Receptor Protein Tyrosine Phosphatase PTPμ
Associates with Cadherins and Catenins In Vivo

Susann M. Brady-Kalnay,* David L. Rimm,‡ and Nicholas K. Tonks*

*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724-2208; and ‡Department of Pathology, Yale University, New Haven, Connecticut 06510-8023

Abstract. The extracellular segment of the receptor-type protein tyrosine phosphatase PTPμ, possesses an MAM domain, an immunoglobulin domain, and four fibronectin type-III repeats. It binds homophilically, i.e., PTPμ on the surface of one cell binds to PTPμ on an apposing cell, and the binding site lies within the immunoglobulin domain. The intracellular segment of PTPμ has two PTP domains and a juxtamembrane segment that is homologous to the conserved intracellular domain of the cadherins. In cadherins, this segment interacts with proteins termed catenins to mediate association with the actin cytoskeleton. In this article, we demonstrate that PTPμ associates with a complex containing cadherins, α- and β-catenin in mink lung (MvLu) cells, and in rat heart, lung, and brain tissues. Greater than 80% of the cadherin in the cell is cleared from Triton X-100 lysates of MvLu cells after immunoprecipitation with antibodies to PTPμ; however, the complex is dissociated when lysates are prepared in more stringent, SDS-containing RIPA buffer. In vitro binding studies demonstrated that the intracellular segment of PTPμ binds directly to the intracellular domain of E-cadherin, but not to α- or β-catenin. Consistent with their ability to interact in vivo, PTPμ, cadherins, and catenins all localized to points of cell–cell contact in MvLu cells, as assessed by immunocytochemical staining. After pervanadate treatment of MvLu cells, which inhibits cellular tyrosine phosphatase activity including PTPμ, the cadherins associated with PTPμ are now found in a tyrosine-phosphorylated form, indicating that the cadherins may be an endogenous substrate for PTPμ. These data suggest that PTPμ may be one of the enzymes that regulates the dynamic tyrosine phosphorylation, and thus function, of the cadherin/catenin complex in vivo.

M ANY cellular processes are regulated by reversible tyrosine phosphorylation, controlled by the balanced and opposing actions of protein tyrosine kinases (PTKs)[1] and protein tyrosine phosphatases (PTPs). PTPs exist in both soluble and transmembrane, receptor-like forms (Charbonneau and Tonks, 1992; Tonks, 1993). In the case of the receptor PTPs (RPTPs), there is the potential for regulating activity by the binding of ligands to the extracellular segment of the protein. Several of the transmembrane PTPs are members of the immunoglobulin superfamily, and display structural motifs in their extracellular segments that are suggestive of a role in cell–cell adhesion (for review see Brady-Kalnay and Tonks, 1994a). To date, two RPTPs, PTPμ and PTPκ, have been shown to mediate aggregation via homophilic binding (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al.,

1994). Thus, the ligand for these two RPTPs is an identical molecule on an apposing cell, suggesting that they may transduce signals in response to cell contact. Information concerning ligands for other RPTPs is also beginning to emerge. PTPζ/β is a chondroitin sulfate proteoglycan (Barnea et al., 1994; Maeda et al., 1994; Shitara et al., 1994). Phosphacan, a splice variant comprising the PTPζ/β extracellular segment, has been shown to interact heterophilically with N-CAM, Ng-CAM, and tenascin (Grumet et al., 1994; Maurel et al., 1994; Milev et al., 1994).

Our studies have focused on PTPμ. We have shown previously that PTPμ can mediate aggregation of cells via homophilic binding (Brady-Kalnay et al., 1993), and that the homophilic binding site resides in the Ig domain (Brady-Kalnay and Tonks, 1994b). The intracellular segment of PTPμ is characterized by two PTP domains and a juxtamembrane segment that is ~70 residues longer than the equivalent segment in most other RPTPs, and displays homology to the entire intracellular domain of members of the cadherin family (for review see Brady-Kalnay and Tonks, 1994a). Cadherins are a major family of calcium-dependent cell–cell adhesion molecules that also bind by a homophilic mechanism and localize to specialized intercel-

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1. *Abbreviations used in this paper.* GST, glutathione-*S*-transferase; IP, immunoprecipitation; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

lular junctions called adherens junctions (for reviews see Grunwald, 1993; Nagafuchi et al., 1993). The cadherins are transmembrane proteins possessing an extracellular calcium-binding segment and an intracellular domain that is highly conserved (~90% identity) among most members of the family. Deletions of the conserved intracellular segment can prevent adhesion even when the extracellular binding domain is intact (Nagafuchi and Takeichi, 1988). The cytoplasmic domain of the cadherins interacts with three molecules termed catenins (α, β, γ), and the complex associates with cortical actin (Rimm et al., 1995b; for reviews see Ranscht, 1994; Cowin, 1994). Two of the catenins have been cloned. α-catenin is homologous to vinculin, a cytoskeletal-associated protein. β-catenin is homologous to plakoglobin, a protein found at desmosomes, and *armadillo*, a segment polarity gene in *Drosophila melanogaster* (Herrenknecht et al., 1991; McCrea et al., 1991; Nagafuchi et al., 1991). Immunochemical data suggest that γ-catenin is identical to plakoglobin (Knudsen and Wheelock, 1992; Pipenhagen and Nelson, 1993). It appears that β-catenin and plakoglobin bind directly to the cytoplasmic domain of E-cadherin while α-catenin binds directly to β-catenin and plakoglobin (Aberle et al., 1994). This interaction between the cadherins and catenins is essential for cadherin-mediated adhesion and association of the complex with the cytoskeleton. Based on the structural similarity between PTPμ and the cadherins, we postulated that PTPμ may interact with cadherins and catenin-like molecules (Tonks et al., 1992).

Here, we have examined protein–protein interactions involving PTPμ, and have demonstrated that the cadherins and α- and β-catenin coimmunoprecipitated with PTPμ from lysates of rat heart, lung, and brain, as well as mink lung MvLu cells. In vitro binding studies indicated that the intracellular segment of PTPμ bound directly to the intracellular domain of E-cadherin, but not to α- or β-catenin. We have demonstrated by immunocytochemistry that PTPμ, cadherins, and catenins were localized at points of cell–cell contact in MvLu cells, and thus are in the appropriate subcellular location to interact in vivo. After pervanadate treatment of MvLu cells, which inhibits cellular tyrosine phosphatase activity, the PTPμ-associated cadherins accumulated in a tyrosine-phosphorylated form. These results indicate that PTPμ may regulate the phosphorylation of tyrosyl residues in cadherins and catenins, and thus may also regulate their function.

Materials and Methods

Cells

MvLu cells were grown as previously described (Brady-Kalnay et al., 1993). The trypsin treatments that cleave and/or preserve the extracellular segment of the cadherins at the cell surface (originally described by Takeichi, 1977) were performed on intact cells as described by Brackenbury et al. (1981). Briefly, three treatments were used: (1) 1 mM EDTA only; (2) LTE (0.002% trypsin plus 1 mM EDTA); (3) TCa^{++} (0.04% trypsin plus 10 mM calcium chloride). Trypsin was inhibited before cell lysis by the addition of lima bean trypsin inhibitor in a sixfold excess by weight. Pervanadate was generated by mixing hydrogen peroxide and sodium vanadate in a 1:1 molar ratio (Maher, 1993). Cells were treated by addition of 10 or 50 μM (final concentration) pervanadate to the cell medium for 1 h at 37°C before lysis. Sf9 cells were infected with recombinant baculoviruses

as described (Brady-Kalnay et al., 1993), harvested 3 d after infection by centrifugation, and lysed in Triton buffer as described below.

Antibodies

An mAb to the intracellular segment of PTPμ (SBK15) and a monoclonal anti-peptide antibody toward the extracellular segment of PTPμ (BK2) have been described previously (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994b). Polyclonal antibodies to glutathione-S-transferase (GST) and α-catenin (RAC) were generated as described (Rimm et al., 1995a). A polyclonal antibody against β-catenin (RBC) was generated using full-length protein fused to GST as the antigen. mAbs to α- (TAC) and β-catenin (TBC) were purchased from Transduction Laboratories (Lexington, KY). Monoclonal (MPan) and polyclonal (RPan) pan cadherin antibodies, which react with the conserved COOH-terminal 24 amino acids of the cadherin cytoplasmic domain, and nonspecific mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Generally, mAbs to the cadherins and catenins were used for immunoprecipitation, while the polyclonal antibodies were used for immunoblotting. Anti-EGF receptor antibodies and monoclonal anti-phosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Antibodies were incubated with protein A or protein G beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 2 h at room temperature, then washed three times with PBS (9.5 mM phosphate, 137 mM NaCl, pH 7.5) before incubation with the lysates. For immunoprecipitation, purified mAbs were used at 0.6 μg of IgG/μl beads, ascites fluid was used at 1 μg of IgG/μl beads, and polyclonal serum was used at 3 μg of IgG/μl beads.

Electrophoresis and Immunoblotting

For most experiments, MvLu cells were lysed in Triton-containing buffer (20 mM Tris, pH 7.6, 1% Triton X-100, 5 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine, 200 μM phenylarsine oxide, 1 mM vanadate, and 0.1 mM molybdate) and scraped off the dish. In selected experiments, cells were lysed in RIPA buffer (20 mM Tris pH 7.5, 1 mM EDTA, 0.15 M NaCl, 10 mM KCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM benzamidine, 200 μM phenylarsine oxide, 1 mM vanadate, and 0.1 mM molybdate). Tissue lysates were prepared by homogenization in Triton-containing buffer. After incubation on ice for 30 min, the lysate was centrifuged at 5,000 g for 5 min, and the Triton-soluble material was recovered in the supernatant. The amount of protein was determined by the Bradford method using BSA as a standard. Immunoprecipitates were prepared from 400 μg of protein from a Triton-soluble lysate using primary antibody that had been coupled to beads. The immunoprecipitates were centrifuged, and the supernatant ("cleared fraction") and the beads were separated. For the supernatant, 4× sample buffer was added (to a final concentration of 1×) and boiled at 95°C for 5 min. The beads were washed four times in Triton- or RIPA-containing buffer, and the bound material was eluted by addition of 100 μl of 2× sample buffer and boiling for 5 min at 95°C. One-fifth of the immunoprecipitation (IP) (20 μl) was loaded per lane of the gel, the proteins were separated by electrophoresis on 6% SDS polyacrylamide gels, and transferred to nitrocellulose for immunoblotting as described previously (Brady-Kalnay et al., 1993).

GST Constructs and Overlay Blots

GST was expressed and purified as described (Brady-Kalnay et al., 1993). GST fusion proteins were generated as follows: a construct encoding the COOH-terminal 151 amino acids of E-cadherin was synthesized by PCR and cloned into pGEX-2T. Full-length cDNA for α-catenin was isolated as described (Rimm et al., 1994), and subcloned into pGEX-KT (Hakes and Dixon, 1992). The entire coding sequence of β-catenin was amplified by RT-PCR using primers based on the sequence in Genbank for human β-catenin, and a fusion protein was made by subcloning into pGEX-KT. Fusion constructs of both the NH$_2$-terminal (residues 1–375) and COOH-terminal (residues 315–781) segments of β-catenin with GST were also generated by RT-PCR, and subcloned into pGEX-KT. Expression of GST-tagged proteins in *Escherichia coli* was induced by isopropylthio-β-D-galactoside (IPTG). The bacteria were collected by centrifugation at 3,000 g for 10 min, and lysed in PBS containing 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM benzamidine, sonicated and centrifuged at 3,000 g for 10 min again to remove debris. The supernatant was passed over glutathione-Sepharose beads (Pharmacia LKB Biotechnology

Inc.) washed, and the bound protein eluted with 10 mM glutathione as described previously (Brady-Kalnay et al., 1993). A GST antibody was used for detection of these fusion proteins. For overlay blots, protein samples were resolved by electrophoresis (on 10% gels), and transferred to nitrocellulose as described above. The immobilized protein was partially renatured by washing the blot three times for a total of 4 h with 5% nonfat dry milk in TBST (660 mM NaCl, 20 mM Tris, pH 7.5, 0.05% Tween). The protein probe was added to the blot at 10 μg/ml in TBST containing 5% nonfat dry milk, and incubated at 4°C overnight. The blot was washed four times in TBST to remove uncomplexed probe. Bound probe was detected immunologically by enhanced chemiluminescence.

Immunocytochemistry

All chemicals were diluted in PBS. Cells were fixed with 2% paraformaldehyde for 15 min at room temperature (Electron Microscopy Sciences, Fort Washington, PA). The cells were permeabilized with 0.5% saponin, blocked with 5% normal goat serum, and incubated with primary antibody for 1 h. The cells were washed with PBS containing 0.5% saponin, and incubated with rhodamine-conjugated secondary antibody (Cappel Research Products, Durham, NC) for 1 h. The fluorescent staining was examined using an oil immersion objective, magnification, 63, of a microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence.

Results

Cadherin and Catenin Expression in a Lung Cell Line

In light of the structural similarity between the juxtamembrane segment of PTPμ and the intracellular segment of the cadherins, we examined whether PTPμ associates with the cadherin/catenin complex in MvLu cells, which express PTPμ endogenously (Brady-Kalnay et al., 1993). The PTPμ protein exists in vivo in both a full-length form and a form that is proteolytically processed into two noncovalently associated fragments, one of which (E subunit) contains most of the extracellular segment while the other fragment (P subunit) contains a small portion of extracellular segment, the transmembrane domain, and the entire intracellular segment (Brady-Kalnay and Tonks, 1994b). The full-length form of PTPμ migrates at ∼200 kD while each of the cleaved fragments migrate at ∼100 kD upon SDS-PAGE. The full-length form and the E subunit reacted with an antibody generated against the extracellular segment of PTPμ (Fig. 1 A).
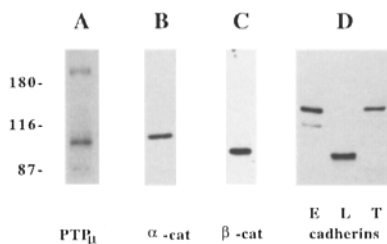
As expected from published observations, antibodies to α-catenin recognized a protein of ∼100 kD (Fig. 1 B), whereas antibodies to β-catenin recognized a protein of ∼90 kD (Fig. 1 C) in immunoblots of MvLu cell lysates. The pancadherin antibody, which was generated against the highly conserved COOH-terminal 24 amino acids of N-cadherin, reacts with most classical cadherins (Geiger et al., 1990). In MvLu cells, this antibody recognized two species (130 and 116 kD) on an immunoblot (Fig. 1 D, lane E), but only immunoprecipitated the 130-kD protein from Triton buffer lysates (see Fig. 2 B, lane C). To verify that these proteins that are immunoreactive to the pancadherin antibody behave as cadherins, we analyzed their sensitivity to trypsinization in the presence or absence of calcium. This test is based on the original description of calcium-independent versus calcium-dependent adhesion molecules (Takeichi, 1977). In this procedure, the extracellular segment of the cadherins (calcium-dependent adhesion molecules) is protected from trypsinization by the presence of calcium, but cleaved in the absence of calcium. The cleavage of the cadherins coincides with these proteins being rendered nonfunctional in aggregation assays (Takeichi, 1977; Brackenbury et al., 1981). As shown in Fig. 1 D (lane L), the proteins that are reactive to the pancadherin antibody are cleaved by trypsin in the absence of calcium. The fragment that appeared in this lane could be resolved into two bands on an 8% gel (data not shown), suggesting that both cadherins are susceptible to tryptic cleavage in the absence of calcium. The 130-kD band is protected from trypsinization in the presence of calcium (lane T). As has been reported for cadherin 5 (Tanihara et al., 1994), the 116-kD form appears to be more sensitive to trypsinization in the presence of calcium, however, analysis of immunoprecipitates indicates that it is partially protected (data not
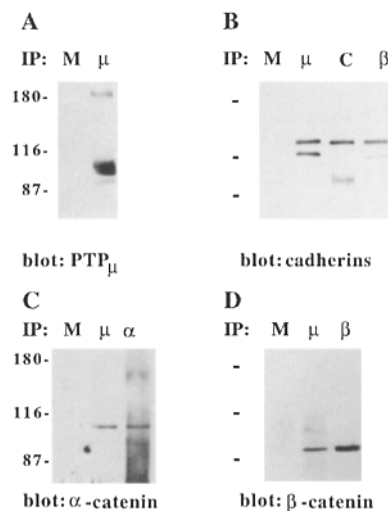

Figure 1. Characterization of PTPμ, cadherin, α-catenin and β-catenin in MvLu cells. This figure shows immunoblots of lysates from MvLu cells. Each lane contains 15 μg of lysate protein. A was probed with an antibody (BK2) to the extracellular segment of PTPμ; B, an antibody (TAC) to α-catenin; C, an antibody (TBC) to β-catenin; D, an antibody (RPan) to cadherin. In panel D, immunoblots of lysates from MvLu cells treated with EDTA alone (E), low trypsin plus EDTA (L), or trypsin plus calcium (T) are presented. The two cadherins were degraded by trypsin in the absence of calcium (L) while the 130-kD band was protected from trypsinization by the presence of calcium (T).

Figure 2. PTPμ, cadherins, and α- and β-catenin are associated in a complex in MvLu cells. This figure illustrates IP immunoblots of lysates from MvLu cells using mouse IgG (M), PTPμ (μ), cadherin (C), α-catenin (α) or β-catenin (β) antibodies for immunoprecipitation. These immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to PTPμ (SK15; A), cadherins (RPan; B), α-catenin (RAC; C), or β-catenin (RBC; D). PTPμ immunoprecipitates contained cadherins and α- and β-catenin.

shown). In light of the reaction with anti-cadherin antibodies and the response to trypsinization, it appears that the 130- and 116-kD proteins are members of the cadherin superfamily.

PTPμ Associates with Cadherins and Catenins In Vivo

We analyzed protein/protein interactions by IP immunoblots, in which proteins are immunoprecipitated with antibodies coupled to beads, the bound material is eluted and resolved by SDS-PAGE, transferred to nitrocellulose, and probed with various antibodies by immunoblotting. Both the full-length (200-kD) and the cleaved (100-kD) forms of PTPμ were immunoprecipitated by antibodies to the extracellular segment of PTPμ (Fig. 2 A, lane μ), but not by mouse IgG (lane M). When immunoprecipitates of PTPμ were probed on immunoblots with anti-pancadherin antibodies, an association between the 130- and 116-kD cadherin and PTPμ was detected (Fig. 2 B, lane μ). Only the 130-kD cadherin was immunoprecipitated by anti-pancadherin antibody (Fig. 2 B, lane C). The lower band in Fig. 2 B, lane C is nonspecific; it cross-reacted with the goat anti–rabbit secondary antibody, and was also seen when other rabbit primary antibodies were used. Immunoprecipitates of β-catenin also contained both the 130- and 116-kD cadherins (Fig. 2 B, lane β) that were not detected when mouse IgG was used for immunoprecipitation (Fig. 2 B, lane M). Similarly, an association between PTPμ and α- and β-catenin was detected when antibodies to α-catenin (Fig. 2 C) or β-catenin (Fig. 2 D) were used as probes in the IP immunoblot. As shown in Fig. 2 C, immunoprecipitates of PTPμ (lane μ) contain a protein that comigrated with the protein recognized by blotting α-catenin immunoprecipitates with α-catenin antibodies (lane α). Similarly, immunoprecipitates of PTPμ contained a protein that comigrated with β-catenin, and was recognized by antibodies to β-catenin (Fig. 2 D, lanes μ and β). Neither α- nor β-catenin were immunoprecipitated with mouse IgG (Fig. 2 C and D, lane M). Based on these data, it appears that we can isolate complexes containing PTPμ, cadherins, α- and β-catenin in MvLu cells which endogenously express these proteins.

We have also observed this complex in rat tissue lysates. Fig. 3 illustrates immunoprecipitates, using mouse IgG (lanes 1, 3, 5) or antibodies to PTPμ (lanes 2, 4, 6), from lung (lanes 1 and 2), heart (lanes 3 and 4), and brain (lanes 5 and 6). A demonstrates that PTPμ was expressed in all three tissues, and was present both in full-length (200-kD), and proteolytically processed (100-kD) forms. The band that runs below 100 kD is not related to PTPμ, as determined by its failure to react with other PTPμ antibodies; it cross-reacted with the secondary antibody on IP immunoblots. As shown in B, proteins of different molecular weights that were reactive to the pan-cadherin antibody coimmunoprecipitated with PTPμ in the three tissues. Using specific antibodies, we have identified two of the cadherins associated with PTPμ in lung as E-cadherin and cadherin 4 (data not shown). Similar to the pattern of association observed in the MvLu mink lung cell line, we also detected α- and β-catenin in immunoprecipitates of PTPμ from rat lung (lane 2, C and D). We only detected β-catenin in the complex with PTPμ and cadherin in heart and
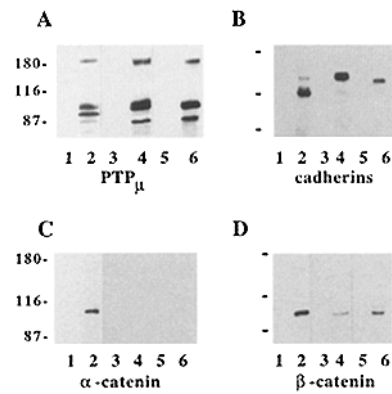

Figure 3. PTPμ, cadherins, α-, and β-catenin are associated in lung, heart, and brain tissue. This figure represents IP immunoblots from lysates of lung (lanes *1* and *2*), heart (lanes *3* and *4*) and brain (lanes *5* and *6*). Lanes *1, 3,* and *5* are immunoprecipitates using mouse IgG. Lanes *2, 4,* and *6* are immunoprecipitates using antibody (BK2) to the extracellular domain of PTPμ. The immunoblot in *A* was probed with antibody (SK15) to PTPμ. The immunoblot in *B* was probed with antibody (RPan) to cadherin. *C* and *D* were probed with antibodies (RAC and RBC) to α- and β-catenin, respectively. PTPμ was found associated with cadherins and β-catenin in all three tissues. α-catenin was only observed to coprecipitate with PTPμ in lung tissue.

brain. As seen in Fig. 3 D, the amount of β-catenin present in the PTPμ immunoprecipitates from the different tissues is variable. The amount of α- and β-catenin associated with PTPμ does not reflect the total amount of these proteins in the tissues. Thus, α-catenin is present at similar levels in all three tissues, and the highest amount of β-catenin associated with PTPμ is observed in lung, even though of the tissues examined, β-catenin is actually most abundant in brain (data not shown). Since α-catenin associates with PTPμ indirectly, through its interaction with β-catenin (see Fig. 6), the presence or absence of α-catenin in the complex may be determined by the level of β-catenin. Whether this reflects differential regulation of the complex between tissues remains to be established.

In the preceding analyses using antibody to the extracellular segment of PTPμ, cadherins and catenins coimmunoprecipitated with the phosphatase. However, we wanted to verify that this reflects the isolation of a multiprotein complex, rather than the presence of a cross-reacting epitope in the various proteins. Therefore, we examined whether the complex could be isolated under conditions of increased stringency after lysis of the cells in a RIPA buffer containing detergents 1% NP-40, 0.1% SDS, and 0.1% deoxycholate. Mouse IgG does not immunoprecipitate PTPμ or the cadherins (Fig. 4, A and B, lanes 1 and 3). Anti-PTPμ antibodies immunoprecipitated both full-length and cleaved forms of the phosphatase from either Triton or RIPA buffer lysates (Fig. 4 A, lanes 2 and 4). Anti-PTPμ immunoprecipitates from Triton buffer lysates also contained cadherins (Fig. 4 B, lane 2), whereas cadherins were not found in the immunoprecipitates from RIPA buffer lysates (Fig. 4 B, lane 4). Fig. 4 C demonstrates that equal amounts of cadherins were present in both the Triton and RIPA cell extracts. Equivalent results were observed for both α- and β-catenin, i.e., they were
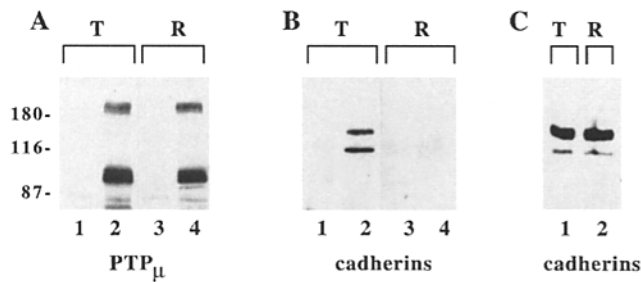
Figure 4. The complex of cadherins and PTPμ is not stable to extraction in SDS-containing RIPA buffer. A and B show immunoblots using antibodies to PTPμ (SK15) or cadherins (RPan), respectively. C is an immunoblot using antibody (RPan) to cadherin. Mouse IgG was used for immunoprecipitation in lanes 1 and 3 (A and B). PTPμ antibody (BK2) was used for immunoprecipitation in lanes 2 and 4. PTPμ was immunoprecipitated in both Triton (A, lane 2) and RIPA buffers (A, lane 4). Cadherins were only associated with PTPμ in Triton buffer (B, lane 2) but not RIPA buffer (B, lane 4). C shows that equal amounts of cadherins were present in both Triton (lane 1) and RIPA (lane 2) buffer lysates.

only recovered in PTPμ immunoprecipitates from Triton buffer lysates, and not when RIPA buffer was used to lyse the cells (data not shown). These data suggest that the PTPμ antibodies do not cross-react with the cadherins, and that the cadherins and catenins were found in immunoprecipitates of the phosphatase because they are associated with PTPμ or an additional protein that also binds to PTPμ.

To determine the fraction of total cadherin that was associated with PTPμ in these cells, we determined how much cadherin remained in the supernatant after immunoprecipitation using various antibodies. In this procedure, IPs were performed, and the proteins that remained in the supernatant after the beads were removed ("cleared fraction") were compared by immunoblotting. When various control antibodies were used, no cadherin was cleared from the supernatant (Fig. 5, lanes 1, 3, 4). However, a substantial amount (~80%) of both cadherins was cleared
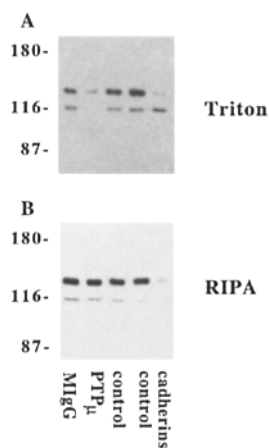

Figure 5. PTPμ associates with >80% of total cellular cadherin. A and B illustrate immunoblots using antibody (RPan) to cadherin. The lysates represent cleared fractions, i.e., the cadherin that remained in the supernatant after immunoprecipitation with the antibodies indicated at the bottom of B. A shows that the amount of cadherin in the Triton lysate was unaffected by mouse IgG or two other nonspecific mAbs (control). Immunoprecipitation with PTPμ cleared ~80% of cadherin from the Triton lysate. The cadherin antibody cleared ~90% of the 130-kD form from the Triton lysate. B shows that only cadherin antibody precipitated cadherin from RIPA buffer lysates. A complex of PTPμ and cadherin was not recovered from lysates prepared in RIPA buffer.

from the Triton buffer lysates (Fig. 5 A, lane 2) but not RIPA buffer lysates (Fig. 5 B, lane 2) after immunoprecipitation with anti-PTPμ antibodies.

Analysis of the Binding Interactions between PTPμ, Cadherins, and Catenins In Vitro

To define which segments of PTPμ, the cadherins, and the catenins were involved in complex formation, we set up the following overlay assays to detect binding interactions in vitro (for review see Carr and Scott, 1992). In overlay assays, proteins are subjected to electrophoresis on SDS-PAGE gels, transferred to nitrocellulose, renatured, and protein probes are added in solution to the blot. The protein probe binds to its partner on the nitrocellulose filter and the bound protein is detected with a specific primary antibody followed by a secondary antibody and chemiluminescence. Fig. 6 A illustrates an immunoblot with anti-GST antibody to demonstrate that the various fusion proteins were expressed and migrated at the appropriate apparent molecular weight after SDS-PAGE. Control overlay blots demonstrated that β-catenin bound both to the intracellular domain of E-cadherin and to α-catenin (B), whereas α-catenin bound
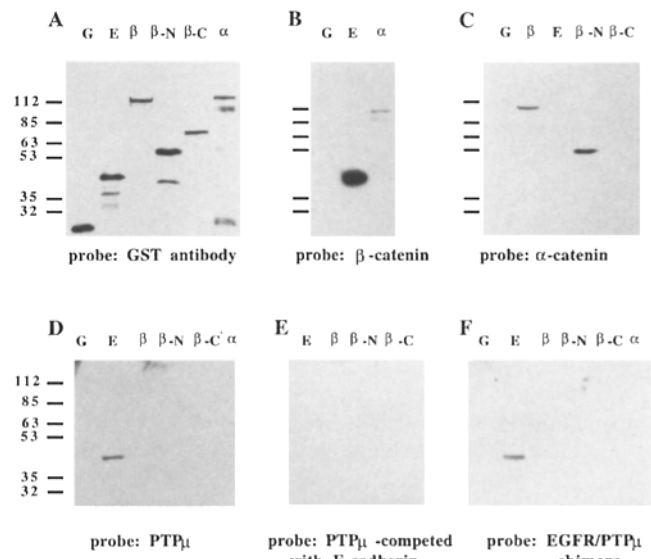

Figure 6. The intracellular domain of PTPμ binds to the intracellular domain of E-cadherin in vitro. A is an immunoblot using anti-GST antibody to demonstrate that the fusion proteins were expressed and migrated at the appropriate apparent molecular weight. Overlay blots (B–F) demonstrate that β-catenin bound to both the intracellular domain of E-cadherin and α-catenin (B) while α-catenin bound only to the NH_2 terminus of β-catenin, which is found in both full-length and the NH_2-terminal construct of β-catenin (C). Overlay blots using PTPμ as the protein probe demonstrated that PTPμ bound to the intracellular domain of E-cadherin (D) and that the binding was blocked by preincubation of the PTPμ probe with soluble E-cadherin (E). If a chimeric molecule, which contained the intracellular segment of PTPμ fused to the extracellular segment of the EGF receptor, was used as the protein probe in the overlay blot, and detected with an antibody to the EGF receptor, it was also observed to bind to E-cadherin (F), indicating that it is the intracellular domain of PTPμ that mediates association. G, GST; E, E-cadherin intracellular domain; β, full-length β-catenin; β-N, NH_2-terminal construct of β-catenin; β-C, COOH-terminal construct of β-catenin; α, α-catenin.

only to β-catenin (C). The binding site for α-catenin was in the NH$_2$-terminal segment of β-catenin (C). When PTPμ was used as the protein probe in the overlay blots, we observed that the phosphatase bound to the intracellular domain of E-cadherin but did not bind α- or β-catenin (D). If PTPμ was preincubated with the intracellular domain of E-cadherin before addition to the overlay blot, the binding to E-cadherin on the blot was blocked (E). A chimeric molecule containing the intracellular segment of PTPμ fused to the extracellular segment of the EGF receptor, was used as the probe in the overlay blot and detected using anti-EGF receptor antibodies. This probe also bound to E-cadherin (F), demonstrating that it is the intracellular segment of PTPμ that binds to the intracellular segment of E-cadherin. These data indicate that PTPμ and cadherins interact directly both in vivo and in vitro.

Immunocytochemistry Confirms the Localization of PTPμ, Cadherins, and Catenins at Points of Cell/Cell Contact

Cadherins and catenins are located at points of cell–cell contact, including adherens junctions. To determine whether PTPμ displayed a similar distribution, we performed immunocytochemistry on MvLu cells. Fig. 7, A and B, shows an immunocytochemical analysis of subconfluent cultures of MvLu cells. PTPμ was localized at points of cell–cell contact. Interestingly, filopodial extensions that contact adjacent cells also contained PTPμ (Fig. 7, A and B). When cells were plated at higher density, PTPμ was restricted to points of cell–cell contact (C and D). Antibodies to the cadherins (E and F) and β-catenin (G and H) also decorated points of cell–cell contact in MvLu cells. Similar staining patterns were observed for α-catenin (data not shown). Double-label immunocytochemistry demonstrated that the distribution of PTPμ, cadherins, and catenins directly overlaps at points of cell–cell contact (data not shown). This similarity in their subcellular distribution confirms that under normal physiological levels of expression, these proteins are in the appropriate location to interact with one another in vivo.

Inhibition of PTPs Leads to an Accumulation of Tyrosine-phosphorylated Cadherins in the Complex with PTPμ

To address the physiological significance of this association, we tested the possibility that cadherins may be physiological substrates of PTPμ. At the present time there are no specific inhibitors of PTPμ available. Therefore, we treated MvLu cells with pervanadate, a broad specificity inhibitor of cellular PTPs, including PTPμ, to examine its effect on the phosphorylation of tyrosyl residues in cadherins. Treatment of cells with 10 μM pervanadate had little apparent effect on PTP activity, as indicated by the very weak signal in anti-phosphotyrosine antibody immunoblots (data not shown). As illustrated in lane 1, only trace amounts of cadherin were immunoprecipitated with anti-phosphotyrosine antibodies when MvLu cells were treated with 10 μM pervanadate (pTYR-IP-10). In contrast, there was a large increase in the level of tyrosine-phosphorylated cadherins, as determined by immunoprecipitation with anti-phosphotyrosine antibody (pTYR-IP-50), after

treatment of the cells with 50 μM pervanadate. After inhibition of PTP activity, the resultant tyrosine phosphorylation of the cadherins correlated with a retardation in their electrophoretic mobility, which was most pronounced for the 116-kD molecule (compare the two lanes designated lysate 10 and lysate 50). Immunoprecipitates of PTPμ from MvLu cell lysates prepared after treatment of the cells with various concentrations of pervanadate, were immunoblotted with the pancadherin antibody. At 10 μM pervanadate, which exerted little effect on PTP activity, the cadherins that coimmunoprecipitate with PTPμ displayed the expected electrophoretic mobility of the dephosphorylated proteins (see lane PTPμ-IP 10). However, upon treatment of the cells with 50 or 100 μM pervanadate, which effectively inhibited PTP activity including PTPμ, the cadherins recovered in the PTPμ immunoprecipitates displayed retarded electrophoretic mobility (lanes PTPμ-IP 50 and 100), similar to that of the tyrosine-phosphorylated protein detected in anti-phosphotyrosine antibody immunoprecipitates (lane PTYR-IP 50). After treatment of the PTPμ immunoprecipitates with either alkaline phosphatase or a tyrosine-specific phosphatase, PTP1B, the retardation in electrophoretic mobility of the associated cadherins was abolished, and they now migrated at the mobility expected for the unphosphorylated proteins (see lane PTPμ-IP PPase). Interestingly, prolonged incubation of the MvLu cells in 100 μM pervanadate resulted in a dissociation of the cadherins from PTPμ (data not shown). These results suggest that when PTPs, including PTPμ, were inhibited, the associated cadherins become tyrosine phosphorylated. Therefore, the cadherins may be endogenous substrates of PTPμ.

Discussion

Cell–cell adhesion molecules of the cadherin superfamily play a critical role in cytoskeletal organization leading to cell-junction formation and epithelial polarization. Cadherins are not only structural proteins, but also are implicated in signal transduction processes. They function as tumor suppressors, i.e., there is often deletion and/or mutation of members of the cadherin/catenin complex in many types of carcinoma. The cadherins associate, through their intracellular segments, with a number of proteins including α-catenin, β-catenin, and γ-catenin/plakoglobin. β-catenin and γ-catenin/plakoglobin bind directly to the E-cadherin cytoplasmic domain, whereas α-catenin binds to both β-catenin and actin directly, thus linking this complex to the cytoskeleton. These associations are essential for the adhesive functions of cadherins. β-catenin also binds to the product of the tumor suppressor gene APC (Rubinfeld et al., 1993; Su et al., 1993; for review see Hulsken et al., 1994a). E-cadherin and APC appear to compete for the internal armadillo-like repeats of β-catenin and plakoglobin, and thus their binding appears to be mutually exclusive (Hülsken et al., 1994b; Rubinfeld et al., 1993).

Destabilization of adherens junctions and cadherin-mediated adhesion correlates with invasion and malignant progression. There is evidence to suggest that phosphorylation of tyrosyl residues in some components of the cadherin/catenin complex leads to loss of adhesive function and breakdown of adherens junctions (Hülsken et al., 1994a).
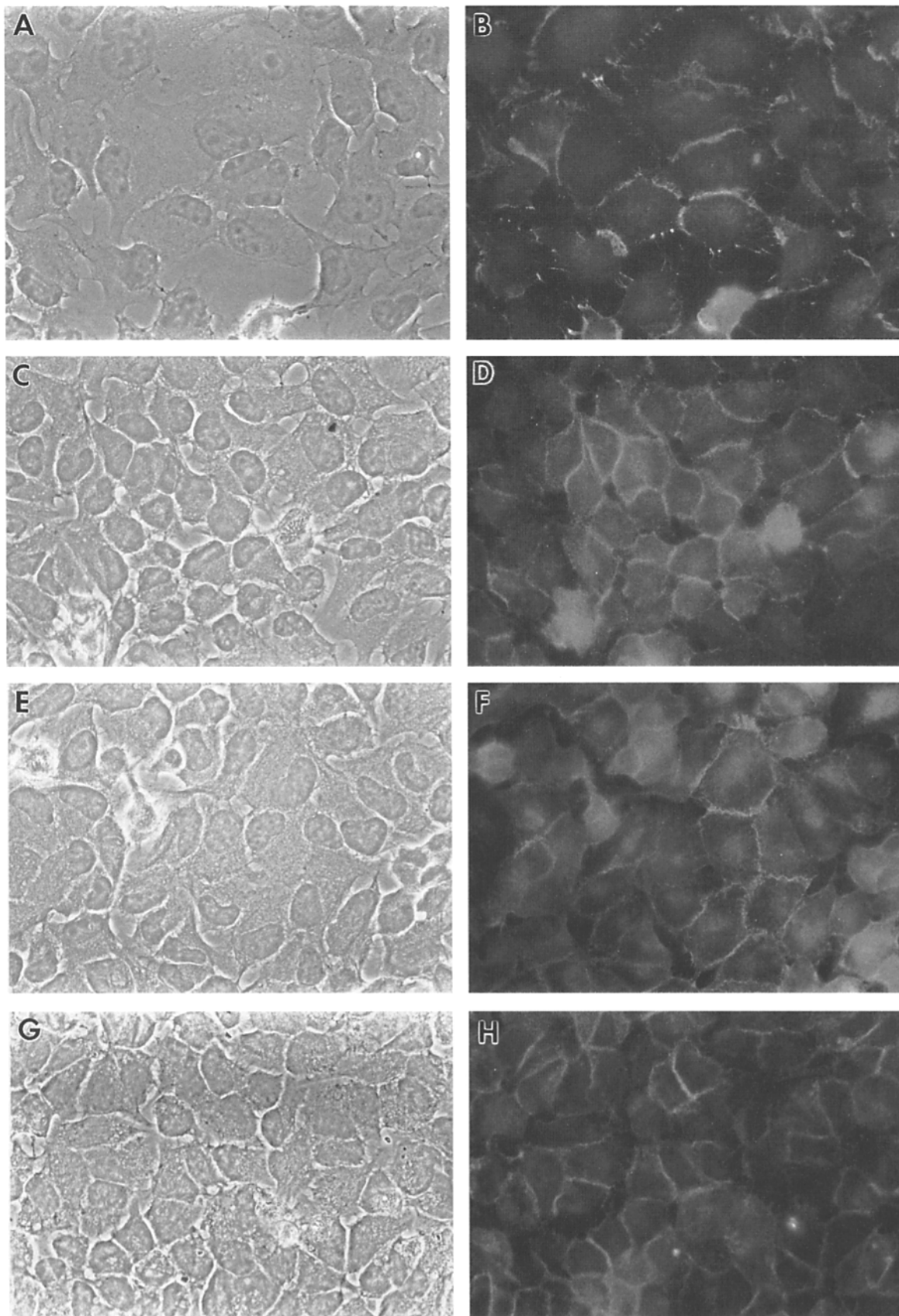
Figure 7. Immunocytochemical localization of PTPμ, cadherins, and β-catenin in MvLu cells. *A, C, E,* and *G* are phase-contrast micrographs of MvLu cells. *B, D, F,* and *H* are fluorescence micrographs. Cells stained with antibodies to PTPμ (SK15, *A* and *B*) show that it is localized to filopodial extensions of the cells and points of cell–cell contact in subconfluent MvLu cells. When the cells were plated at higher density, PTPμ was also localized at points of cell–cell contact (SK15, *C* and *D*). Antibodies to cadherins (MPan, *E* and *F*) and β-catenin antibody (TBC, *G* and *H*) also showed localization to cell contact sites.

Presumably, these proteins are normally maintained in a dephosphorylated state through the action of a phosphatase, which is crucial for the stabilization of adhesion and adherens junctions. Here we demonstrate that PTPμ, cadherins, and catenins exist in a complex, and localize to points of cell–cell contact. Furthermore, treatment of MvLu cells with pervanadate, which is a general inhibitor of PTP activity including PTPμ, leads to an accumulation of tyrosine-phosphorylated cadherin in the complex with PTPμ, indicating that the cadherins may be an endogenous substrate of PTPμ. Our results suggest that PTPμ may be one of the enzymes that regulate the dynamic tyrosine phosphorylation of the cadherin/catenin complex, and thus its interaction with the cytoskeleton.

The most commonly used model systems for analysis of cadherin function include MDCK, A431, MCF-7, and L cells. However, as assessed by immunoblotting, these cells do not express significant levels of PTPμ (data not shown). PTPμ displays a restricted tissue distribution. It is particularly prevalent in lung, hence our choice of MvLu cells in which to examine physiological function. Nevertheless, we have also observed complex formation between PTPμ, cadherins, and catenins in primary rat tissues including lung, heart, and brain, which also express PTPμ endogenously. In fact, in lung we have identified two of the cadherins as E-cadherin and cadherin 4. Furthermore, we have demonstrated direct binding in vitro between the intracellular segments of PTPμ and E-cadherin. Our data clearly suggest that at least in these tissues in which it is expressed, PTPμ may regulate the function of the cadherin/catenin complex. The implication is that there may be

other PTPs regulating tyrosyl phosphorylation of cadherins and catenins in other cell types. In this regard, it is interesting to note that a homologue of PTPμ, termed PTPκ, has been described, that displays 77% overall similarity at the amino acid level, and a much broader expression pattern than PTPμ (Jiang et al., 1993). Whether PTPκ also interacts with cadherins and catenins remains to be established.

There is now a substantial body of information that points to the importance of tyrosine phosphorylation in the control of the integrity of cell junctions and the function of the cadherin/catenin complex. The cadherins localize to adherens junctions which are points of cell–cell contact at which the actin cytoskeleton is anchored (for review see Geiger and Ayalon, 1992). Cell junctions are dynamic structures, and are areas of rapid phosphotyrosine turnover in normal cells (Volberg et al., 1991). PTKs such as src localize to these areas and phosphorylate junctional proteins in both normal and transformed cells (Maher et al., 1985; Tsukita et al., 1991). Aberrant tyrosine phosphorylation induced by expression of v-src causes loss of adherens junctions, although tight junctions and desmosomes appear to be unaffected (Warren and Nelson, 1987; Volberg et al., 1992). However, the adherens junctions were able to reform after addition of tyrphostins (PTK inhibitors) to the src-transformed cells (Volberg et al., 1992). Furthermore, inhibition of PTPs in MDCK cells by treatment with pervanadate caused dramatic accumulation of phosphotyrosyl proteins at adherens junctions (Volberg et al., 1992). This led to a deterioration of the adherens junctions with concomitant increase in the number and size of focal contacts, thus indicating differential effects of phosphorylation on junctional structures (Volberg et al., 1992). Thus, the stability of adherens junctions depends upon the balance between the action of PTKs and PTPs. PTKs, such as members of the src family, have the potential to disrupt tissue architecture by phosphorylating cell junction or cytoskeletal proteins. The PTPs that antagonize these effects will also be crucial components of the systems for control of junctional integrity. The structural features of PTPμ and its association with cadherins and catenins suggest that this RPTP may localize to adherens-type junctions and serve such a regulatory function.

Several studies have shown that tyrosine phosphorylation of cadherins and catenins suppresses cadherin-mediated adhesion and suggest that phosphorylation may play a role in association of the complex with the cytoskeleton (for review see Kemler, 1993). In rat fibroblasts transformed either by v-src or v-src in combination with v-fos, β-catenin was heavily phosphorylated on tyrosyl residues (Matsuyoshi et al., 1992). This coincided with a change in the morphology of aggregates of these cells from a compact, firmly connected appearance in the untransformed state to a much looser association of the transformed cells. Interestingly, treatment of these cells with herbimycin A, a PTK inhibitor, inhibited β-catenin phosphorylation, and induced tighter aggregation of the transformed cells, whereas vanadate, a PTP inhibitor, inhibited cadherin-mediated aggregation (Matsuyoshi et al., 1992). A similar observation was made in chick embryo fibroblasts transformed with Rous sarcoma virus. Expression of p60^{v-src} suppressed cadherin-mediated adhesion, an effect that correlated with the

pTYR-IP   lysate   PTPμ-IP

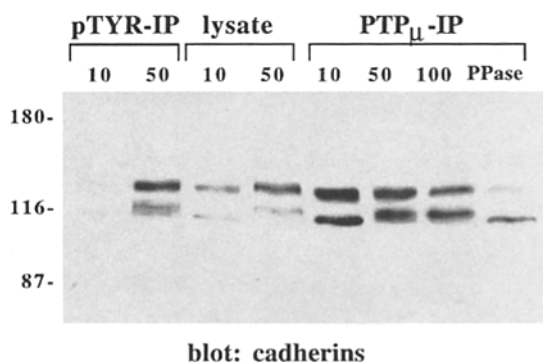10   50   10   50   10   50   100   PPase

180-

116-

87-

blot: cadherins

Figure 8. Inhibition of cellular PTPs by pervanadate resulted in an accumulation of tyrosine-phosphorylated cadherin associated with PTPμ. This figure is an immunoblot, probed with cadherin antibody (RPan), of lysates and IPs from MvLu cells treated with 10 μM (10), 50 μM (50) or 100 μM (100) pervanadate. Anti-phosphotyrosine antibodies only precipitated cadherin from cells treated with 50 μM pervanadate in which cellular PTPs had been inhibited. Lysates from cells treated with 10 and 50 μM pervanadate show that there is a retardation in the electrophoretic mobility of the cadherins that correlates with tyrosine phosphorylation (lysate 50 μM). Immunoprecipitates of PTPμ from cells treated with 50 or 100 μM pervanadate contain cadherins that display retarded electrophoretic mobility (lanes 50 and 100 under PTPμ-IP). Treatment of the immunoprecipitates with either alkaline phosphatase or PTP1B restored the normal electrophoretic mobility of the cadherins (PPase). Thus, when cellular PTPs were inhibited, the cadherin associated with PTPμ was tyrosine phosphorylated.

tyrosine phosphorylation of *N*-cadherin and α- and β-catenin (Hamaguchi et al., 1993). Again these effects were reversed by herbimycin A. In addition, kinase-active but transformation-defective mutants of p60$^{v\text{-}src}$ neither suppressed cadherin-mediated adhesion, nor phosphorylated *N*-cadherin and the associated catenins. A temperature-sensitive v-*src* mutant induced tyrosine phosphorylation of E-cadherin and β-catenin at the permissive temperature in MDCK cells that correlated with a loss of cell–cell contacts and an increase in invasion (Behrens et al., 1993). Thus, the suppression of adhesion and the correlative *src*-induced tyrosine phosphorylation of cadherins and catenins appears to be highly specific for transformation. These studies indicate a critical role for the balanced action of PTKs and PTPs in regulating the adhesive function of cadherins, suggesting that a PTP, such as PTPμ, may stabilize adhesion by promoting dephosphorylation of the cadherins and catenins.

Tyrosine phosphorylation of the cadherin/catenin complex has been observed not only in transformed cells as described above, but also under normal physiological conditions. Stimulation of tyrosine phosphorylation by hepatocyte growth factor or EGF results in the tyrosine phosphorylation of β-catenin and plakoglobin (Shibamoto et al., 1994). Furthermore, the EGF receptor has been shown to bind directly to β-catenin in vitro, and to associate with the cadherin/catenin complex in epithelial cells (Hoschuetzky et al., 1994). Recently it was shown that the c-erbB-2 gene product also associates with catenins in cancer cells (Ochiai et al., 1994). Our demonstration of the association of PTPμ with cadherins suggests a regulatory balance to the tyrosine kinases to permit rapid reversible tyrosine phosphorylation and modulation of the strength of cadherin driven adhesive reactions during processes such as migration and cell division. In addition, our data and others suggest that, for adhesion in normal cells, the cadherins are normally maintained in a dephosphorylated state, at least in part by PTPμ. The potential importance of a cadherin associated PTP similar to PTPμ is clear when one considers that tyrosine phosphorylation of the cadherin/catenin complex can lead to loss of cell–cell adhesion, transformation, and metastasis.

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