin

actin

wt1 wt2 mut1 mut2 Co

B Immunoblots

anti-chkPTP

55 —

39 —

Co Wt Mut

anti-PTP(UBI)

55 —

39 —

Co Wt Mut

Figure 3. Expression of N-cadherin and chick PTP1B in transfected cells as detected by RT-PCR and immunoblot. (*A*) RT-PCR was performed using primers specific to tagged chkPTP1B, N-cadherin, and actin to ensure that comparable amounts of material were used. (*B*) Expression of chick PTP1B peptide in transfected cells. Cell lysates were fractionated by SDS-PAGE, transferred to PVDF membrane, and then immunoblotted with the indicated antibodies. *Co*, parental N-cadherin–expressing cells; *Wt*, LN cells transfected with wild-type PTP1B; *Mut*, LN cells transfected with mutant PTP1B. *Left*, numbers indicate the migration of the molecular mass standards.

increase over control values (Fig. 3 *B*). Similar analysis of the band at ~40 kD reveals an even greater increase; however, we find more variability in this ratio.

RT-PCR was also used to evaluate the expression of N-cadherin; neither wt- nor mut-chkPTP1B had any effect on expression of N-cadherin (Fig. 3 *A*). In addition, cell surface expression of N-cadherin was not altered by transfection of LN cells with the PTP1B constructs, as determined by FACS (data not shown). Similarly, expression of β-catenin was not altered by transfection with the chkPTP1B, as determined by immunoblots (data not shown). Furthermore, expression of the transfected wild-type or mutant chkPTP1B does not cause a noticeable change in the overall pattern of tyrosine phosphorylation, as determined by immunoblot of cell lysates separated by SDS-PAGE with an antiphosphotyrosine antibody (data not shown).

Cadherin-mediated Cell–Cell Adhesion Is Lost in Cells Expressing Catalytically Inactive PTP1B

LN cells expressing wild-type and mutant PTP1B were assayed for their ability to adhere to LN cells and to wells coated with affinity-purified N-cadherin or anti–N-cadherin antibody, as described previously (Balsamo et al., 1991). The advantage of the latter assay is that it unambiguously measures only adhesion due to N-cadherin. The results are comparable in all assays; two independently isolated clones of LN cells expressing mut-chkPTP1B show markedly reduced N-cadherin–mediated adhesion when compared with untransfected LN cells or to LN cells trans-

A

B
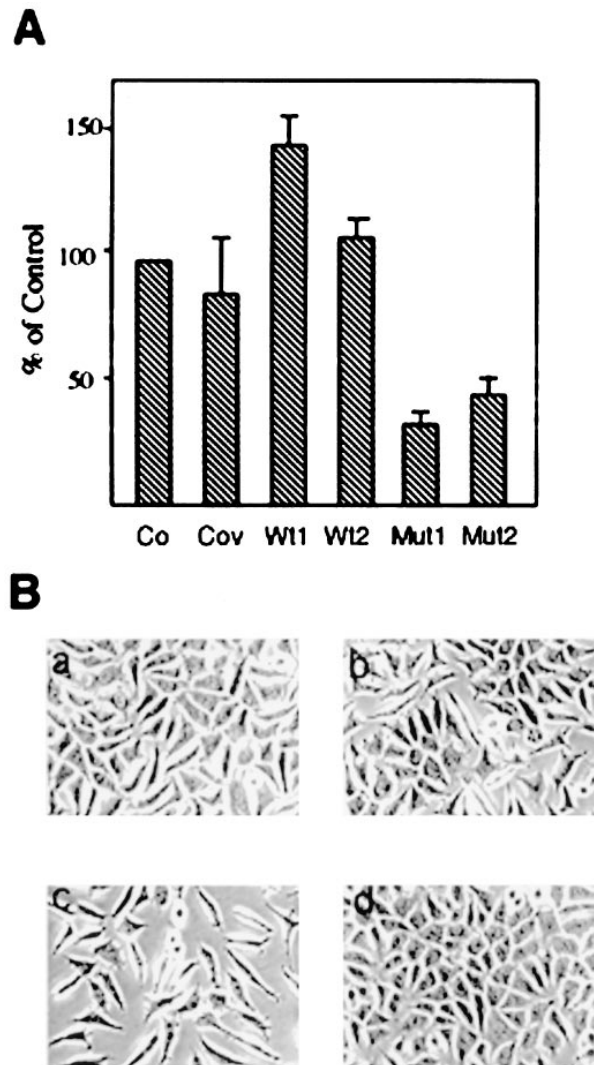
Figure 4. Expression of mutant PTP1B inhibits N-cadherin–mediated adhesion. (*A*) Adhesion assays. Cells were harvested using 0.1% trypsin (GIBCO BRL) in buffer containing 1 mM Ca^{2+}. Single cells were plated on 96-well plates coated with anti–N-cadherin antibody NCD-2. Cells adhering to the substrate were measured after 45 min by staining with crystal violet. *Co*, LN cells; *Cov*, LN cells transfected with vector lacking insert; *Wt*, LN cells transfected with the wild-type PTP1B; *Mut*, LN cells transfected with the mutant PTP1B. Adhesion is plotted as percent of control values. Each point is the average of three measurements. The results presented are from one experiment representative of many. (*B*) Phase–contrast micrographs showing the phenotype of control LN cells (panel *a*), LN cells transfected with vector alone (panel *b*), LN cells transfected with mutant PTP1B (panel *c*), and LN cells tranfected with wild-type PTP1B (panel *d*).

fected with control vector (Fig. 4 *A*). In contrast, two independent isolates of LN cells transfected with wt-chk-PTP1B show slightly increased adhesion (Fig. 4 *A*).

The loss in cell–cell adhesion among LN cells transfected with mutant chkPTP1B is reflected in their phenotype (Fig. 4 *B*). Twofold overexpression of the wild-type chkPTP1B has no detectable effect on the phenotype of the transfected cells (Fig. 4 *B*, panel *d*; compare with control cells with and without vector in panels *a* and *b*). On the other hand, LN cells transfected with mutant chkPTP1B have a spindle-like morphology (Fig. 4 *B*, panel *c*).

Catalytically Inactive PTP1B Correlates with Increased Levels of Tyrosine Phosphate Residues on β-Catenin and Loss of the Cadherin–Actin Connection

To determine if loss of cadherin-mediated adhesion after transfection with mut-chkPTP1B correlates with increased phosphorylation of tyrosine residues on β-catenin and loss of its association with N-cadherin, cell lysates prepared in buffer containing neutral detergent were immunoprecipitated with anti–N-cadherin antibody NCD-2, separating the lysate into N-cadherin–bound and –free fractions. The N-cadherin–bound fraction has greatly reduced levels of β-catenin after transfection with the mutant, as compared with wild-type chkPTP1B (Fig. 5 *A*), and in neither case is β-catenin phosphorylated on tyrosine residues (data not shown). Consistent with this, analysis of the free fraction reveals an increase in β-catenin in cells transfected with mut-chkPTP1B (Fig. 5 *B*). Furthermore, in the N-cadherin–free fraction, more β-catenin is phosphorylated on tyrosine residues in cells transfected with mut-chkPTP1B as compared with cells transfected with wild-type chk-PTP1B (Fig. 5 *B*). Thus, transfection with the dominant-negative, catalytically inactive PTP1B, but not wild-type PTP1B, results in retention of phosphate on tyrosine residues of β-catenin and loss of its association with cadherin.

β-catenin, through α-catenin, serves as the link between N-cadherin and the actin-containing cytoskeleton, an association essential for N-cadherin function. In retina cells, this linkage is reflected in the coprecipitation of actin with N-cadherin only when β-catenin is not phosphorylated on tyrosine residues (Balsamo et al., 1995, 1996). In either control LN cells or LN cells transfected with wt-chk-PTP1B, actin also coprecipitates with N-cadherin (Fig. 6 *A*). However, in LN cells transfected with the mut-chk-PTP1B, the amount of actin associated with N-cadherin is

IP: NCD-2

A

Blot: anti-actin

57 —

36 —

← actin

Co Mut Wt
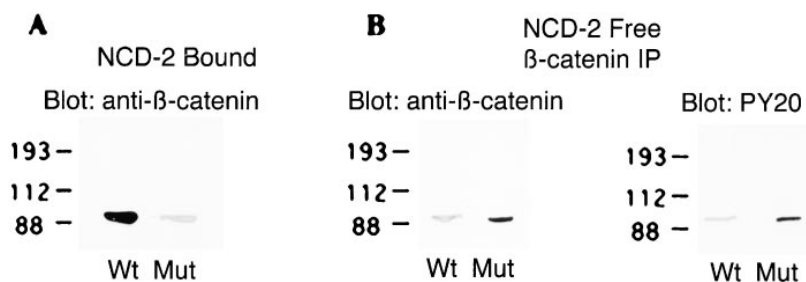
B

Blot: NCD-2

181 —

116 —

← N-cad

Co Mut Wt
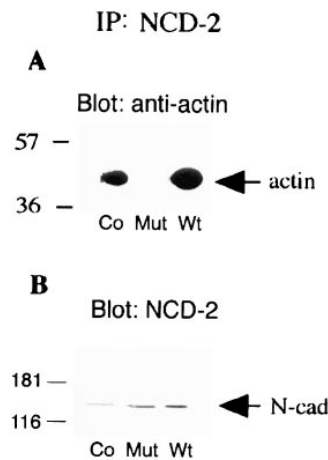
Figure 6. The association of N-cadherin with actin is disrupted in cells transfected with mutant PTP1B. LN cells were transfected with wild-type (*Wt*), or mutant (*Mut*) chkPTP1B or with vector alone and immunoprecipitated with anti–N-cadherin antibody NCD-2. The immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with (*A*) anti-actin antibody or (*B*) NCD-2. *Left*, numbers indicate the migration of the molecular mass standards.

greatly reduced (Fig. 6 *A*). In both cases the amount of N-cadherin precipitated remains constant (Fig. 6 *B*).

PTP1B Associates both In Vivo and In Vitro with N-Cadherin and This Association Is Dependent on Its Tyrosine Phosphorylation
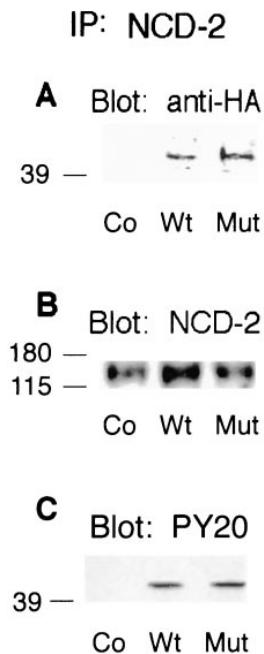
In neural retina cells, PTP1B associates with N-cadherin and is localized at points of cell–cell contact (Balsamo et al., 1996). To confirm the association of chkPTP1B with the N-cadherin complex in transfected cells, homogenates from cells transfected with wt- and mut-chkPTP1B were immunoprecipitated with the anti–N-cadherin antibody NCD-2. NCD-2 precipitates the ∼40-kD chicken-specific PTP1B (Fig. 7 *A*), along with N-cadherin (Fig. 7 *B*). Furthermore, N-cadherin–associated PTP1B is phosphorylated on tyrosine residues (Fig. 7 *C*). As we expected, substitution of cysteine 215 with serine had no effect on the phosphorylation of the mutant PTP1B or its association with N-cadherin (Fig. 7 *C*). The association of the chk-PTP1B with the N-cadherin complex is blocked by the tyrosine kinase inhibitor Genistein (data not shown), supporting our earlier observation in chick retina cells (Balsamo et al., 1996).

To determine the cellular expression pattern of the transfected chkPTP1B, LN cells were transiently transfected with wt- or mut-chkPTP1B with enhanced green flourescent protein (GFP) fused in frame at the NH_2 terminus (GFP–chkPTP1B). At 72 h the cellular distribution

A

NCD-2 Bound

Blot: anti-ß-catenin

193 —

112 —

88 —

Wt Mut

B

NCD-2 Free
ß-catenin IP

Blot: anti-ß-catenin

193 —

112 —

88 —

Wt Mut

Blot: PY20

193 —

112 —

88 —

Wt Mut

Figure 5. β-catenin shows increased levels of tyrosine-phosphate in LN cells transfected with mutant PTP1B. LN cells transfected with either wild-type (*Wt*) or mutant (*Mut*) PTP1B were lysed in neutral detergent, immunoprecipitated with anti–N-cadherin antibody NCD-2, and then separated into bound and free fractions. (*A*) The NCD-2–bound fractions were separated by SDS-PAGE and immunoblotted with anti–β-catenin antibody. (*B*) The NCD-2–free fractions were immunoprecipitated with anti–β-catenin antibody, separated by SDS-PAGE, and then immunoblotted with anti–β-catenin antibody or antiphosphotyrosine antibody PY20. *Left*, numbers indicate the migration of the molecular mass standards.

IP: NCD-2

A Blot: anti-HA

39 —

Co Wt Mut

B Blot: NCD-2

180 —
115 —

Co Wt Mut

C Blot: PY20

39 —

Co Wt Mut

Figure 7. ChkPTP1B associates with N-cadherin in transfected LN cells. LN cells transfected with HA-tagged chick PTP1B were homogenized in neutral detergent-containing buffer and immunoprecipitated with anti–N-cadherin antibody NCD-2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with (*A*) anti-HA antibody, (*B*) anti–N-cadherin antibody NCD-2, and (*C*) anti-phosphotyrosine antibody PY20. *Co*, LN cells transfected with vector alone; *Wt*, LN cells transfected with the wild-type PTP1B; *Mut*, LN cells transfected with the mutant PTP1B. *Left*, numbers indicate the migration of the molecular mass standards.

of GFP was examined by laser confocal microscopy. LN cells transfected with vector containing GFP alone show uniform fluorescence throughout the cytoplasm and the characteristic morphology of N-cadherin–transfected cells, including localization of β-catenin at cell boundaries (Fig. 8, *A* and *B*). For optimal visualization of potential membrane localization, fields with isolated GFP-expressing cells were chosen. As expected, cells expressing the wt or mut GFP–chkPTP1B show extensive perinuclear localization characteristic of ER (Fig. 8, *C* and *E*) (Arregui et al., 1998). Under these conditions it is clear that cells expressing wt-GFP–chkPTP1B are labeled at sites of cell–cell contact (Fig. 8, *C* and *D*). This is not apparent in cells expressing mut-GFP–chkPTP1B (Fig. 8, *E* and *F*). In the LN cells bearing mutant PTP1B, N-cadherin is still expressed at the cells surface as analyzed by FACS (data not shown) and the ∼40-kD form of PTP1B is associated with N-cadherin (Figs. 7 and 9). The fact that there is no well-defined concentration of fluorescence at the cell periphery may be attributed to the diffuse distribution of N-cadherin due to lack of regions of cell–cell contact.

To insure that the GFP–chkPTP1B was actually associated with the N-cadherin complex, populations of cells transfected with the GFP–PTP1B were lysed in nonionic detergent and immunoprecipitated with anti–N-cadherin antibody. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-GFP antibody (Fig. 9 *A*). The GFP–chkPTP1B fusion protein associated with N-cadherin migrates at an apparent molecular mass of ∼70 kD, consistent with the combined molecular mass of GFP (27 kD) and the N-cadherin–associated PTP1B (∼40 kD). A similar protocol was used to demonstrate that transfected chkPTP1B actually displaces endogenous PTP1B from its association with N-cadherin. Identical Western transfers were blotted with the pan-specific anti-PTP1B antibody: in this case the larger molecular mass of the GFP fusions facilitates the distinction between endog-

enous PTP1B and the chkPTP1B. Both endogenous and transfected chkPTP1B are found associated with the N-cadherin complex; however, endogenous PTP1B is reduced in the transfectants as compared with the controls (Fig. 9 *B*).

Finally, to determine if chkPTP1B interacts directly with N-cadherin, we assayed the interaction between purified N-cadherin and recombinant chkPTP1B in vitro. The purified N-cadherin was biotinylated and analyzed by SDS-PAGE to insure that it was free of contaminating proteins, particularly those components known to be associated with cadherin complexes (see Balsamo et al., 1996). The c-myc–tagged mut chkPTP1B was produced as a GST fusion protein in a tyrosine kinase positive bacterial strain (TKB1). Purified N-cadherin was incubated with the tagged mut-chkPTP1B and the mixture immunoprecipitated with anti–c-myc antibody. The presence of N-cadherin in the immunoprecipitates was determined by immunoblot with NCD-2 after separation by SDS-PAGE (Fig. 10). N-cadherin is immunoprecipitated by the anti–c-myc antibody after incubation with mut chkPTP1B that is phosphorylated on tyrosine residues. When mut chkPTP1B is treated with a tyrosine phosphatase before incubation with N-cadherin, no interaction is observed (Fig. 10).

Discussion

Our data indicate that PTP1B plays a role in maintaining cadherin in a functional state. This state is characterized by the integrity of the cadherin–β-catenin–α-catenin–actin connection. Displacement of active PTP1B from the cytoplasmic domain of N-cadherin by transfection of cells with an inactive form of PTP1B results in enhanced tyrosine phosphorylation of β-catenin, and loss of its association with cadherin and the actin-containing cytoskeleton. Thus, PTP1B plays an important role in controlling cadherin function by dephosphorylating tyrosine residues on β-catenin, and maintaining the cadherin–cytoskeletal linkage.

The association of PTP1B with cadherin is regulated by its tyrosine phosphorylation. This, in turn, is regulated by the interaction between a chondroitin sulfate proteoglycan with its cell surface receptor, a cell surface glycosyltransferase (GalNAcPTase). This interaction initiates a signal which blocks phosphorylation of PTP1B resulting in increased tyrosine phosphorylation of β-catenin and loss of cadherin function (Balsamo et al., 1996).

PTP1B appears to be involved in many different cellular functions; these diverse functions must depend on specific targeting of the enzyme inside the cell. The enzyme is localized to the cytoplasmic face of the ER via the last 35 amino-acid residues at the COOH terminus (Frangioni et al., 1992). It is cleaved by calpain in response to integrin stimulation in platelets, with concomitant relocation from the ER to the cytosol (Frangioni et al., 1993). A cleaved, ∼42-kD form of PTP1B, as well as the full-size enzyme, have also been localized to the platelet cytoskeleton in response to thrombin stimulation (Ezumi et al., 1995). Although cleavage appears to be one mechanism through which PTP1B is retargeted within the cell, the intact ∼50-kD enzyme does interact with the insulin receptor upon insulin stimulation (Seely et al., 1996; Kenner et al., 1996; Bandyopadhyay et al., 1997) and the EGF receptor (Milarski et al., 1993; Flint et al., 1997; Liu and Chernoff, 1997).
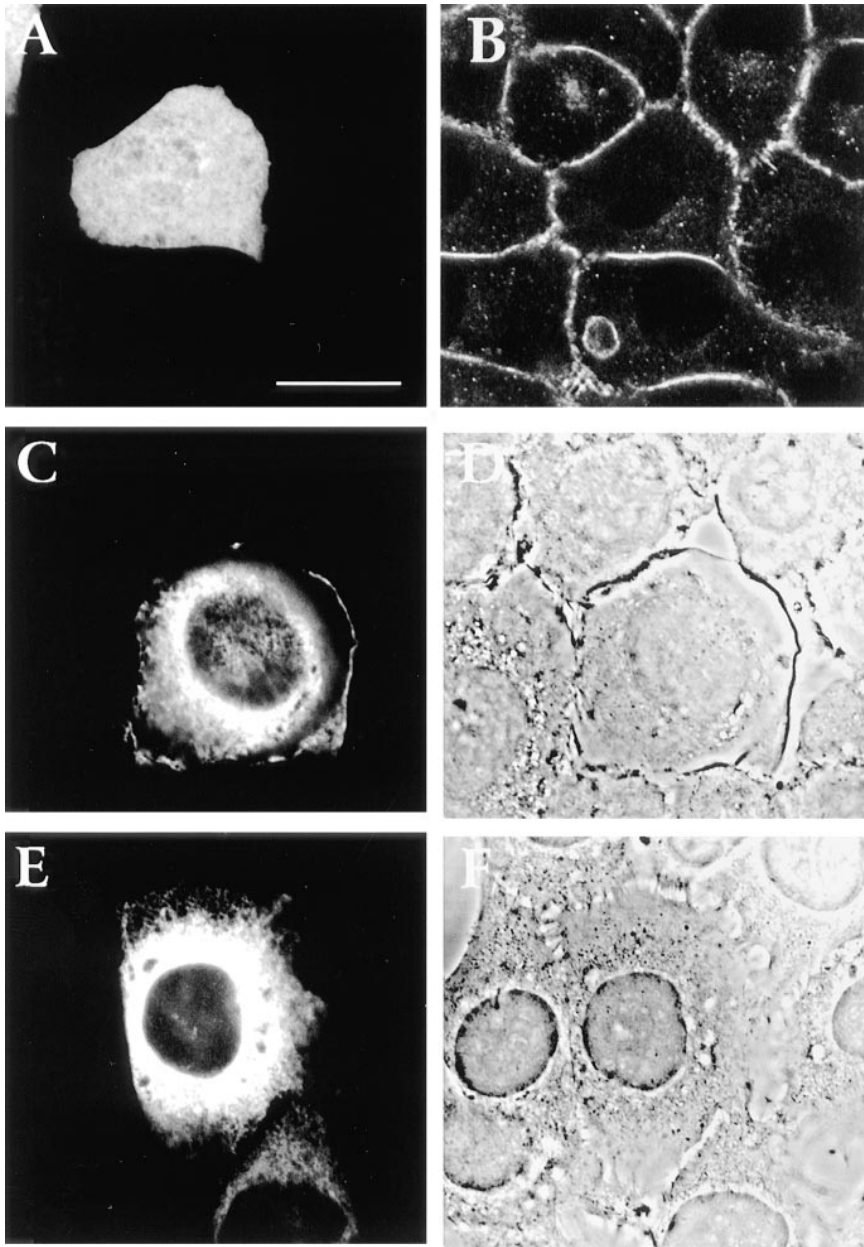
Figure 8. Cellular distribution of chk-PTP1B in LN cells. LN cells transfected with vector containing GFP alone, wild-type GFP–chkPTP1B, or mutant GFP–chkPTP1B were immunostained with anti-GFP antibody and analyzed by laser confocal microscopy. LN cells expressing GFP alone show diffuse GFP (*A*). The same cells stained with anti–β-catenin antibody show the characteristic morphology of L cells expressing N-cadherin with β-catenin localized at sites of cell–cell contact (*B*). LN cells expressing either wild-type or mutant GFP–chkPTP1B show extensive perinuclear (ER) localization (*C* and *E*). Wild-type GFP–chkPTP1B is also localized to areas of cell-cell contact (*C* and *D* [phase]). LN cells expressing mutant GFP–chkPTP1B show little or no fluorescence at the cell periphery (*E* and *F* [phase]) and lack the well-defined borders seen in control cells and cells expressing wild-type GFP–chkPTP1B. Bar, 20 μm.

Overexpression and activation of the EGF receptor results in phosphorylation of β-catenin and loss of E-cadherin mediated adhesion (Fujii et al., 1996; Hazan and Norton, 1998). The EGF receptor has also been shown to directly phosphorylate β-catenin (Hoschuetzky et al., 1994). In addition to the EGF receptor, its close relative, the transmembrane protein tyrosine kinase c-erbB-2 (Ochiai et al., 1994; Kanai et al., 1995) is also found associated directly with β-catenin and may play such a role. These observations suggest an alternative possibility for the effect of the dominant-negative PTP1B; it could maintain the EGF receptor in an activated state, resulting in hyperphosphorylation of β-catenin. Among LN cells this mechanism does not appear to be operative, as there is no detectable EGF receptor (Balsamo, J., unpublished data).

Although phosphorylation of β-catenin is consistently correlated with loss of cadherin function, (for review see Lilien et al., 1997), loss of the cadherin–β-catenin connection is not always detected (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Papkoff, 1997; Hazan and Norton, 1998). It is notable that although increased tyrosine phosphorylation of β-catenin after activation of the EGF receptor does not result in dissociation of the cadherin–β-catenin interaction, it does result in dissociation of the cadherin–actin linkage (Hazan and Norton, 1998). Thus, in spite of the fact that the detailed molecular associations detected differ, possibly reflecting subtle differences between cell types or slight but important differences in the procedures used, phosphorylation/dephosphorylation of β-catenin appears to be a specific feature of the cadherin complex of proteins regulating its association with actin. Global changes in the actin cytoskeleton also ramify on cadherin function, and this may be the basis for altered cadherin function in L cells expressing a cadherin–
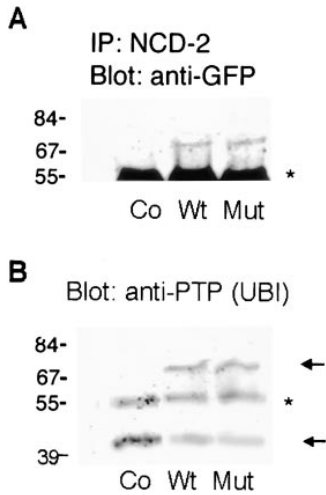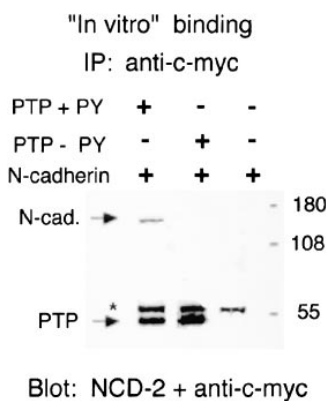
Figure 9. Association of GFP–chkPTP1B fusion protein with N-cadherin. LN cells transfected with GFP–chkPTP1B were homogenized in neutral detergent-containing buffer and immunoprecipitated with anti–N-cadherin antibody NCD-2. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (*A*) Anti-GFP antibody, (*B*) anti-PTP1B antibody. *Co*, LN cells transfected with vector alone; *Wt*, LN cells transfected with the wild-type PTP1B; *Mut*, LN cells transfected with the mutant PTP1B. *Asterisk*, position of IgG heavy chain. *Left*, numbers indicate the migration of the molecular mass standards. *Arrows*, position of GFP–PTP1B at ∼70 kD and endogenous PTP1B at ∼40 kD.

α-catenin fusion after expression of v-src (Takeda et al., 1995).

Tyrosine phosphatases other than PTP1B may also play a role in regulating cadherin function through dephosphorylation of β-catenin. Two transmembrane protein tyrosine phosphatases, hPTPκ and LAR-PTP, have been reported to act on β-catenin and may play a role similar to that of PTP1B in regulating cadherin–cytoskeletal interactions. hPTPκ has been localized at adherens junctions in a human mammary tumor cell line (Fuchs et al., 1996). This

Figure 10. In vitro association of PTP1B with N-cadherin. Phosphorylated, catalytically inactive, GST–c-myc-chkPTP1B fusion protein was purified on a glutathione–Sepharose affinity column. One aliquot was treated with 0.2 U recombinant protein tyrosine phosphatase (*Yersinia enterolitica*, Calbiochem-Novabiochem, La Jolla, CA) and the reaction stopped with 4 mM vanadate. Purified N-cadherin was incubated with control or dephosphorylated PTP1B for 20 min at room temperature. Putative complexes were precipitated with anti–c-myc antibody and separated by SDS-PAGE. PTP1B-bound N-cadherin was detected with the anti–N-cadherin antibody NCD-2. Phosphorylated and dephosphorylated c-myc PTP1B were detected with anti–c-myc antibody. The molecular mass of the bacterial PTP1B fusion protein, 50 kD, represents the entire ORF. *Asterisk* at ∼55 kD, position of immunoglobulin heavy chain derived from the anti–c-myc antibody. *Left*, migration of N-cadherin and PTP1B. *Right*, numbers indicate the migration of the molecular mass standards.

transmembrane tyrosine phosphatase interacts directly with both β- and γ-catenin, but not α-catenin, suggesting that the armadillo motifs are essential for the interaction (Fuchs et al., 1996). LAR-PTP is found associated with the β-catenin–cadherin complex in PC12 cells (Kypta et al., 1996) and appears to interact directly with the NH$_2$-terminal domain of β-catenin. LAR may also play a role in regulating cell–matrix interactions as it has been reported to colocalize with a LAR-interacting protein (LIP.1) at focal adhesions (Serra-Pages et al., 1996). The receptor tyrosine phosphatase, RPTPμ, associates directly with the COOH-terminal region of the cadherin molecule itself (Brady-Kalnay et al., 1995, 1998). In this case, the role of the cadherin–phosphatase interaction is not well understood and no definitive cellular substrates have yet been reported.

Cadherin function is regulated by its association with the actin containing cytoskeleton. This association, or the stability of the association, is regulated by tyrosine phosphorylation/dephosphorylation of β-catenin. PTP1B appears to play a central role in this process. There are several pathways regulating the activity and/or association of tyrosine kinases and phosphatases with the cadherin–catenin complex. Activation of these pathways by environmental cues adds a new dimension to the potential for spatial and temporal control of morphogenesis.

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