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The Protein Tyrosine Phosphatase Pez Is a Major Phosphatase of Adherens Junctions and Dephosphorylates β-Catenin

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Cell-cell adhesion regulates processes important in embryonal development, normal physiology, and cancer progression. It is regulated by various mechanisms including tyrosine phosphorylation. We have previously shown that the protein tyrosine phosphatase Pez is concentrated at intercellular junctions in confluent, quiescent monolayers but is nuclear in cells lacking cell-cell contacts. We show here with an epithelial cell model that Pez localizes to the adherens junctions in confluent monolayers. A truncation mutant lacking the catalytic domain acts as a dominant negative mutant to upregulate tyrosine phosphorylation at adherens junctions. We identified β-catenin, a component of adherens junctions, as a substrate of Pez by a "substrate trapping" approach and by in vitro dephosphorylation with recombinant Pez. Consistent with this, ectopic expression of the dominant negative mutant caused an increase in tyrosine phosphorylation of β-catenin, demonstrating that Pez regulates the level of tyrosine phosphorylation of adherens junction proteins, including β-catenin. Increased tyrosine phosphorylation of adherens junction proteins has been shown to decrease cell-cell adhesion, promoting cell migration as a result. Accordingly, the dominant negative Pez mutant enhanced cell motility in an in vitro "wound" assay. This suggests that Pez is also a regulator of cell motility, most likely through its action on cell-cell adhesion.

INTRODUCTION

Cell-cell adhesion regulates diverse cellular functions including cell proliferation, migration, and apoptosis (Vleminckx and Kemler, 1999). One important cell-cell adhesion system, the adherens junction (AJ), is mediated by a family of homophilic receptors, the cadherins (Steinberg and McNutt, 1999). The strength of cadherin-mediated adhesion is regulated by lateral clustering of cadherin molecules at the plasma membrane and also through the linkage of its intracellular cytoplasmic tail to the actin cytoskeleton. β-Catenin, a structural component of AJs and signal transducer of the wnt signaling pathway, is crucial for cross-linking cadherins to the actin cytoskeleton through another intermediate, α-catenin (Gumbiner, 1995;Cowin and Burke, 1996).

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Reversible tyrosine phosphorylation, catalyzed by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), is an important mechanism for regulating the linkage of cadherins to the cytoskeleton. A number of PTKs and PTPs have been found to be associated with AJs (Steinberg and McNutt, 1999). Inhibitors of PTPs have been shown to disrupt cell-cell adhesion, suggesting that PTPs play a critical role in maintaining the integrity of AJs (Ayalon and Geiger, 1997). The observation that phosphorylation of a critical tyrosine residue, Tyr654, on β-catenin results in its dissociation from E-cadherin (Roura *et al.*, 1999), verifies that tyrosine phosphorylation is an important mechanism for regulating the E-cadherin-catenin linkage. Tyrosine phosphorylation has also been reported to disrupt the β-catenin–α-catenin linkage (Ozawa and Kemler, 1998), although the critical tyrosine(s) in this case has not been determined. These observations suggest that multiple targets for tyrosine phosphorylation exist to regulate cell-cell adhesion.

The PTP Pez (PTPD2/PTP36) is a 130-kDa cytosolic (nontransmembrane) PTP (Smith *et al.*, 1995) expressed in a number of tissues. It is a member of the FERM (four-point-one,

ezrin, radixin, moesin) family of PTPs characterized by a conserved N-terminal FERM domain (Chishti *et al.,* 1998) and a C-terminal PTP catalytic domain separated by an intervening region. We recently showed that the subcellular localization of Pez is regulated in both HeLa and human umbilical vein endothelial cells (HUVEC); in cells grown to confluence Pez is localized to the cytosol, where it is concentrated at intercellular junctions, but it is predominantly nuclear in sparsely plated cells that have not yet formed extensive cell-cell contacts (Wadham *et al.,* 2000). Other factors also regulate the subcellular localization of Pez, including TGFβ, which inhibits translocation of Pez from the cytosol to the nucleus, and serum, which promotes the accumulation of Pez in the nucleus (Wadham *et al.,* 2000). Together these findings suggest that Pez could have multiple roles, involving the dephosphorylation of different substrates depending on whether it is in the nucleus or at intercellular junctions. Its presence at the intercellular junctions of confluent monolayers suggests that it may regulate the assembly or disassembly of adhesion complexes.

To elucidate the function of Pez, we used a "substrate trapping" approach (Flint *et al.,* 1997) in combination with the generation and overexpression of a dominant negative form of Pez to identify its substrates. We identified β-catenin as a substrate and show that the dominant negative Pez enhances both tyrosine phosphorylation of adherens junctions and cell motility.

MATERIALS AND METHODS

Tissue Culture and Cell Lines

MDCK and HEK 293 cell lines were cultured in DMEM supplemented with 10% FBS. HUVECS were obtained from discarded umbilical cords and cultured in M199 medium supplemented with 20% FBS and endothelial growth factors as previously described (Wall *et al.,* 1978). Transient transfections of HEK293 cells were carried out using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Stable MDCK clones were generated by transfection by standard calcium phosphate coprecipitation and transfectants selected by G418 (Promega, Madison, WI) resistance.

Antibodies

The polyclonal Pez antibody had previously been characterized (Wadham *et al.,* 2000). All other antibodies used were purchased: monoclonal Flag epitope antibody (M2) from AMRAD Biotech (Victoria, Australia), β-catenin, γ-catenin, and E-cadherin monoclonal antibodies from Transduction Laboratories, α-catenin mAb from either Transduction Laboratories (Lexington, KY) or Zymed (San Francisco, CA), monoclonal antiphosphotyrosine antibody (PY100) from New England Biolabs (Beverley, MA), and polyclonal ZO-1 and monoclonal p120catenin antibody from Zymed.

Generation of Mutant Pez Constructs

Isolation of the human Pez cDNA and generation of a Flag epitope-tagged construct in the mammalian expression vector, pcDNA3 (Invitrogen) has been described (Wadham *et al.,* 2000). The $D_{1079}A$ and R1127M mutations in Pez cDNA were made by site-directed mutagenesis using PCR. ΔFERM (amino acids 337-1187)- and ΔPTP (amino acids 1–932)-Pez were generated by PCR using the appropriate primers to remove the entire FERM or PTP domain, respectively. All constructs were tagged with the Flag epitope. The sequences of all mutated constructs were verified by sequencing from both the sense and antisense directions.

Generation of GST-Pez Fusion Proteins

wt-Pez and ST-Pez coding sequences were excised from the pcDNA3 constructs described above and cloned into the pGEX 4T-1 vector (Amersham Biosciences, Piscataway, NJ) to generate GST-fusion Pez proteins. The constructs were transformed into BL21-Codon Plus (DE3)-RIL *Escherichia coli* (Stratagene, La Jolla, CA) for protein expression. Cultures were induced with 0.15 mM IPTG for 2 h at ambient temperature, and the GST-fusion proteins were purified on glutathione sepharose. The amounts of full-length fusion proteins produced were determined by Coomassie blue staining after PAGE. Equal amounts of GST-wt-Pez and GST-ST-Pez protein were used for in vitro dephosphorylation of β-catenin.

Substrate Trapping

Newly confluent HUVEC lysates, used as a source of tyrosine-phosphorylated proteins, were prepared as described (Flint *et al.,* 1997). Briefly, the cells were incubated for 30 min with 50 μM sodium pervanadate to enrich for tyrosine-phosphorylated proteins, washed in phosphate-buffered saline (PBS), and lysed in ST buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 150 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail [P2714, Sigma, St Louis, MO]) containing 1 mM sodium orthovanadate at 4°C. The lysates were incubated on ice for 30 min in the presence of 5 mM iodoacetic acid to irreversibly inactivate endogenous PTPs. Unreacted iodoacetic acid was inactivated with 10 mM DTT. The lysates were then frozen on liquid nitrogen and stored at −70°C.

Flag-tagged wt-Pez or ST-Pez was transiently transfected into HEK293 cells and transfectants lysed in ST buffer 48 h after transfection. Equal amounts of protein from each lysate were immunoprecipitated (in the absence of orthovanadate) with an anti-Flag (M2) antibody precoupled to protein A sepharose beads. The Pez immunoprecipitates were washed three times in ST buffer, added to the phosphotyrosine enriched HUVEC lysates, and rocked at 4°C for 2 h. The beads were washed three times with ST buffer, boiled in Laemmli sample buffer, and bound proteins resolved by 8% SDS-PAGE. To detect tyrosine-phosphorylated proteins "pulled-down" by either wt or ST Pez, Western blots were performed using an antiphosphotyrosine antibody (PY100).

Immunoprecipitations and Western Blots

Immunoprecipitations were carried out after lysis of cells in ice-cold ST buffer containing 1 mM orthovanadate. Lysates were precleared with 20 μl of protein A-sepharose for 30 min at 4°C. Protein concentration was assayed using Bradford Reagent from Bio-Rad (Hercules, CA). Equal amounts (1–5 mg) of protein were incubated with 2 μg of primary antibodies supplemented with 20 μl packed protein A-sepharose for 1 h at 4°C. After washing, bound proteins were eluted by boiling in Laemmli sample buffer for 5 min separated by 8% SDS-PAGE and transferred to PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) for Western blotting. Western blotting was carried out after blocking with 5% milk, 0.1% Triton X-100 in PBS using the indicated antibodies and developed using HRP-conjugated secondary antibody (Immunotech, Marseille, France) and ECL (Amersham Pharmacia Biotech). For quantitation, Western blots were developed with ECL-Plus (Amersham Pharmacia Biotech), and fluorescence intensity was imaged using a Molecular Dynamics Typhoon 9410 (Amersham Biosciences, United Kingdom) variable mode imager.

Immunofluorescence

MDCK stable cells lines expressing either wt-Pez, ΔPTP-Pez, or ΔFERM-Pez were plated at confluent density onto fibronectin

coated glass LabTek chamber slides (Nalge, Nunc International, Naperville, IL) and incubated for 2–3 d before staining. The cells were fixed in 4% paraformaldehyde/PBS for 10 min, quenched with 10 mg/ml sodium borohydride for 15 min, and then permeabilized by treatment with 0.1% Triton X-100. Primary antibodies were used at 1:100 dilution and binding detected by incubation with either fluorophore-coupled secondary antibodies or biotinylated secondary antibodies followed by fluorophore-conjugated streptavidin, as indicated (Molecular Probes, Eugene, OR).

Epifluorescence microscopy was performed on an Olympus BX-51 microscope equipped with excitation filters for Alexa Fluor 594/Texas red and fluorescein (494 nm), acquired to a Cool Snap FX, charge-coupled device (CCD) camera (Photometrics, Phoenix, AZ). Images were adjusted for brightness and contrast with V^{++} software (Digital Optics Ltd., Auckland, New Zealand). The line-profiling feature of this software was used to plot the intensity vs. position of different fluorophores along a path through the cell monolayer, in cells that had been costained for two proteins. Confocal microscopy was performed using a 60× oil-immersion objective on an Olympus IX70 inverted microscope linked to a Bio-Rad Radiance 2100 confocal microscope. Sequential scans of each fluorophore separately were carried out for two-color colocalization studies.

Wounding Assay

MDCK stable cell lines were plated onto six-well trays at densities that would give confluent monolayers after 24 h. Confluent monolayers were incubated a further 48 h to allow intercellular junctions to mature before being serum-starved for 24 h. A linear wound was generated on the monolayers by scraping with the edge of a cell scraper. Unattached cells were washed off with agitation. Cells were photographed at the same point on a grid at the time of scraping and again at 24 h later. The difference in width of the wound between the two edges at the time of scraping and 24 h later was measured and represents the distance migrated. Each line was plated and wounded in triplicate.

RESULTS

Pez Colocalizes with E-cadherin at AJs

We previously observed in confluent endothelial monolayers that endogenous Pez localizes to the intercellular junctions (Wadham *et al.*, 2000). Here, we investigate the localization of Flag-tagged-Pez stably expressed in MDCK cells, a polarized epithelial cell line in which cell-cell adhesion is well characterized. For subsequent investigations of the function of Pez, truncation mutants of Pez that lack either the catalytic (ΔPTP-Pez) or FERM (ΔFERM-Pez) domain (Figure 1A) were created and their subcellular localization when stably expressed in MDCK epithelial cell lines were also examined. wt-Pez and both truncated Pez mutants, examined by epifluorescent microscopy, were similarly localized to what appears to be the intercellular junctions (Figure 1B). To further confirm that the localization of Pez was indeed at intercellular junctions rather than at the cell surface, optical sectioning using a confocal microscope was performed on wt-Pez-MDCK cells that had been costained for the Flag-epitope (on Pez) and E-cadherin (a marker of AJs). The data showed that Pez precisely colocalized with E-cadherin at basolateral membranes both along the *z*-axis (Figure 1, C and D) and in the *x-y* plane (Figure 1E), confirming that it is localizing to the AJs.

ΔPTP-Pez Is a Potential Dominant Negative Mutant of Pez That Causes an Increase in Tyrosine Phosphorylation at AJs

Because ΔPTP-Pez is devoid of the catalytic domain and therefore not enzymatically active, but retains the ability to localize to intercellular junctions, it can potentially act as a dominant negative mutant. If Pez is an AJ PTP that regulates the level of tyrosine phosphorylation at AJs, then overexpression of a dominant negative mutant of Pez should result in an increase in tyrosine phosphorylation of AJ proteins. This was investigated using confluent monolayers of MDCK cells overexpressing ΔPTP-Pez. Cells were serum-starved followed by 10 min serum stimulation before staining with an antiphosphotyrosine antibody. Epifluorescence microscopy showed that there was a markedly higher level of tyrosine phosphorylation at intercellular junctions (marked by costaining with an anti–ZO-1 antibody) in ΔPTP-Pez–transfected cells compared with the empty vector control or wt-Pez transfectants (Figure 2A). Under these experimental conditions no tyrosine phosphorylation was detected at the intercellular junctions of empty vector- and wt-Pez–transfected cells. This is best demonstrated when the fluorescence intensities resulting from both the phosphotyrosine and ZO-1 antibodies were quantitated across several cell boundaries (Figure 2A, right column).

To determine the exact location of the tyrosine-phosphorylated proteins induced by overexpression of the putative dominant negative mutant ΔPTP-Pez, optical sectioning with a confocal microscope was performed on confluent ΔPTP-Pez–transfected cells costained with both the antiphosphotyrosine and anti–E-cadherin antibodies. As with Pez, the tyrosine phosphorylation induced by ΔPTP-Pez precisely colocalized with E-cadherin both along the *Z*-axis (Figure 2, B and C) and in the *x-y* plane, confirming that the increased tyrosine phosphorylation occurred at AJs. In addition, these data also suggest that the tyrosine-phosphorylated substrates remained in the proximity of the plasma membrane and did not translocate to other parts of the cell. Induction of tyrosine phosphorylation at intercellular junctions has also been confirmed using another potential dominant negative mutant, the R1127M point-mutant (Figure 2D; R1127 of Pez is the equivalent of R221 of PTP1B, which when mutated leads to inactivation of its PTP activity (Flint *et al.*, 1997).

The AJ Protein β-Catenin Is a Substrate of Pez and Coimmunoprecipitates with Pez

Data obtained so far suggested that Pez was an AJ PTP and in concordance with this hypothesis, a putative dominant negative mutant of Pez caused an increase in tyrosine phosphorylation of AJs. A number of components of the AJ complex can be tyrosine phosphorylated, leading to alterations in their functions (Steinberg and McNutt, 1999). We therefore used a substrate trapping approach to identify substrates of Pez at the AJ.

Asp181 of PTP1B is an essential residue for catalytic activity of PTP1B, which when mutated to Ala results in a catalytically inactive substrate trapping (ST) mutant (Flint *et al.*, 1997). Sequence alignment of the phosphatase domains of Pez and PTP1B indicate that Asp1079 of Pez corresponds to Asp181 of PTP1B. To generate a ST mutant of Pez (denot-

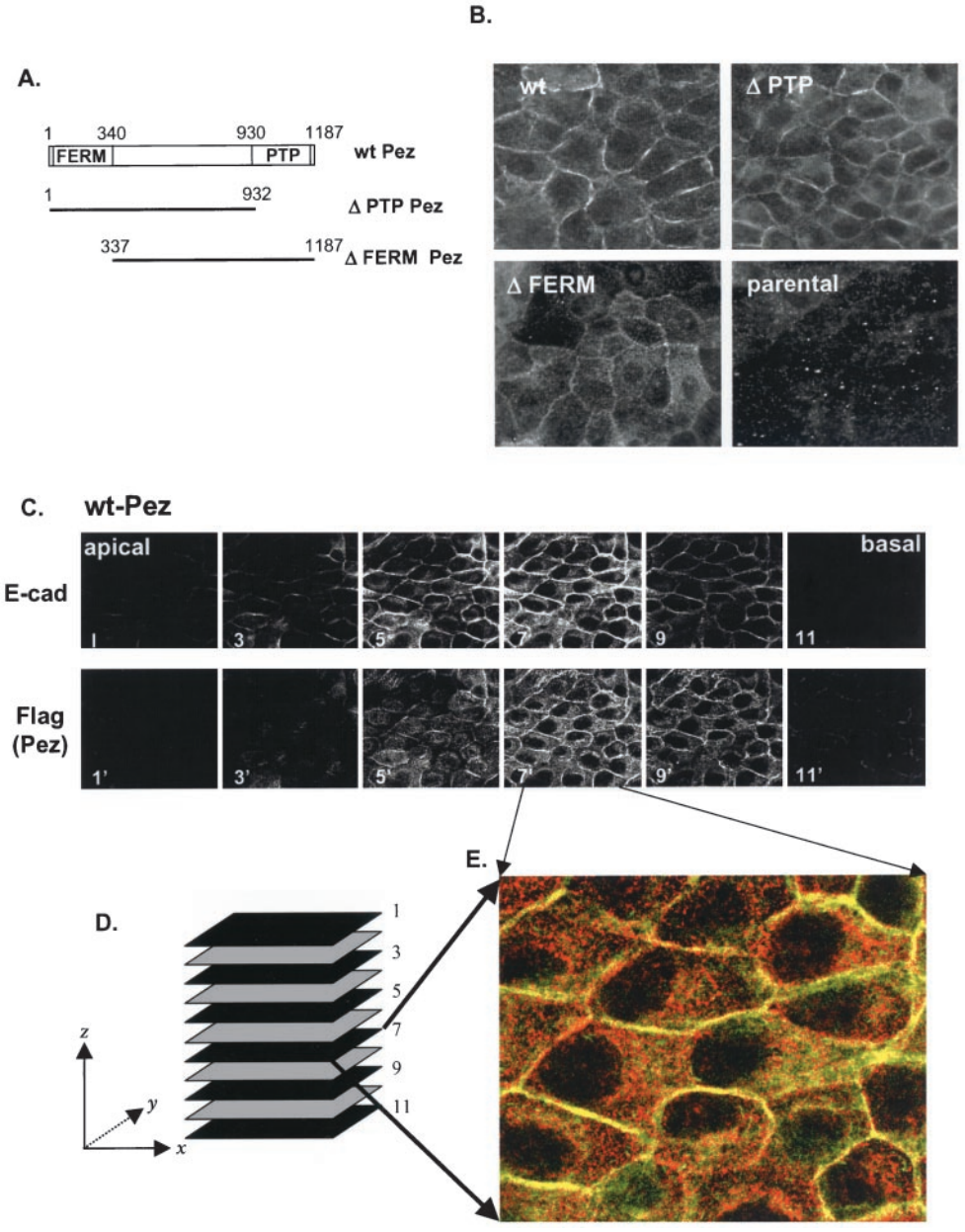

Figure 1. Flag-tagged wt-Pez and truncation mutants of Pez localize to the AJs of confluent monolayers of MDCK cells. (A) Schematic showing the structure of wt-, ΔPTP-, and ΔFERM-Pez. (B) Epifluorescence microscopy of confluent monolayers of parental MDCK cells and stable MDCK cell lines expressing wt-, ΔPTP-, and ΔFERM-Pez, labeled with an antibody against the Flag epitope and detected by indirect immunofluorescence using FITC-conjugated anti-mouse antibody. Specific labeling was observed in all Pez-expressing cell lines at the intercellular junctions. No staining was observed in the parental cells. (C) Z-series obtained by confocal laser scanning microscopy showing wt-Pez–transfected MDCK cells double-stained with the anti–E-cadherin (top row) and anti-Flag (bottom row) antibodies to indicate colocalization of Pez with E-cadherin along the *z*-axis. Anti–E-cadherin antibody was detected with phycoerythrin-conjugated anti-mouse IgG2a and anti-Flag antibody with biotinylated anti-mouse IgG1 antibody and FITC-conjugated streptavidin. Z-steps were carried out at 0.5-μm intervals, and alternate optical sections are shown. Top and bottom panels from each row show E-cadherin and Pez staining, respectively, from the same optical section. (D) Diagrammatic representation of the optical sections in the Z-series shown in C, indicating *x*, *y*, and *z* axes. (E) High-resolution merged image of one optical section (represented by panels 7 and 7′ from C) showing colocalization (yellow) in the *x*-*y* plane of Pez (green) and E-cadherin (red).

ed ST-Pez), we mutated D1079 of Pez to Ala and verified using an in vitro assay with a tyrosine-phosphorylated peptide that the catalytic activity of ST-Pez was significantly reduced compared with that of wt-Pez (between 10–20% of wt-Pez activity; unpublished data).

Without prior knowledge of the substrates of Pez, specific agonists could not be used to trigger their phosphorylation. Therefore, treatment with pervanadate, an inhibitor of PTPs, was used to upregulate tyrosine phosphorylation of proteins, including potential Pez substrates, in vivo (Figure 3A, panel 1). After lysis, endogenous PTPs in the HUVEC lysate were subsequently irreversibly inactivated by treatment with iodoacetic acid. The phosphotyrosyl-enriched HUVEC lysate devoid of endogenous PTP activities was incubated with either Flag-tagged wt- or ST-Pez (expressed in HEK 293 cells) immunoprecipitates bound to protein A-sepharose beads (Figure 3A, panel 6). Tyrosine-phosphorylated proteins that could interact with the wt- or ST-Pez immunoprecipitates, either directly or indirectly, were pulled-down and detected by Western blotting with an antiphosphotyrosine antibody. A number of tyrosine-phosphorylated proteins of similar staining intensities were pulled-down by both wt- and ST-Pez immunoprecipitates (Figure 3A, panel 2) but not immunoprecipitates from vector-transfected cells (panel 7). However, Band 1 was barely detectable in wt-Pez pull-downs but was clearly present when associated with ST-Pez. This could be because Band 1 did not

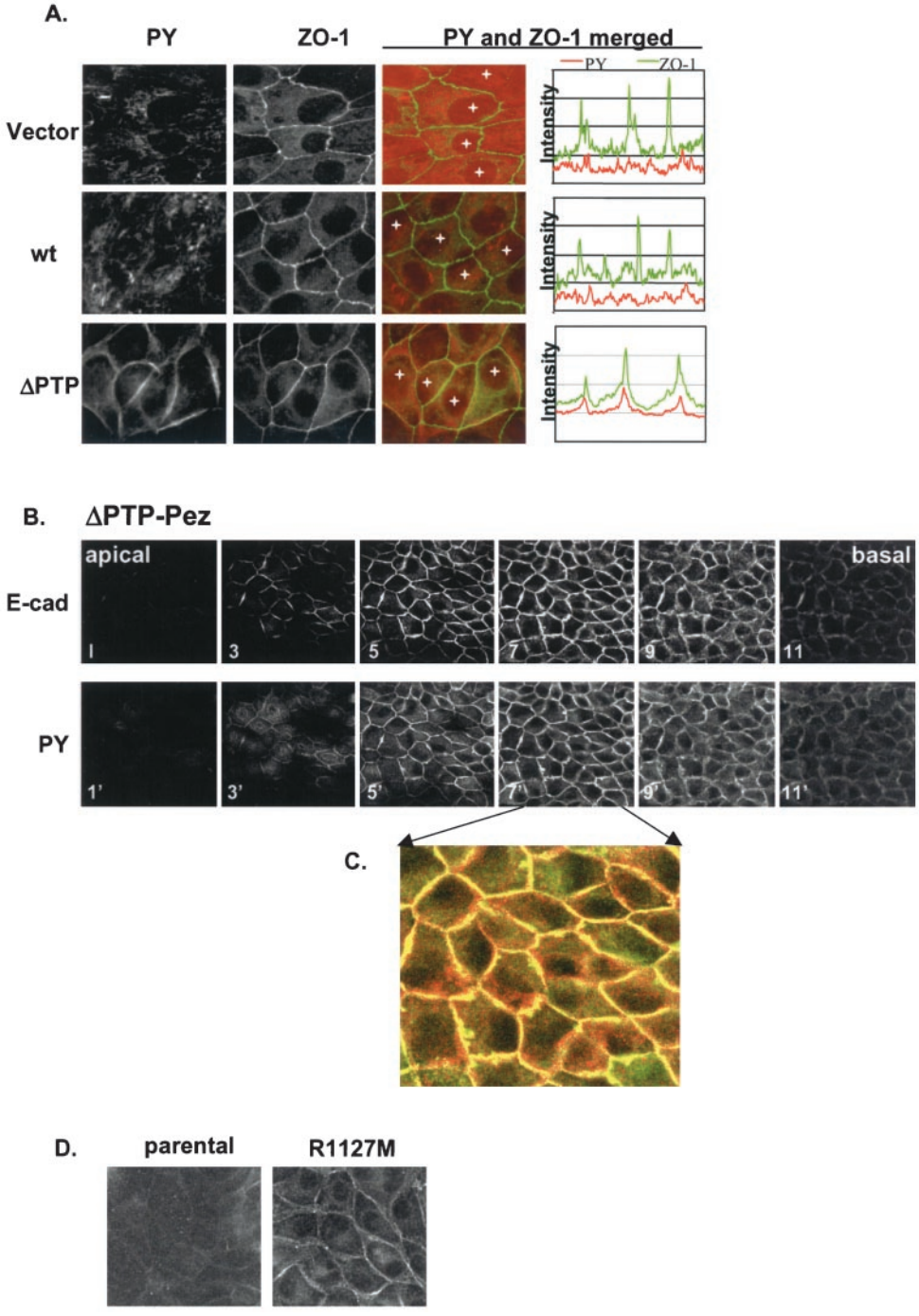

Figure 2. ΔPTP-Pez enhances tyrosine phosphorylation of AJs. (A) Epifluorescence microscopy showing confluent monolayers of MDCK cells stably expressing vector, wt- or ΔPTP-Pez, double-labeled with phosphotyrosine (PY) and ZO-1 antibodies and detected by indirect immunofluorescence using biotinylated anti-mouse antibody followed by Texas Red–conjugated streptavidin and Alexa fluor 350–conjugated anti-rabbit antibody, respectively. From left to right columns: phosphotyrosine antibody staining, ZO-1 staining to show positions of cell-cell contacts, merged image of PY staining (false colored red) and ZO-1 staining (false colored green), and quantitation of PY and ZO-1 fluorescent intensities taken along a line connecting the crosses shown in the merged image. (B) Z-series obtained by confocal laser scanning microscopy showing ΔPTP-Pez–transfected MDCK cells double-stained with the anti–E-cadherin (top row) and antiphosphotyrosine (bottom row) antibodies to show colocalization of phosphotyrosines with E-cadherin along the *z*-axis. Anti–E-cadherin antibody was detected with phycoerythrin-conjugated anti-mouse IgG2a, and antiphosphotyrosine antibody was detected with biotinylated anti-mouse IgG1 antibody and FITC-conjugated streptavidin. Z-steps were carried out at 0.5-μm intervals, and alternate optical sections are shown. Top and bottom panels from each row show E-cadherin and phosphotyrosine staining, respectively, from the same optical section. (C) High-resolution merged image of one optical section (represented by panels 7 and 7′ of B) showing colocalization (yellow) in the *x-y* plane of phosphotyrosine (green) and E-cadherin (red) staining. (D) Epifluorescence microscopy of confluent monolayers of parental MDCK or a stable MDCK cell-line expressing a Pez mutant, R1127M, stained with the phosphotyrosine antibody.

bind sufficiently stably to wt-Pez to be pulled-down or because the associated protein had been dephosphorylated by the catalytically active wt-Pez but not the inactive ST-Pez. Both causes for its absence in the wt-Pez pull-downs are consistent with Band 1 being a specific substrate of Pez.

Because the molecular weight of Band 1 is similar to that of the AJ protein β-catenin, we probed parallel lanes with a β-catenin antibody. β-Catenin was found to be pulled-down by both wt- and ST-Pez (Figure 3A, panel 3) and furthermore comigrated exactly with Band 1, suggesting that the substrate at this position could indeed be β-catenin. Thus, if indeed Band 1 is β-catenin, it was only tyrosine phosphorylated when in association with inactive ST-Pez, suggesting that the lack of tyrosine phosphorylation in the wt-Pez–associated protein was due to dephosphorylation by wt-Pez.

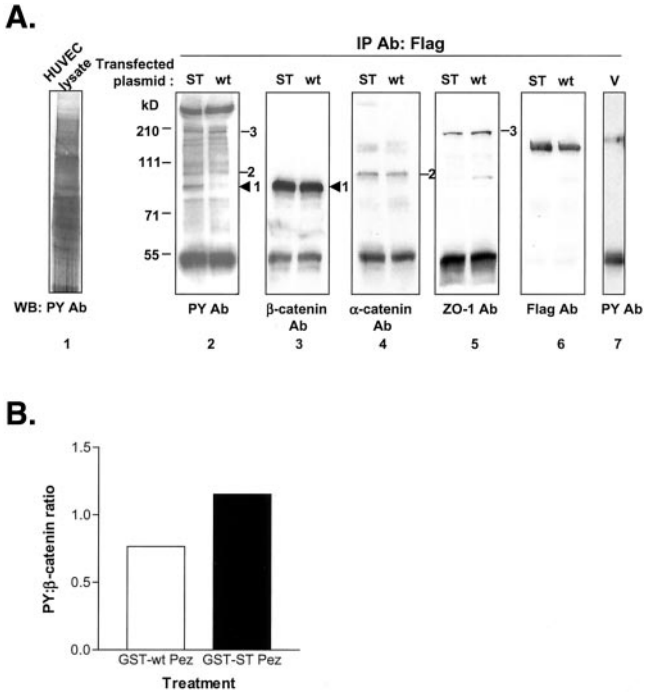

Figure 3. Identification of Pez substrates and interacting proteins by substrate trapping and by in vitro dephosphorylation. (A) Substrate trapping. HUVEC lysate enriched for tyrosine-phosphorylated proteins (panel 1) was incubated for 2 h with Sepharose-bound anti-Flag immunoprecipitates from cells transfected with wt-Pez, ST-Pez, or empty vector as shown. The beads were then washed, and bound proteins were eluted and immunoblotted with various antibodies as indicated. (B) In vitro dephosphorylation of β-catenin by GST-Pez. HUVEC lysate enriched for tyrosine-phosphorylated proteins (as in A) was incubated with either GST-wt-Pez or GST-ST-Pez bound to glutathione sepharose beads. After removal of the GST-Pez fusion proteins, β-catenin was immunoprecipitated, and the immunoprecipitates were Western blotted with an antiphosphotyrosine antibody. The amount of tyrosine-phosphorylated β-catenin was quantitated by fluorimaging. The blots were then stripped, counterblotted with anti–β-catenin antibody, and quantitated for total β-catenin. The ratio of tyrosine-phosphorylated β-catenin to total β-catenin for each GST-Pez treatment is shown (one representative of three experiments).

After stripping and reprobing the filters with antibodies to other components of junctional adhesion complexes, we identified a number of other junctional proteins, including α-catenin (band 2, Figure 3A, panel 4), ZO-1 (band 3, Figure 3A, panel 5), and plakoglobin (unpublished data), which interact with Pez but do not appear to be substrates for its PTPase activity. β-Catenin, α-catenin, and plakoglobin are all components of the AJ complex. ZO-1 is normally associated with tight junctions in polarized epithelial and endothelial monolayers but in newly confluent HUVEC, which have not formed bona fide tight junctions, it is also associated with AJs (Stevenson and Keon, 1998), suggesting that a complex of AJ proteins may be binding to Pez.

The phosphotyrosyl-enriched HUVEC lysate that was the source of phospho-β-catenin used in the substrate-trapping approach was made devoid of any active PTPs by iodoacetate treatment. However, there is a possibility that the wt-Pez immunoprecipitates incubated with the lysate may contain other active PTPs in addition to Pez. To demonstrate that phospho-β-catenin can be directly dephosphorylated by Pez, we carried out a similar experiment using recombinant Pez, prepared as a GST fusion protein from *E. coli*. As a control, we performed the same reaction with the inactive GST-ST-Pez. After incubation of recombinant Pez with the HUVEC lysate, β-catenin was immunoprecipitated with anti–β-catenin antibody and the amount of tyrosine-phosphorylated β-catenin remaining was quantitated by fluorimager analysis of phosphotyrosine immunoblots. Although the GST fusion proteins expressed poorly in *E. coli* (>90% of the products were degraded, presumably because the large fusion protein [~160 kDa] was poorly folded), the data showed that GST-wt-Pez, but not GST-ST-Pez, dephosphorylated the β-catenin in the lysate, removing about one third of the tyrosylphosphates on the β-catenin (Figure 3B). GST-Pez proteins lacking the FERM domain were also expressed and assayed, showing that the forms with wt but not ST catalytic domains dephosphorylate β-catenin to a similar extent as full-length GST-wt-Pez (unpublished data).

These experiments indicated that Pez can directly dephosphorylate β-catenin in vitro. To further assess whether Pez and β-catenin interact in vivo, we investigated whether Pez can be coimmunopreciptated with β-catenin. Immunoprecipitation of endogenous β-catenin from cell lysates containing either endogenous or ectopically expressed Pez was carried out, followed by Western blotting to determine if Pez is coimmunoprecipitated. Endogenous Pez was detected in β-catenin immunoprecipitates when a β-catenin antibody, but not a control antibody, was used to immunoprecipitate β-catenin from confluent HUVEC monolayers (Figure 4A). Similarly, ectopically expressed Pez coimmunoprecipitated with β-catenin in cells that were transfected with an expression vector bearing Pez cDNA but not empty vector (Fig, 4B), confirming that Pez and β-catenin interact in vivo.

Although β-catenin coimmunoprecipitated with both endogenous and ectopic Pez, the coimmunoprecipitation appeared to be relatively weak. This could have a number of possible explanations. First, the coimmunoprecipitations were carried out in 1% Triton X-100 with a brief pulse of sonication to maximize recovery of Pez. These conditions may be too harsh for the proteins to remain bound. In preliminary experiments to test the strength of the interaction between Pez and β-catenin, we have observed better coimmunoprecipitations if the sonication step was omitted although more striking increases in coimmunoprecipitation were observed by reducing the concentration of detergent used in the lysis buffer from 1% to 0.5% Triton X-100 (Figure 4B, right panel). This suggests that the complex formed may be detergent labile. Such detergent lability has been well documented with p120 catenin (p120ctn)-cadherin interactions, whereby only 5–20% of the p120ctn in detergent lysates is associated with cadherin in contrast to its almost complete localization to AJs or membrane fraction under detergent-free conditions (reviewed in Anastasiadis and Reynolds, 2000). Finally, it is likely that not all the Pez within the cell is localized to the AJ. This has certainly been observed in endothelial cells where Pez expression is observed in the cytosol away from the cell junctions, even when the monolayer is confluent (Wadham *et al.*, 2000). This is also

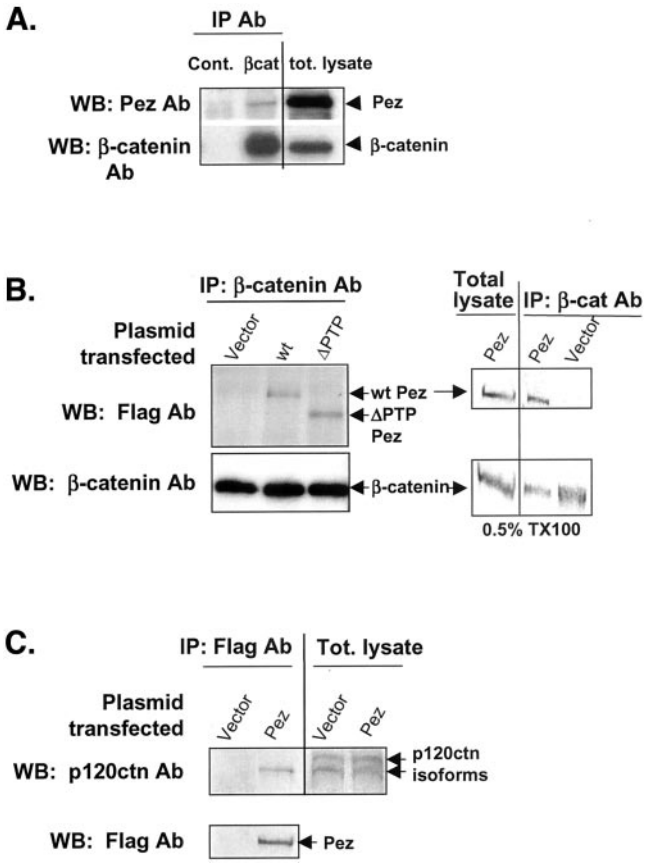

Figure 4. Coimmunoprecipitations of endogenous Pez and ectopically expressed wt- and ΔPTP-Pez with endogenous β-catenin and p120ctn. (A) HUVEC lysate was incubated with β-catenin antibody or control isotype-matched irrelevant antibody, and immunoprecipitates were Western blotted with Pez or β-catenin antibodies as indicated; right panel shows Western blots of total lysate. (B) Left panel: Empty vector, wt-Pez, or ΔPTP-Pez were transiently transfected into HEK293 cells. β-Catenin was immunoprecipitated from the lysates of transfectants and Western blotted using a Flag-epitope antibody to detect ectopically expressed Pez. The blot was stripped and reblotted with a β-catenin antibody showing the presence of β-catenin in all three immunoprecipitations. Right panel as in left panel, except that lysis was carried out in 0.5% instead of the 1% Triton X-100, which was used in all the other immunoprecipitations shown. (C) Flag epitope-tagged Pez was immunoprecipitated with an anti-Flag antibody from HEK 293 cells stably transfected with either empty vector or Flag epitope-tagged Pez. The immunoprecipitates were Western-blotted with an anti-p120ctn antibody and reblotted with the anti-Flag antibody.

particularly true when Pez is ectopically expressed in HEK 293 cells (unpublished data).

Because a number of AJ proteins were also pulled-down together with β-catenin (Figure 3A) and because of their structural relatedness, we also investigated whether p120ctn coimmunoprecipitates with Pez. Immunoprecipitates from lysates of vector- or Pez-transfected stable HEK 293 cell lines carried out using the Flag-epitope antibody were Western blotted with a p120ctn antibody. p120ctn coimmunoprecipitated with Pez that was immunoprecipitated with the Flag antibody (Figure 4C). Western blots of total lysates from both the vector- and Pez-transfected cells showed that HEK 293 cells expressed two major p120ctn isoforms of similar abundance (Figure 4C), with the larger isoform corresponding to full-length p120ctn (isoform 1). Interestingly, only the smaller MW isoform (~95 kDa) coimmunoprecipitated with Pez. Isoforms 1 and 3 are the most commonly expressed (reviewed in Anastasiadis and Reynolds, 2000), hence the smaller isoform coimmunoprecipitating with Pez is most likely isoform 3, although this remains to be confirmed. It is unclear why Pez is only associated with one isoform of p120ctn, and it is also unkown at this stage whether p120ctn interacts directly with Pez and whether it is a substrate. These will be the subject of future studies. What is clear, however, is that the cell junctional proteins that are pulled-down with Pez are likely to be specific because the coimmunoprecipitation discriminated between the two highly related isoforms of p120ctn.

ΔPTP-Pez Interacts with and Induces Tyrosine Phosphorylation of β-Catenin

If ΔPTP-Pez acts as a dominant negative mutant of Pez (as suggested by its localization to the AJ [Figure 1B] and by its ability to induce tyrosine phosphorylation of proteins at the AJ [Figure 2]), and if β-catenin is a bona fide substrate of Pez, then ΔPTP-Pez should interact with β-catenin in vivo to increase its tyrosine phosphorylation. To investigate the ability of ΔPTP-Pez to interact with β-catenin in vivo, β-catenin was immunoprecipitated from HEK293 cells stably transfected with ΔPTP-Pez. The β-catenin immunoprecipitates were then Western blotted with an anti-Flag antibody to detect ΔPTP-Pez that has coimmunoprecipitated with β-catenin. The data showed that ΔPTP-Pez was able to coimmunoprecipitate with β-catenin to the same extent as wt-Pez (Figure 4B, left panel).

To examine whether ΔPTP-Pez can induce tyrosine phophorylation of Pez substrates through a dominant negative effect, antiphosphotyrosine Western blots were performed on extracts from confluent monolayers of MDCK cells stably expressing empty vector, wt-Pez, or ΔPTP-Pez. Cells were serum-starved for 24 h before addition of serum for 10 min to induce tyrosine phosphorylation. To see specific tyrosine phosphorylation of Pez substrates, cells were not pretreated with pervanadate, which would have caused a global increase in tyrosine phosphorylation. Thus, in concordance with other studies (Ayalon and Geiger, 1997), the basal level of tyrosine-phosphorylated proteins in vector-transfected cells was very low. Two bands that were specifically phosphorylated in extracts from ΔPTP-Pez but not empty vector or wt-Pez transfected cells (Figure 5A, closed arrowheads) were observed. The lower MW band comigrated with β-catenin when the filter was counterblotted with a β-catenin antibody, suggesting that one of the proteins that is tyrosine phosphorylated through overexpression of ΔPTP-Pez is β-catenin. Immunoprecipitation of β-catenin followed by Western blotting with an antiphosphotyrosine antibody confirmed that β-catenin was indeed phosphorylated in ΔPTP-Pez–transfected but not empty vector-transfected cells (Figure 5B). These data indicate that ΔPTP-Pez acts as a dominant negative mutant of Pez leading to an increased level of β-catenin tyrosine phosphorylation. The finding that ΔPTP-Pez could interact with and increase

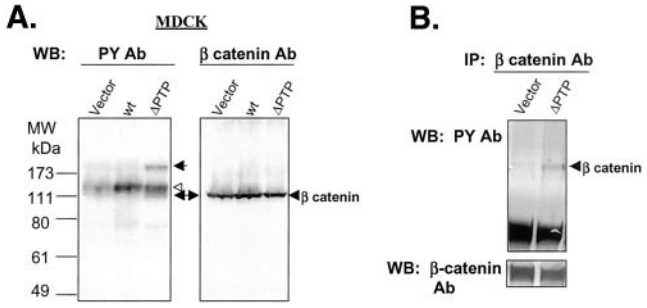

Figure 5. ΔPTP-Pez enhances tyrosine phosphorylation of β-catenin. (A) MDCK cell lines stably expressing vector, wt-Pez, or ΔPTP-Pez were serum-starved for 24 h followed by 10-min stimulation with 10% FBS to induce tyrosine phosphorylation. Lysates were Western blotted with a phosphotyrosine (PY) antibody (left). Arrowheads show two proteins specifically tyrosine phosphorylated in the ΔPTP-Pez transfected cells, one of which comigrates with β-catenin (double arrowhead) when the blot was stripped and reblotted with a β-catenin antibody (right). (B) Lysates from empty vector- or ΔPTP-Pez–transfected MDCK cells were immunoprecipitated with a β-catenin antibody followed by Western blotting of the immunoprecipitates with an antiphosphotyrosine antibody (top). The blot was stripped and counterprobed with a β-catenin antibody (bottom).

the tyrosine phosphorylation status of β-catenin further reinforces our conclusion that β-catenin is a bona fide Pez substrate. The presence of another protein with increased tyrosine phosphorylation in ΔPTP-Pez but not wt-Pez or empty vector-transfected cells (Figure 5A, open arrowhead) suggests that there are other Pez substrates that were not identified by substrate trapping. Any additional substrates are likely to also be located at the intercellular junctions because the tyrosine phosphorylation induced by ΔPTP-Pez was highly specific to intercellular junctions (Figure 2A). The presence of other substrates at intercellular junctions in addition to β-catenin would also account for the dramatic increase in tyrosine phosphorylation at intercellular junctions in ΔPTP-Pez MDCK cells. Similarly, the tyrosine-phosphorylated band comigrating with β-catenin in Figure 5A may be comprised of more than one protein of the same relative mobility. The higher degree of tyrosine phosphorylation relative to β-catenin protein in the ΔPTP-Pez lysate shown in Figure 5A compared with that in the β-catenin immunoprecipitates in Figure 5B (where the observed phosphorylation is due solely to β-catenin) suggests that this may be the case.

Overexpression of the Dominant Negative Mutant (ΔPTP-Pez) Enhances Cell Migration

Tyrosine phosphorylation of β-catenin has been correlated with increased cell migration in a number of studies (Liu *et al.*, 1997;Muller *et al.*, 1999;Hollande *et al.*, 2001). We used an in vitro "wound" assay to investigate whether the increase in tyrosine phosphorylation of β-catenin that results from overexpression of the dominant negative mutant, ΔPTP-Pez, could affect rates of cell migration. In this assay, a linear scratch was made on a confluent monolayer of MDCK cells to generate a linear denuded area, after which cells from the edge of the wound could migrate into the denuded area to repopulate it. After 24 h, cells overexpressing ΔPTP-Pez had migrated further into the wound than cells overexpressing either empty vector or wt-Pez (Figure 6A). Measurements of the distances migrated after 24 h (Figure 6B) showed that the average distance migrated by the ΔPTP transfectants was significantly greater ($p = 0.02$) than the distance migrated by either wt-Pez or vector control cells. There was no significant difference between the distances migrated by the wt-Pez cells and empty vector-transfected cells ($p = 0.08$). Higher resolution images of the "wound" shows that the cells at the edge are migrating into the wound characterized by protrusions into the wound and formation of pseudopodia (Figure 6C). Our observation that the dominant negative mutant, ΔPTP-Pez, enhanced cell motility suggests that Pez is a regulator of cell motility, most likely through its role in regulating cell-cell adhesion.

DISCUSSION

Regulation of the integrity of AJs in response to external cues is important for proper tissue and organ formation during embryonal development and for wound healing in adults. The participation of tyrosine phosphorylation in regulation of AJ function is apparent from the involvement of PTKs such as Src (Behrens *et al.*, 1993;Fujita *et al.*, 2002), EGF receptor, and HGF/scatter factor receptor (Shibamoto *et al.*, 1994) in phosphorylation of AJ proteins, leading to decreased cell-cell interaction and concomitant enhancement of cell migration. A number of PTPs have also been found to be components of the AJ complex, including the receptor PTPs μ, κ, and LAR and the cytosolic PTP, PTP1B (reviewed in (Steinberg and McNutt, 1999).

In this study, we have identified the PTP Pez as a novel PTP of AJs. A truncation mutant of Pez lacking the catalytic domain acted as a dominant negative mutant to enhance tyrosine phosphorylation of AJs and promote cell migration. Analysis of the proteins that are tyrosine phosphorylated as a result of the overexpression of the dominant negative mutant suggested that there are at least two Pez substrates in epithelial cells, one of which is the AJ protein β-catenin. Using a substrate trapping mutant to isolate potential Pez substrates followed by in vitro dephosphorylation of β-catenin by recombinant Pez, we confirmed that β-catenin is indeed a substrate of Pez. Dephosphorylation of β-catenin by recombinant Pez in the absence of any other active PTP also demonstrated that Pez could directly dephosphorylate β-catenin. Both endogenous and ectopically expressed Pez coimmunoprecipitated with endogenous β-catenin, indicating that they interact in vivo, providing further evidence that Pez is a physiological regulator of β-catenin tyrosine phosphorylation.

The highly similar complement of AJ proteins pulled-down by both wt-Pez and ST-Pez, the observation that wt-Pez and ST-Pez can pull-down β-catenin equally well, and the observation that wt-Pez can pull-down unphosphorylated β-catenin all suggest that Pez is likely to be a component of the AJ complex. This raises a number of questions. First, although we have demonstrated that Pez could directly dephosphorylate β-catenin, the question remains as to whether its association with the AJ complex is solely through binding to β-catenin or through binding to some

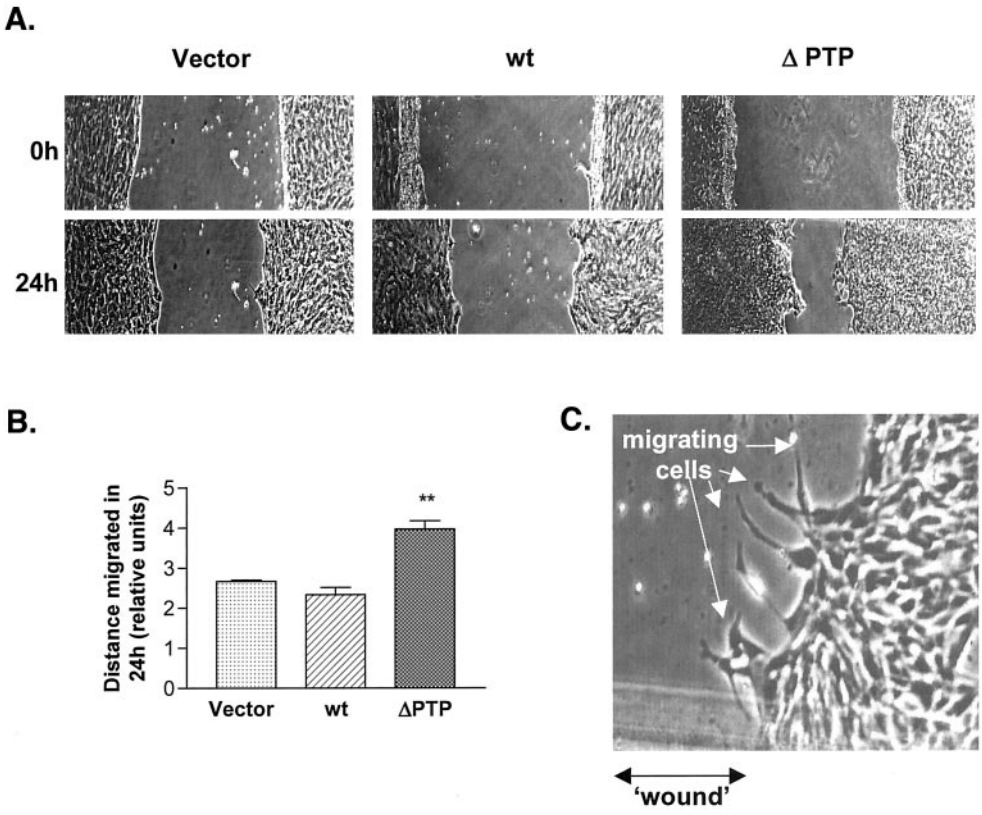

Figure 6. ΔPTP-Pez enhances cell motility. (A) Confluent monolayers of MDCK cells stably expressing empty vector, wt-Pez, or ΔPTP-Pez were wounded using the edge of a cell scraper and photographed immediately (0h). Each wound was photographed again at the same spot 24 h later. (B) The distance between the wound edges was measured at the same point on each wound at 0 h and at 24 h. The difference in distance between the two edges at 24 h and 0 h was taken to be the distance migrated in 24 h. Each cell line was plated and wounded in triplicate and the experiment performed at least twice with similar outcomes; data from one representative experiment are presented. **p = 0.02 when distance migrated by ΔPTP- expressing cells was compared with cells expressing empty vector. (C) Higher magnification image of ΔPTP-Pez MDCK cells at the wound edge 24 h after wounding.

other component of the AJ complex. Second, if Pez is a component of the AJ through direct binding to β-catenin or some other protein, than one might expect that any tyrosine phosphorylation of β-catenin at the AJ will be very transient. To achieve longer-term loosening of the AJ might then require downregulation of Pez activity.

Of the PTPs that have previously been shown to be localized to the AJ, PTP LAR and PTP 1B have been shown to dephosphorylate β-catenin, whereas the substrates of PTPμ are yet to be identified. There are potentially many reasons for multiple PTPs to be associated with AJs. These include cell type-specific expression of some PTPs, different degrees of responsiveness to external stimuli and different substrate specificities exhibited by different PTPs. In the case of β-catenin, the crystal structure indicates there are potentially up to 14 tyrosines that are accessible for phosphorylation. One of these, Tyr654, has been demonstrated to regulate the binding of β-catenin to E-cadherin (Roura *et al.*, 1999), but the phospho-tyrosine that interrupts α-catenin binding is yet to be determined. It is conceivable that dephosphorylation of different tyrosines that mediate different functions on the one molecule may be regulated by different PTPs. A comprehensive analysis of substrate specificity and responses to external stimuli for individual PTPs, which to date has not been carried out, is essential to fully elucidate the role of tyrosine phosphorylation in regulating AJ functions.

Finally, some studies have reported that after the tyrosine phosphorylation of β-catenin and its dissociation from intercellular junctions, it is translocated into the nucleus where it can, under some circumstances, interact with the LEF-1/Tcf transcription factor to alter gene expression (Adam *et al.*, 2001; Kim and Lee, 2001; Monga *et al.*, 2002). What is not clear from these studies is whether β-catenin is translocated into the nucleus in its tyrosine-phosphorylated form and if so, whether the tyrosine-phosphorylated form can interact with LEF-1/Tcf. Intriguingly, under conditions where cell-cell adhesion is disrupted, we have shown that Pez also translocates into the nucleus (Wadham *et al.*, 2000). It would be particularly important to determine whether Pez and β-catenin interact in the nucleus and if so, what the functional consequence of this interaction is.

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