Essential Tyrosine Residues for Interaction of the Non-receptor Protein-tyrosine Phosphatase PTP1B with N-cadherin*

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Expression of a dominant-negative, catalytically inactive form of the nonreceptor protein-tyrosine phosphatase PTP1B in L-cells constitutively expressing N-cadherin results in loss of N-cadherin-mediated cell-cell adhesion. PTP1B interacts directly with the cytoplasmic domain of N-cadherin, and this association is regulated by phosphorylation of tyrosine residues in PTP1B. The following three tyrosine residues in PTP1B are potential substrates for tyrosine kinases: Tyr-66, Tyr-152, and Tyr-153. To determine the tyrosine residue(s) that are crucial for the cadherin-PTP1B interaction we used site-directed mutagenesis to create catalytically inactive PTP1B constructs bearing additional single, double, or triple mutations in which tyrosine was substituted by phenylalanine. Mutation Y152F eliminates binding to N-cadherin in vitro, whereas mutations Y66F and Y153F do not. Overexpression of the catalytically inactive PTP1B with the Y152F mutation in L-cells constitutively expressing N-cadherin has no effect on N-cadherin-mediated adhesion, and immunoprecipitation reveals that the mutant Y152F PTP1B does not associate with N-cadherin in situ. Furthermore, among cells overexpressing the Y152F mutant endogenous PTP1B associates with N-cadherin and is tyrosine-phosphorylated.

Members of the cadherin family of cell-cell adhesion molecules are key players in morphogenetic processes, and regulation of cadherin function, as opposed to transcription and translation, is thought to be responsible for many of the rapid changes that occur during development. Classic cadherins are characterized by a highly conserved intracellular domain that interacts with the actin-containing cytoskeleton, an interaction essential for function. This interaction is mediated by α- and β-catenin (1–4); β-catenin associates directly with a 20-amino acid domain near the carboxyl terminus of cadherin (5, 6) and with α-catenin, which, in turn, interacts with actin, either directly (7) or indirectly, through α-actin (8). β-catenin not only performs a bridging role between cadherin and actin, but free β-catenin can be translocated to the nucleus where it regulates transcription of cadherin and other gene products (9, 10). Thus, the regulation of free β-catenin is of critical importance, and, consequently, the interaction of β-catenin with cadherin has multiple ramifications on cellular function (11, 12).

Regulation of the interaction of β-catenin with N-cadherin is mediated by the phosphorylation of tyrosine residues on β-catenin (13, 14). In embryonic chick neural retina cells, hyperphosphorylation of β-catenin is correlated with loss of its association with N-cadherin and loss of cadherin function (13, 14). Enhanced phosphorylation of β-catenin has also been correlated with loss of E-cadherin function (15–19). These data suggest that tyrosine kinases and/or phosphatases must play a critical role in maintaining β-catenin association with cadherin and/or its ability to mediate the cytoskeletal linkage. We have reported that the nonreceptor protein-tyrosine phosphatase PTP1B binds to the cytoplasmic domain of N-cadherin and regulates its function by dephosphorylating β-catenin (13, 14). Furthermore, transfection of mouse L-cells constitutively expressing N-cadherin with a catalytically inactive PTP1B (substitution of cysteine 215 for serine) abolishes the ability of these cells to form N-cadherin-mediated adhesions. The mutant PTP1B associates with N-cadherin displacing endogenous PTP1B, resulting in dissociation of the cadherin-actin connection and accumulation of cadherin-free tyrosine-phosphorylated β-catenin (14).

PTP1B is targeted to many distinct cellular locations based on specific residues or domains in the molecule. The largest single pool is localized to the cytoplasmic face of the endoplasmic reticulum through a carboxyl-terminal domain (20). PTP1B also interacts with the insulin receptor and the EGF receptor and is phosphorylated on tyrosine residues in response to receptor stimulation (21–23). We have also reported that PTP1B is physically and functionally associated with focal adhesion complexes (24). This association may depend on binding to p130ncos through a proline-rich site (25). Binding of PTP1B to N-cadherin requires that PTP1B itself be phosphorylated on tyrosine residues (13, 14). In this study we show that the in vitro and in situ interaction between PTP1B and N-cadherin depends on phosphorylation of tyrosine residue 152.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal mouse anti-PTP1B antibody was purchased from Calbiochem. Anti-N-cadherin antibodies were NCD-2, a rat monoclonal antibody (Sigma). A rabbit polyclonal anti-pan-cadherin (Sigma). Monoclonal rabbit anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Anti-HA antibody was from Babco, Richmond, CA). HRP-conjugated anti-mouse and anti-rat secondary antibodies were from Organon Teknika Co. (Durham, NC). Goat-HRP anti-rabbit antibody and fluorescein isothiocyanate-conjugated anti-rat IgG were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Antibodies

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1 The abbreviations used are: HRP, horseradish peroxidase; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione S-transferase; PAG, polyacrylamide gel electrophoresis; cyt-N-cad, cDNA fragment corresponding to the cytoplasmic domain of N-cadherin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LN-cells, L-cells constitutively expressing N-cadherin; PVDF, polyvinylidene difluoride; ER, endoplasmic reticulum.
conjugated to magnetic beads, used in immunoprecipitations, were from PerSeptive Biosystems (Farmingham, MA).

**Site-directed Mutagenesis**—All mutant forms of PTP1B were generated using recombinant PCR. For bacterial expression in pGEX-KG (Amersham Pharmacia Biotech), we added a SmaI and an XhoI restriction site (or 3 end of the cytoplasmic sequence of N-cadherin, cDNA). The oligonucleotide primers were used as follows: forward primer, 5'-TCCCCGGGGGGACATGAGGAGGAGGAAGAGGATGGTCC-3'; reverse primer, 5'-CCGGCTGAAGGCGGCACTGAAATACACCTCCG-3'. The underlined bases indicate the stop codon. For expression in eukaryotic cells, the forward primer included a KpnI restriction site and an HA tag at the 5' end, and the reverse primer contained an XhoI restriction site at the 3' end to facilitate cloning into the pcDNA3.1 (+) eukaryotic mammalian expression vector (Invitrogen, Carlsbad, CA). The oligonucleotide primers used were as follows: 5' primer with a KpnI restriction site, 5'-GGGATCCATGCTCACTGTCGAGTAGTATCGAGGAGGAGGATGGTCC-3'; reverse primer, 5'-CCGGCTGAAGGCGGCACTGAAATACACCTCCG-3'. A diagram of all the constructs is shown in Fig. 1.

**Purification of GST-PTP1B**—The recombinant bacterial proteins were separated from insoluble residue by centrifugation at 15,000 × g for 30 min. Aliquots containing equivalent amounts of protein were incubated overnight at 4 °C with 1 μl of rabbit anti-HA tag antibody (1 mg/ml). 10 μl of goat anti-rabbit IgG conjugated to magnetic beads were then added to the supernatant, and the mixture was incubated for 1 h at 4 °C with mixing. The magnetic beads were then washed three times with a washing buffer (1% BSA, 0.01% sodium azide, 0.05% Tween 20) and resuspended in the same buffer containing 0.1% BSA, 10 μg/ml DNase, and 0.4 mM AesfB (Calbiochem). Approximately four to tenfold more proteins were added to each well of the plate. The plate was incubated for 45 min at 37 °C with washing 4 times with HBSGKCa. The cells remaining on the wells were solubilized in 0.5% SDS, and radioactivity was determined by liquid scintillation.

**RESULTS**

**Tyrosine Residues 66, 152, and 153 in PTP1B Are Targets for Phosphorylation**—The amino acid sequence of chick PTP1B has eleven tyrosine residues; however, only three of those fit the consensus substrate site for most protein-tyrosine kinases (26). To determine the residues essential for interaction between N-cadherin and PTP1B, we used the catalytically inactive C215S PTP1B mutant to create point mutations substituting phenylalanine for tyrosine residues 66, 152, and 153. This substitution is the most conservative, maintaining the structure and size of the amino acid, but eliminating the phosphorylation site. A diagram of all the constructs is shown in Fig. 1. The mutated PTP1B cDNAs were subcloned into pGEX-KG and expressed as GST fusion proteins in the bacterial strain TKB1, which expresses a tyrosine kinase with broad specificity, in the presence of [3H]methionine. The GST fusion proteins were analyzed for reactivity with anti-PTP1B and anti-phosphoantibodies (Fig. 2A). All PTP1B fusion proteins migrate as multiple bands on SDS-PAGE, with apparent molecular masses between 60 and 76 kDa (Fig. 2A), reflecting the added masses of GST (26 kDa) and PTP1B (50 kDa). The multiple bands do not appear to reflect differential
phosphorylation, as immunoblotting with an anti-phosphotyrosine antibody reveals only two major bands. The triple mutant, Y66F/Y152F/Y153F, does not show any reactivity with anti-phosphotyrosine antibody, demonstrating that these tyrosine residues are indeed the only substrate sites for Src-like tyrosine kinases. The wild-type enzyme also shows minimal tyrosine phosphorylation as compared with the C215S mutants because of its phosphotyrosine phosphatase activity.

**Tyr-152 Is the Crucial Residue for PTP1B Binding to the Cytoplasmic Domain of N-cadherin in Vitro**

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**FIG. 1.** Diagrammatic representation of PTP1B showing all the mutations analyzed in these studies, the relative position of the catalytic domain, and the targeted tyrosine residues.

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**FIG. 2.** Immunoblots of PTP1B and N-cadherin fusion proteins. A, Western transfers of SDS-PAGE of wild-type PTP1B (WT), catalytically inactive PTP1B (CS), and catalytically inactive PTP1B containing single, double (indicated by residue numbers), and triple (Tp) mutations at tyrosine residues were blotted with anti-PTP1B (top) and anti-phosphotyrosine (bottom). GST indicates fusion produced from vector lacking an insert. B, Western transfers of SDS-PAGE of biotinylated N-cadherin fusion protein (bio) blotted with a pan-cadherin antibody (left) and with HRP-avidin (right).

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**FIG. 3.** Binding of wild-type (WT) and catalytically inactive PTP1B containing each of the single, double, and triple mutants (indicated by residue numbers) to N-cadherin fusion protein. A, 50 µg/ml of PTP1B fusion protein was added to wells containing the immobilized N-cadherin cytoplasmic domain. Asterisks indicate binding groups, within which there is no statistical difference (*p* < 0.01). The difference between binding of 66 and catalytically inactive (CS) is not statistically significant (*p* > 0.05). B, binding of increasing concentrations of wild-type (WT), catalytically inactive (CS), or PTP1B bearing mutations at all three tyrosines Tp, (Y66F/Y152F/Y153F) to the immobilized N-cadherin cytoplasmic domain. Data are graphed as a percentage of control (CS at 50 µg/ml).
the most critical determinant of PTP1B binding to N-cadherin 

in vitro. In agreement with this, the C215S double mutants 

containing a 152 mutation (Y66F/Y152F and Y152F/Y153F) 

also show reduced binding, whereas the C215S Y66F/Y153F 

double mutant binds as well as the unsubstituted C215S (Fig. 

3). These results are true over a wide concentration range (Fig. 

3B); concentrations of C215S PTP1B that show saturation 

binding still fail to show binding of the Y152F mutant. It is 

interesting to note that the Y66F mutant actually facilitates 

binding.

Tyr-152 Is Essential for PTP1B Interaction with N-cadherin—To determine the interaction of the PTP1B mutants 

with N-cadherin in cells, the several PTP1B cDNA constructs 

were subcloned into the pcDNA3.1(+)zeo vector and trans-

fected into LN-cells (14). A 9-amino acid sequence coding for 

the hemagglutinin sequence was added to the amino terminus 

of the PTP1B sequence to facilitate detection of the transfected 

enzyme. Stable cell clones were established by culturing in the 

presence of Zeocin and Geneticin (for stable N-cadherin expres-

sion). Cells were grown to near confluency, lysed with nonionic 

detergent in the presence of tyrosine phosphatase inhibitors, 

and immunoprecipitated with anti-HA antibody. Immunopre-

cipitated material was fractionated by SDS-PAGE and trans-

ferred to PVDF membranes, and the membranes were probed 

with anti-N-cadherin antibody (NCD-2) and anti-PTP1B anti-

body (Fig. 4 A). In agreement with what we observed in the in 

vitro binding assays, the Y152F mutation alone is enough to 

eliminate binding to N-cadherin (Fig. 4 A). Furthermore, all 

combinations of mutant tyrosine residues that include Tyr-152 

behave identically (not shown), whereas mutation at tyrosine 

residues 66 and 153 alone (Fig. 4 A) or in combination (not 

shown) have no effect on binding of PTP1B to N-cadherin.

As in embryonic chick retina cells (13), endogenous PTP1B is 

associated with N-cadherin in control LN-cells (transfected

FIG. 6. Morphology and localization of N-cadherin among LN-cells transfected with catalytically inactive PTP1B mutated at key tyrosine residues and visualized with anti-N-cadherin antibody. WT, wild-type; C215S, catalytically inactive; Y66F, Y152F, Y153F, and Y6/2/3F (triple mutant), catalytically inactive forms containing mutations at the indicated tyrosine residues. Note that among the forms bearing mutations at tyrosine residues, only cells transfected with forms mutated at Tyr-152 revert to a tightly adherent population with N-cadherin present at cell-cell boundaries.

FIG. 5. Adhesion of LN-cells expressing each of the PTP1B constructs to N-cadherin. The data are expressed as the percentage of input cells adhering to the substrate. WT, wild-type PTP1B; CS, catalytically inactive PTP1B; numbers indicate mutations at the indicated tyrosine residues; +NCD indicates adhesion in the presence of the function blocking antibody NCD2; IP, immunoprecipitate; Vec, vector; Tp, Y66F/Y152F/Y153F.

FIG. 3. In situ interaction of N-cadherin with PTP1B. Neutral detergent extracts of LN-cells transfected with HA-tagged PTP1B mutants were immunoprecipitated with anti-HA antibody (A) or anti-N-cadherin antibody (B), separated by SDS-PAGE, transferred to PVDF, and blotted with the indicated antibodies. CS, cells expressing the C215S mutant; 66, 152, and 153, cells expressing the C215S mutant in conjunction with mutations at each of the indicated tyrosine residues; Vec, cells transfected with empty vector.
with vector alone) and is phosphorylated on tyrosine residues (Fig. 4B). Expression of the dominant-negative C215S mutant PTP1B in LN-cells prevents the association of endogenous PTP1B with N-cadherin (see Fig. 4B and Ref. 14). In contrast, expression of PTP1B carrying both the C215S and the Y152F mutations does not alter the association of endogenous PTP1B with N-cadherin. Thus tyrosine 152 is critical for in situ binding and displacement of endogenous PTP1B from cadherin.

The Y152F Mutation Reverses the C215S Dominant-Negative Effect on N-cadherin-mediated Adhesion—The catalytically inactive C215S PTP1B mutant acts as a dominant-negative when introduced into LN-cells, inhibiting N-cadherin-mediated cell interaction (14). By introducing a mutation that eliminates binding to N-cadherin in the C215S PTP1B, the dominant-negative effect should be abolished; this is indeed the case (Fig. 5). N-cadherin-mediated cell adhesion is abolished in the C215S mutants but restored in the C215S mutants that also have a Y152F mutation. In comparison, mutations in tyrosine residues 66 and 153 alone or in combination have no effect (Fig. 5). This effect on N-cadherin-mediated adhesion is reflected in the cells phenotype; LN-cells grow in clusters of tightly adherent cells because of expression of N-cadherin (Fig. 6A; see also Ref. 14). In the dominant-negative C215S mutant this phenotype is lost because of inactivation of N-cadherin (compare Fig. 6, A and B) but recovered in the C215S mutant bearing the Y152F mutation (Fig. 6C). In contrast, mutation of either tyrosine 66 or 153 has little or no effect on the dominant-negative phenotype.

DISCUSSION

Our laboratory has demonstrated that PTP1B interacts directly with N-cadherin and that phosphorylation of PTP1B on tyrosine residues is necessary for this association (13, 14). We now identify tyrosine residue 152 in PTP1B as the critical residue for PTP1B-N-cadherin interaction. PTP1B mutants that have tyrosine 152 replaced by phenylalanine do not interact with N-cadherin in vitro binding assays. Moreover, in L-cells expressing N-cadherin and HA-tagged PTP1B carrying the Y152F and C215S double mutation, HA-PTP1B does not coimmunoprecipitate with N-cadherin, indicating a lack of association between the two molecules in situ. This is also reflected in the loss of the dominant-negative effect on adhesion of the C215S mutation on N-cadherin function. Furthermore, in LN-cells expressing the Y152F mutation endogenous PTP1B is associated with N-cadherin, and it is tyrosine-phosphorylated.

The multiple intracellular roles played by PTP1B require interactions with many different intracellular partners. The needed binding specificity appears to be achieved by compartmentalization or by targeting mediated by specific domains. The carboxyl terminus of PTP1B directs its localization to the cytoplasmic face of the endoplasmic reticulum, thus restricting the number of potential interactors (20). In platelets and activated T-cells, proteolytic cleavage in the ER targeting domain results in translocation of PTP1B to the cytoskeletal/membrane fraction (27-29). This cleavage is dependent on integrin engagement, resulting in increased Ca+2 levels and, consequently, activation of calpain. We also find that PTP1B associated with N-cadherin in vivo migrates faster on SDS-PAGE than the intact ~50-kDa enzyme, suggesting cleavage (13, 14). The N-cadherin-associated PTP1B represents a small fraction of the total and colocalizes with N-cadherin in sites of cell-cell contacts and at the tips of growing neurites (14, 30). Elimination of the ER localization signal does not alter the interaction of PTP1B with N-cadherin, suggesting that targeting of PTP1B to the N-cadherin complex does not depend on prior targeting to the ER. Furthermore, targeting to specific plasma membrane locations does not appear to depend on cleavage of the ER targeting sequence, as the PTP1B associated with focal adhesion complexes (24) and the insulin receptor (22) have an apparent molecular mass of ~50 kDa.

Phosphorylation on tyrosine residues is important for targeting of PTP1B to at least two of its interacting partners. As we demonstrate here, phosphorylation of tyrosine 152 is critical for binding to N-cadherin. Additionally, interaction of PTP1B with the insulin receptor results in phosphorylation of tyrosine residues 66 and 152/153. Phosphorylation of these residues further promotes binding to the receptor. Tyrosine 66 is the major target for phosphorylation of PTP1B by the insulin receptor, creating a site essential for downstream signaling (22). In contrast, tyrosine phosphorylation on PTP1B does not appear to play a role in the binding of PTP1B to p130cas (25). This interaction, which probably mediates targeting of PTP1B to the integrin complex, is mediated by a proline-rich, SH3-binding domain in PTP1B (25). These differences highlight the fact that even though PTP1B is a ubiquitous enzyme, it plays a pivotal role in regulating many cellular functions through specific protein-protein interactions.

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