PTP1B Modulates the Association of β-Catenin with N-cadherin through Binding to an Adjacent and Partially Overlapping Target Site*

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The nonreceptor tyrosine phosphatase PTP1B associates with the cytoplasmic domain of N-cadherin and may regulate cadherin function through dephosphorylation of β-catenin. We have now identified the domain on N-cadherin to which PTP1B binds and characterized the effect of perturbing this domain on cadherin function. Deletion constructs lacking amino acids 872–891 fail to bind PTP1B. This domain partially overlaps with the β-catenin binding domain. To further define the relationship of these two sites, we used peptides to compete in vitro binding. A peptide representing the most NH$_2$-terminal 8 amino acids of the PTP1B binding site, the region of overlap with the β-catenin target, effectively competes for binding of β-catenin but is much less effective in competing PTP1B, whereas two peptides representing the remaining 12 amino acids have no effect on β-catenin binding but effectively compete for PTP1B binding. Introduction into embryonic chick retina cells of a cell-permeable peptide mimicking the 8 most COOH-terminal amino acids in the PTP1B target domain, the region most distant from the β-catenin target site, prevents binding of PTP1B, increases the pool of free, tyrosine-phosphorylated β-catenin, and results in loss of N-cadherin function. N-cadherin lacking this same region of the PTP1B target site does not associate with PTP1B or β-catenin and is not efficiently expressed at the cell surface of transfected L cells. Thus, interaction of PTP1B with N-cadherin is essential for its association with β-catenin, stable expression at the cell surface, and consequently, cadherin function.

β-Catenin occupies a central position in cell physiology and development as both a transcriptional co-activator regulated by Wnt signaling (reviewed in Refs. 1 and 2) and a bridge between the cytoplasmic domain of cadherin cell-cell adhesion molecules and the actin-containing cytoskeleton (reviewed in Ref. 3), a connection that is crucial to function (reviewed in Refs. 4 and 5). It is noteworthy that distinct gene products may carry out these two key roles in Caenorhabditis elegans (6). However, in Drosophila and vertebrates, the same gene products appear to assume both roles (7). It is not surprising, then, that in the absence of Wnt stimulus the pool of free β-catenin is subject to rapid degradation (8, 9), possibly preventing spurious interference between these two key functions. Whereas the details of how these two roles of β-catenin are regulated to integrate function and maintain the integrity of the two pathways are not completely clear, tyrosine phosphorylation of β-catenin may be one key regulatory determinant.

Phosphorylation of tyrosine residues on β-catenin has repeatedly been correlated with loss of cadherin adhesive function (reviewed in Refs. 10 and 11). This, in turn, is correlated with instability of the β-catenin-cadherin bond (12), uncoupling of cadherin from the actin-containing cytoskeleton (13, 14), and an increase in the free cytosolic pool of tyrosine-phosphorylated β-catenin (15, 16). Tyrosine phosphorylation of β-catenin also increases the interaction of β-catenin with basal transcription factor and increases transcriptional activity of the β-catenin-Tcf complex (12, 17). The same tyrosine residue, 654, is critical for both instability of the β-catenin-cadherin bond and for enhanced binding to basal transcription factor (12, 17), suggesting that the two functions of β-catenin may be coordinated through the creation of a pool of free β-catenin following tyrosine phosphorylation and dissociation of the β-catenin-cadherin link and a concomitant increase in the potential of this pool to participate in transcription.

Our laboratory has focused on the role of the nonreceptor tyrosine phosphatase PTP1B in regulating the phosphorylation of tyrosine residues on β-catenin in N-cadherin-expressing cells. Introduction into L-cells constitutively expressing N-cadherin of a catalytically inactive, dominant-negative construct of PTP1B results in hyperphosphorylation of tyrosine residues on β-catenin, an increase in the free cytosolic pool of tyrosine-phosphorylated β-catenin, and dissociation of the cadherin-actin connection concomitant with loss of cadherin function (13, 14). This dominant negative PTP1B construct also inhibits neurite extension on N-cadherin substrates (18). Consistent with these observations, PTP1B is present at adherens junctions and localizes to growth cones (14, 18). Furthermore, PTP1B binds directly to the cytoplasmic domain of N-cadherin (13, 14). Because of the key position PTP1B occupies, it is not surprising that its binding to N-cadherin is also regulated. Indeed, we have previously shown that PTP1B must be tyrosine-phosphorylated on tyrosine 152 in order to bind to N-cadherin (13, 14, 19).

In this paper, we define the target domain for PTP1B on N-cadherin and show that it is adjacent to, and partially overlaps with, the binding site for β-catenin. Introduction into primary embryonic chick neural retina cells of a cell-permeable, “Trojan” peptide that mimics the most distant, nonoverlapping portion of the PTP1B binding site in N-cadherin results in loss of cadherin function and an increase in the free pool of tyrosine-phosphorylated β-catenin. Furthermore, L-cells transfected with N-cadherin lacking the entire site or the portion that does not overlap with the β-catenin binding site show loss of N-cadherin expression at the cell surface. We suggest that
the proximity of β-catenin and PTP1B binding sites allows for continuous and rapid removal of phosphate from tyrosine residues, stabilizing and maintaining the integrity of the cadherin-actin cytoskeletal linkage and cadherin function.

**MATERIALS AND METHODS**

**Antibodies**—The anti-N-cadherin antibody NCD-2 (20) was purified from hybridoma culture medium in our laboratory as described previously (21). Anti-phosphotyrosine (PY20) monoclonal antibody was from Transduction Laboratories (Lexington, KY), anti-FLAG was from Stratagene (La Jolla, CA), and anti-phosphoheparine was from Zymed Laboratories Inc. (San Francisco, CA). Anti-PTP1B antibodies were obtained from Transduction Laboratories, Calbiochem, or Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-β-catenin antibodies were a mouse monoclonal IgG from Transduction Laboratories and a rabbit polyclonal antibody directed against a synthetic 15-amino acid sequence (22). HRP-conjugated secondary antibodies were purchased from Organon Teknika Co. (Durham, NC), and antibodies conjugated to magnetic beads, used in immunoprecipitations, were from Polysciences Inc. (Warrington, PA).

**Preparation of N-cadherin Constructs and GST Fusion Proteins**—cDNAs encoding the full cytoplasmic region of N-cadherin (residues 752–912) and truncated constructs were generated by PCR using specific oligonucleotide primers flanked by EcoRI and NcoI restriction sites and chick N-cadherin cDNA as a template. The PCR products were subcloned in PGEX-KG (Amersham Biosciences). To create N-cadherin containing the extracellular, transmembrane and cytoplasmic domain carrying deletions of amino acids 872–891, 878–891, or 884–891 (Genemed, San Francisco, CA). An antennapedia sequence fused to the N-cadherin was immobilized on 300 μl of glutathione-Sepharose 4B and washed in CKII kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 200 mM NaCl, 0.1 mM ATP) or GSK-3β kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, and 0.2 mM ATP). The proteins were phosphorylated in a total volume of 500 μl for 30–60 min at 30 °C with 10 units of casein kinase II (Promega, Madison, WI) or 1 unit of glycogen synthase kinase-3β (Calbiochem). The beads were then washed with PBS, and bound protein was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 6.0, containing 1% protease inhibitor mixture (Roche Appl. Sci.). The eluted protein was biotinylated with Sulfo-NHS-LC-Biotin for immobilization, and the presence of phosphorylated serine residues was determined by immunoblots using anti-phosphoantibody.

**Cell Lines and Transfections**—Mouse L cells were used for both stable and transient transfections with N-cadherin constructs. Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen) and transfected with pCMV-ΔS72–891, pCMV-Δ878–891, or pCMVΔ884–891. Stable clones were selected with 500 μg/ml G418. L cells transfected with empty pCMV-FLAG-4A were used as control. Expression of N-cadherin was analyzed by immunoblot using N-Cadherin antibody as a control.

**Expression of N-cadherin at the Cell Surface**—Cell surface proteins were labeled with the cell membrane-impermeable reagent, NHS-LC-Biotin (Pierce) at room temperature for 30 min. Cells were washed three times with ice-cold PBS (pH 8.0) to remove any remaining biotinylated reagent and lysed in cold 1% O/N2-2 as described above, fractionated by SDS-PAGE, and transferred to PVDF membranes. The membranes were immunoblotted with the antibody indicated in the figure.
the substrate for adhesion, purified N-cadherin-Fc (23) was immobi-
lized on 96-well plates precoated with anti-mouse IgG (BD Biosciences; BD Biosciences, Bedford, MA) or poly-L-lysine (Sigma). Plates were washed and blocked with 2% BSA for 1 h. Approximately 10^4 cells were added to each well in 50 μl of HBSSGKCa, in the presence of the indicated peptide at 4 μg/ml. After 1 h at 37°C, nonadherent cells were removed, the wells were washed gently four times with HBSSGKCa, and adherent cells were quantified by staining with crystal violet.

Substrates for neurite growth were prepared by coating eight-well slides with polylysine followed by N-cadherin-Fc or laminin, followed by washing and blocking with 2% BSA. The presence of neurites was quantitatively assessed in sparse single cell cultures of E8 chick neural retina. Peptides were added at 8 μg/ml, 2 h after plating. After overnight culture in Dulbecco’s modified Eagle’s medium containing 1% insulin/transferring/selenium (Invitrogen), cells were fixed in 4% p-formaldehyde, and ~200 cells were evaluated for the presence of neurites. Neurite growth was visualized using phase optics.

**RESULTS**

**Mapping the PTP1B Binding Domain on N-cadherin—**

PTP1B is ubiquitous in the cell and can interact with many different partners. We have previously shown that PTP1B binds directly to the N-cadherin cytoplasmic domain, controlling the association of N-cadherin with β-catenin and therefore its function (13, 14). Furthermore, binding is dependent on phosphorylation of tyrosine 152 of PTP1B (19). To be able to specifically perturb the cadherin-PTP1B association, we sought to identify the sequence in the cytoplasmic domain of N-cadherin that is necessary for binding of PTP1B. The full-length cytoplasmic domain of N-cadherin and several deletion mutants were expressed as GST fusion proteins (Fig. 1A), and the purified fusion proteins were biotinylated and immobilized on streptavidin-coated wells. Catalytically inactive GST-PTP1B, phosphorylated on tyrosine residues (Fig. 1B), was then added, and binding was measured using anti-PTP1B antibody in an enzyme-linked immunosorbent assay. We began with two constructs, C2 (amino acids 752–878) and C3 (amino acids 872–912), which overlap by 7 amino acids. Binding of PTP1B to either construct shows approximately the same dose dependence as binding to the full-length cytoplasmic domain (C1; Fig. 1C), suggesting that the binding site for PTP1B encompasses the COOH terminus of C2 and the NH2 terminus of C3. A deletion of 8 amino acids from the COOH terminus of C2 (C4; amino acids 752–870) eliminates binding to PTP1B (Fig. 1C), mapping the NH2 terminus of the binding domain between amino acid residues 871 and 878. Sequential deletions from the NH2 terminus of C3 set the COOH terminus of the binding region at amino acid residues 887–891; construct C5 (amino acids 887–912) is positive for binding, whereas C6 (amino acids 891–912) is not (Fig. 1C). These results establish the boundaries of the PTP1B binding region at amino acids 872 and 891 on the cytoplasmic domain of N-cadherin. Construct C7 containing just this sequence (amino acids 872–891) binds PTP1B as well as the full-length cytoplasmic domain C1 (Fig. 1C), whereas C8, consisting of the cytoplasmic domain of cadherin lacking this core sequence (C8; Δ872–891) does not bind (Fig. 1C).

**The PTP1B and β-Catenin Binding Sites in the Cytoplasmic Domain of N-cadherin Are Adjacent and Overlapping—** The same cadherin constructs used above to evaluate PTP1B binding were also evaluated for β-catenin binding (not shown). The results are consistent with prior studies by Simcha et al. (24) (see also Refs. 25–27) placing the PTP1B binding region NH2-terminal to the β-catenin binding region, with an overlap of about 7 amino acids. To better understand the relationship between these two binding domains, we used synthetic peptides as competitors for the in vitro binding between PTP1B and N-cadherin and between β-catenin and N-cadherin. Each peptide mimics an 8-amino acid stretch of the PTP1B binding sequence, from the NH2 to the COOH terminus, overlapping with the next sequence by 2 amino acids (see diagram in Figs. 2A and 9). Binding of PTP1B to the cytoplasmic domain of N-cadherin is reduced to background levels at a concentration of 0.8–1.0 μg/well of peptides 2 or 3, corresponding to the COOH-terminal two-thirds of the PTP1B binding sequence (Fig. 2B). The reverse sequence of peptide 3, P3R, has no effect on binding. At similar concentrations, peptide 1, corresponding to the NH2 terminus of the PTP1B binding sequence and the portion overlapping the β-catenin-binding domain, inhibits only about 20–30% of PTP1B binding to cadherin (Fig. 2B). In
contrast, peptide 1 is an effective competitor of the binding of β-catenin to N-cadherin at a concentration of 0.8 μg/well (Fig. 2C), whereas peptides 2 and 3 and the control peptide 3R, at this same concentration, have no effect on the β-catenin-N-cadherin interaction (Fig. 2C). Peptide P4, corresponding to the sequence NH2-terminal to the putative PTP1B-binding domain and well within the reported β-catenin binding region, does not compete for PTP1B binding to N-cadherin but completely abolishes β-catenin binding to N-cadherin (Fig. 2, B and C).

These results suggest that, although the sequence corresponding to amino acids 872–878 in the cytoplasmic tail of N-cadherin has the potential to interact with both β-catenin and PTP1B, the interaction with PTP1B is of lower affinity. This is confirmed by comparing the time course of binding of PTP1B to deletion construct C2, containing the region of overlap between the PTP1B and β-catenin binding sites, with the full-length cytoplasmic domain of cadherin (C1), the full putative PTP1B-binding domain (C7), or the deletion constructs C3 and C5. PTP1B binds to C2 at a slower rate than to C1 or C7 or to the COOH-terminal region of the PTP1B binding domain (C3 and C5): 25% of control values after 30 min for C2, as compared with about 60% for C1, C3, C5, and C7 (Fig. 2D). In addition, β-catenin and PTP1B do not compete for binding to C1 (Fig. 3A). However, β-catenin does compete for binding of PTP1B to the NH2-terminal or overlapping portion of PTP1B binding region (C2) (Fig. 3B).

There are 11 serine and threonine residues in the combined β-catenin/PTP1B binding site in N-cadherin and 7 in the PTP1B site alone. This suggests the possibility that serine/threonine phosphorylation could modulate the binding of either effector. We do see an increase in binding of β-catenin to the full-length cytoplasmic domain of N-cadherin after in vitro phosphorylation of serine residues as reported (24, 27) but no effect in the binding of PTP1B (Fig. 4, A and B).

Peptides Mimicking the PTP1B Binding Site on N-cadherin Inhibit N-cadherin-mediated Adhesion and Neurite Outgrowth among Embryonic Chick Neural Retina Cells—To be able to perturb the interaction between N-cadherin and PTP1B in cells that express endogenous N-cadherin, we designed two cell membrane-permeable peptides containing the sequences be-
PTP1B Binding Site on N-cadherin

Fig. 3. PTP1B and β-catenin do not compete for binding to the cytoplasmic domain of N-cadherin. A, increasing concentrations of GST-PTP1B have no effect on binding of β-catenin to C1 (left). Likewise, increasing concentrations of GST-β-catenin have no effect on binding of PTP1B to C1 (right). Results are represented as percentage of binding in the absence of competitor and are the mean ± S.E. of triplicate wells. B, binding of PTP1B and β-catenin to N-cadherin lacking the COOH-terminal two-thirds of the PTP1B binding sequence (N-cadherin Δ878–891). Binding of β-catenin to N-cadherin Δ878–891 is not affected by the presence of PTP1B (left). However, increasing concentrations of β-catenin efficiently compete for binding of PTP1B to the Δ878–891 construct (right). Results are represented as percentage of binding in the absence of competitor and are the mean ± S.E. of triplicate wells.

between amino acids 878–891 and 884–891 (AP2/3 and AP3, respectively; see Figs. 2A and 9) of N-cadherin covalently linked to the antennapedia "Trojan" peptide (28, 29). These peptides mimic only the portion of the PTP1B binding domain that does not overlap the β-catenin binding domain. To confirm that peptides AP2/3 and AP3 can indeed prevent the stable association of PTP1B with N-cadherin, E8 chick neural retina cells were incubated with AP2/3, AP3, or a control peptide consisting of the antennapedia permeation sequence fused to the reverse of P3 (AP3R) for 4 h; cell lysates were immunoprecipitated with NCD-2 and analyzed by Western blot with antipTP1B antibody (Fig. 5A). Immunoprecipitates from the cells incubated with AP3, but not AP3R, have greatly reduced N-cadherin-associated PTP1B (Fig. 5A). Among cells treated with AP3, the amount of β-catenin associated with N-cadherin is also reduced (Fig. 5A). Furthermore, the pool of β-catenin phosphorylated on tyrosine residues is enriched in cells treated with AP3 but not AP3R (Fig. 5B). The amount of cell surface N-cadherin is not affected during the time course of the experiment (Fig. 5C); thus, the cell-permeable peptide AP3, by competing for binding of PTP1B to N-cadherin, effectively uncouples or destabilizes the association between N-cadherin and β-catenin. Furthermore, this occurs without compromising the actual β-catenin target site on N-cadherin. Similar results were obtained when cells were treated with peptide AP2/3 (not shown).

Loss of association between β-catenin and N-cadherin is correlated with loss of N-cadherin-mediated adhesion and neurite outgrowth among embryonic neural retina cells (10). E8 chick neural retinas were assayed for their ability to adhere to immobilized N-cadherin (Fc-N-cadherin chimera) in the presence of AP2/3 or AP3. Both peptides result in loss of N-cadherin-mediated adhesion (Fig. 6A). To assay for N-cadherin-mediated neurite outgrowth, E8 chick neural retina cells were plated on Fe-N-cadherin-coated slides, and cell-permeable peptides were added at 2 h when all or a great majority of the cells were adherent, and the cells were incubated for another 10–12 h (25, 30). Both AP2/3 and AP3 significantly reduce N-cadherin-mediated neurite growth, whereas control peptides consisting of the Antennapedia permeation sequence alone, the reverse of P3 (COP and AP3R, respectively; Fig. 6, B and C), or the Antennapedia sequence fused to an unrelated sequence in the cytoplasmic domain of N-cadherin (SBP (see Ref. 25); not shown). The loss of N-cadherin-mediated adhesion and neurite outgrowth is not due to a significant reduction in the amount of cell surface N-cadherin during the assay period (not shown) and therefore is most likely due to loss of N-cadherin function. In addition, AP2/3 (not shown) and AP3 have no effect on neurite outgrowth on a laminin-coated substrate (Fig. 6C).

Deletion of the PTP1B Binding Domain Results in Reduced Expression of N-cadherin at the Cell Surface and Loss of β-Catenin from the N-cadherin Complex—We next looked at the effect of eliminating the PTP1B binding domain in live cells by transfecting L cells with the cDNAs for full-length N-cadherin (FL cells) and N-cadherin lacking the complete PTP1B binding sequence (Δ872–891) or the 8 carboxyl-terminal amino acids (Δ884–891), the residue most distant from the β-catenin binding domain. N-cadherin expression at the cell surface and association with PTP1B and β-catenin were analyzed in both stable lines and cells transiently expressing these constructs,
with similar results (Fig. 7 shows results for transient cultures). To determine whether PTP1B is present in N-cadherin complexes, the cells were lysed in nonionic detergent and immunoprecipitated with NCD-2, and the immunoprecipitates were assayed for the presence of PTP1B by immunoblot (Fig. 7A). PTP1B is not detected in N-cadherin precipitates lacking the complete PTP1B binding region (Δ872–891) or the carboxyl-terminal portion of the PTP1B binding region (Δ884–891; Fig. 7B; total amounts of PTP1B are shown as a loading control). The absence of the entire PTP1B binding domain or the carboxyl-terminal portion of the domain also correlates with loss of β-catenin from the N-cadherin complex (Fig. 7B). Furthermore, there is a notable increase in the reactivity of the pool of free or unbound β-catenin with anti-phosphotyrosine antibodies when PTP1B is absent from the N-cadherin complex (Fig. 7B). Thus, in the absence of even the most carboxyl-terminal 8 amino acids of the PTP1B binding domain, the region most distant from the β-catenin binding site, N-cadherin does not efficiently associate with β-catenin.

To examine the expression of N-cadherin at the cell surface, the cells were biotinylated with a cell-impermeable reagent and then lysed and immunoprecipitated with NCD-2. Only cells transfected with the full-length construct express N-cadherin at the cell surface, although N-cadherin is present in approximately equal levels in all cell types (Fig. 7C). Thus, in the absence of bound PTP1B, N-cadherin does not associate with β-catenin and does not reach the cell surface and/or is rapidly degraded.

In order to visualize the distribution of N-cadherin lacking the PTP1B binding domain, we transfected L cells with GFP fusions of the full-length N-cadherin and the N-cadherin deletion construct lacking the 8 COOH-terminal amino acids of the PTP1B-binding domain (Δ884–891). After 24 h, cells were fixed and reacted with anti-β-catenin followed by fluorescent second antibody. Cells expressing full-length N-cadherin show co-localization of β-catenin and N-cadherin at the cell periphery and areas of cell-cell contact. In the absence of the 8 COOH-terminal amino acids of the PTP1B binding domain, N-cadherin is largely localized intracellularly, as is β-catenin, showing only minimal overlap (Fig. 8; compare A, C, and E with B, D, and F).

**DISCUSSION**

In this paper, we characterize the binding site on the cytoplasmic domain of N-cadherin for the nonreceptor tyrosine phosphatase PTP1B and characterize the biochemical and biological ramifications of interfering with this site through the use of cell-permeable competitor peptides and deletion constructs. At the same time, these analyses further define the COOH-terminal limits of the β-catenin target site. Fig. 9 is a
Each treatment, and results are expressed as the mean of at least three dishes. Cells were plated on six-well slides coated with Fc-N-cadherin. After 2 h, E8 retina cells were cultured on Fc-N-cadherin- or laminin-coated slides in the presence of control peptide (COP, the antennapedia cell permeation sequence only; AP2/3, the antennapedia cell permeation sequence fused to the sequence from amino acid 878 to 891 on the cytoplasmic domain of N-cadherin; AP3, the antennapedia cell permeation sequence fused to the sequence from amino acid 884 to 891; AP3R, the antennapedia sequence fused to the reverse of peptide 3 (amino acids 891–891), Fig. 9) results in competition between 27). Analysis of the PTP1B target site also leads us to conclude that the binding site for PTP1B, encompassing amino acids 872–891, and overlaps with a portion of the COOH-terminal 8 amino acids (Δ884–891) of the PTP1B binding sequence. A, N-cadherin lacking the PTP1B target sequence fails to associate with PTP1B. L cells expressing each N-cadherin construct were lysed in nonionic detergent and immunoprecipitated with anti-cadherin antibody. The immunoprecipitates were fractionated by SDS-PAGE, transferred to PVDF membranes, and blotted with anti-PTP1B. Aliquots of total lysate that were also immunoblotted with anti-PTP1B as a loading control. B, lack of PTP1B binding results in loss of β-catenin from the N-cadherin complex. The NCD-2 immunoprecipitates above were also immunoblotted with anti-β-catenin antibody (top). Supernatants from the above NCD-2 precipitations were further immunoprecipitated with anti-β-catenin, and the precipitates treated as above and blotted with anti-phospho-tyrosine antibody PY20 (bottom). C, expression of cell surface N-cadherin. Intact cells were labeled with biocytin, and cell extracts were immunoprecipitated with NCD-2, fractionated by SDS-PAGE, transferred to PVDF membranes, and reacted with HRP-avidin. L cells transfected with Δ872–891 or with the partial PTP1B binding region deletion (Δ884–891) do not express N-cadherin at the cell surface. The same precipitates were immunoblotted with NCD-2 to determine total expression of N-cadherin in transfected cells.

Fig. 6. Introduction into embryonic chick neural retina cells of peptides that mimic the PTP1B binding site on N-cadherin results in loss of cadherin-mediated adhesion and neurite outgrowth. Peptides that mimic the carboxyl-terminal regions of the PTP1B binding motif on N-cadherin, AP2/3 and AP3, fused to the antennapedia cell permeation sequence, were introduced into retina cells, and cadherin-mediated adhesion and neurite outgrowth were assayed. A, adhesion to Fc-N-cadherin. E8 embryonic chick neural retina cells were plated on 96-well plates coated with Fc-N-cadherin extracellular domain chimera in the presence of the indicated peptide. After 1 h, adherent cells were quantified using crystal violet. Results are shown as percentage of control (adhesion to Fc-N-cadherin in the absence of control peptide (COP)); each value represents the mean ± S.E. of triplicate wells. COP, control peptide consisting of the antennapedia cell-permeable sequence only; AP2/3, the antennapedia cell permeation sequence fused to the sequence from amino acid 878 to 891 on the cytoplasmic domain of N-cadherin; AP3, the antennapedia cell permeation sequence fused to the sequence from amino acid 884 to 891; AP3R, the antennapedia sequence fused to the reverse of peptide 3 (amino acids 891–891). B, quantitation of neurite outgrowth. E8 retina cells were plated on six-well slides coated with Fc-N-cadherin. After 2h, the indicated peptide was added, and the cells were cultured for an additional 12 h. Cells bearing neurites longer than two cell diameters were counted as positive. A minimum of 200 cells were analyzed for each treatment, and results are expressed as the mean ± S.E. C, images of E8 retina cells cultured on Fc-N-cadherin- or laminin-coated slides in the presence of the indicated peptides.

β-catenin and PTP1B for binding to N-cadherin. Thus, the target sites for β-catenin and PTP1B overlap by -6 amino acids. We show that introduction into primary embryonic chick neural retina cells of a cell-permeable peptide carrying an 8-amino acid sequence mimicking the region of the PTP1B binding domain in N-cadherin that is most distant from the β-catenin target site (AP3, Fig. 9) results in loss of N-cadherin-mediated adhesion and neurite outgrowth. Concomitant with inhibition of adhesion, β-catenin is no longer associated with N-cadherin, and the pool of free, tyrosine-phosphorylated β-catenin is increased. Among these primary embryonic neural retina cells, with a full complement of cell surface N-cadherin, we do not find that the amount of N-cadherin at the cell surface is significantly reduced during 4 h of exposure to the peptide, a time period exceeding that required to complete the adhesion
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 assay. Thus, in retina cells we can conclude that compromising the binding of PTP1B to N-cadherin affects its function through retention of phosphate on tyrosine residues of β-catenin, reduced binding of β-catenin to N-cadherin, and thus loss of the crucial link to the cytoskeleton. Furthermore, either deletion of the entire PTP1B target domain (Δ872–891, Fig. 9) or of the most distant nonoverlapping portion (Δ884–891, Fig. 9) results in a reduction in bound β-catenin and reduced expression at the cell surface. It is interesting to note that introduction of the cell-permeable PTP1B competitor peptide into a population of cells already expressing N-cadherin at the cell surface results in loss of PTP1B, β-catenin, and thus cadherin function without immediately affecting cell surface expression; however, de novo synthesized N-cadherin lacking even a portion of the PTP1B target site compromises expression at the cell surface. This suggests that PTP1B, through its role in maintaining β-catenin in a dephosphorylated state, and thus bound to N-cadherin, plays two distinct roles, one involving the functional connection of cell surface N-cadherin to the cytoskeleton and a second involving transport to the cell surface (31). This reduction in cell surface expression may be due to reduced transport in the absence of β-catenin (31) and/or enhanced degradation due to the absence of bound β-catenin (32).

Alignment of several type I and type II cadherin cytoplasmic domains shows a high degree of conservation of the PTP1B binding domain (10). This suggests that PTP1B interacts with many different cadherins and may regulate function in a manner analogous to that shown for N-cadherin (13, 14). We are at present testing this supposition. It is interesting to note that PTPμ, the sole transmembrane tyrosine phosphatase thus far shown to interact directly with cadherins, binds to the carboxy-terminal 38 amino acids of E-cadherin (33), a region that includes most of the PTP1B binding domain.

Our working hypothesis is that PTP1B is constitutively associated with N-cadherin and possibly other cadherins and ensures the integrity of the cadherin-mediated adhesions through continuous dephosphorylation of β-catenin. However, the rapid uncoupling of cadherins from the cytoskeleton and the concomitant loss of function may be essential aspects of development. For example, during the conversion of epithelial cells to a motile mesenchymal phenotype, cadherin-mediated adhesions are lost, and transcriptional regulation may be too slow to effect such a change in a timely fashion (10). Similarly, N-cadherin-mediated neurite outgrowth guidance may depend on rapid inactivation of N-cadherin-mediated adhesions at pathway boundaries (34). Regulation of phosphotyrosine content of β-catenin is one means of affecting such changes. Several kinases and phosphatases have the potential to alter the phosphotyrosine content of β-catenin, suggesting that multiple kinases and phosphatases are involved, possibly depending on the tissue and time of development. Overexpression of the nonreceptor tyrosine kinases Src (35–37) and Fer (38) has been demonstrated to target β-catenin and increase its tyrosine phosphorylation. Furthermore, a dominant negative Src that interferes with Src function or an Src-specific tyrosine kinase inhibitor induces cell-cell adhesion (39). Two transmembrane tyrosine kinases, the epidermal growth factor receptor (40–42) and hepatocyte growth factor/scatter factor receptor (43), have also been shown to target β-catenin. Additionally, treatment of a squamous carcinoma cell line (44) or a mammary carcinoma cell line (45) with epidermal growth factor results in reduced cadherin-mediated adhesion and increased phosphorylation of β-catenin. Furthermore, suppression of the association of the epidermal growth factor receptor with β-catenin increases the

FIG. 8. The cellular distribution of GFP-full-length N-cadherin, GFP-N-cadherinΔ884–891, and β-catenin by confocal microscopy. L cells were transfected with GFP-full-length N-cadherin (A, C, and E) or GFP-N-cadherinΔ884–891 (B, D, and F) and visualized after 24 h. A and B, distribution of β-catenin. Cells were fixed and incubated with anti-β-catenin monoclonal antibody followed by Alexa Fluor 568-conjugated secondary antibody. C and D, distribution of wild type or mutant GFP-N-cadherin. E and F, merged images. Each image represents the projection of four confocal sections (0.4 μm each) near the equator of the cells. Note that GFP-full-length N-cadherin colocalizes with β-catenin at the cell surface, whereas GFP-N-cadherin Δ884–891 does not colocalize with β-catenin.

FIG. 9. Diagramatic representation of the β-catenin and PTP1B target region of the cytoplasmic domain of N-cadherin, the peptides used to analyze in vitro binding (P1, P2, P3, and P4), and those used as antennapedia fusions for introduction into embryonic chick neural retina cells (AP2/3 and AP3). Shown is a diagram of the deletion constructs used for analysis of cell surface expression of N-cadherin following transfection into L cells.
association of β-catenin with cadherin (46) and suppresses 
in vitro and in vivo invasion of a gastric cancer cell line, presum-
ably by increasing cadherin-mediated adhesions (47). Finally,
activated Ras, often coupled to transmembrane tyrosine ki-
nases, also results in an increase in the phosphorylation of
β-catenin and reduces the stability of the cadherin-β-catenin
bond (15, 48).

There are also a number of phosphatases that have the po-
tential to alter the state of phosphorylation of β-catenin.
Members of three distinct families of receptor PTPs (RPTP)
have been reported to interact with β-catenin and/or be corre-
lated with the state of phosphorylation of cadherin itself: LAR-
PTP, the chondroitin sulfate proteoglycan PTPβR, and the
MAM (Meprin/A5/Mu) domain-containing family members: κ,
λ, and μ. LAR-PTP (16, 49) has been shown to interact with and
dephosphorylate β-catenin. Additionally, overexpression of
LAR-PTP correlates with prevention of β-catenin phosphoryla-
tion and inhibition of epithelial cell migration (16). Similarly,
PTPβR interacts with and dephosphorylates β-catenin (50), and
interaction with its ligand, pleiotrophin, results in inactivation
of intrinsic catalytic activity and enhanced tyrosine
phosphorylation of β-catenin (51). The RPTPs κ, λ, and μ are
very closely related (51), and thus it is interesting that they
appear to play two different roles with respect to cadherin
function. PTPκ (52) and PTPλ (53) interact directly with β-
catenin, and PTPκ has been shown to dephosphorylate β-catenin
(52). PTPμ does not interact directly with β-catenin but does
commonprecipitate with the N-, E-, and R-cadherin com-
plexes (33, 54). Furthermore, it interacts directly with E-
cadherin through a 38-amino acid carboxyl-terminal region. PTPμ
does not alter the phosphorylation of β-catenin, but, under
conditions where E-cadherin is tyrosine-phosphorylated, PTPμ
is no longer associated with the cadherin complex of proteins
(33).

Placing the many tyrosine kinases and phosphatases shown
to have the potential to alter the phosphorytose content of
β-catenin in developmental context will present some signifi-
 cant challenges, since most of the signaling intermediates in-
volved in regulating phosphorytose content are used in mul-
tiple pathways at many times during development. We believe
that perturbing specific protein–protein interactions through
the use of peptide competitors provides one means of disrupting
specific interactions without affecting others. Consistent with
this, our goal has been to develop such competitors that can
be added to specific tissues at specific developmental times for
each of the effectors regulating cadherin function.

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