Polycystin-1 Induces Cell Migration by Regulating Phosphatidylinositol 3-kinase-dependent Cytoskeletal Rearrangements and GSK3 β -dependent Cell–Cell Mechanical Adhesion

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Polycystin-1 (PC-1) is a large plasma-membrane receptor encoded by the *PKD1* gene mutated in autosomal dominant polycystic kidney disease (ADPKD). Although the disease is thought to be recessive on a molecular level, the precise mechanism of cystogenesis is unclear, although cytoarchitecture defects seem to be the most likely initiating events. Here we show that PC-1 regulates the actin cytoskeleton in renal epithelial cells (MDCK) and induces cell scattering and cell migration. All of these effects require phosphatidylinositol 3-kinase (PI3-K) activity. Consistent with these observations *Pkd1*^{−/−} mouse embryonic fibroblasts (MEFs) have reduced capabilities to migrate compared with controls. PC-1 overexpressing MDCK cells are able to polarize normally with proper adherens and tight junctions formation, but show quick reabsorption of ZO-1, E-cadherin, and β -catenin upon wounding of a monolayer and a transient epithelial-to-mesenchymal transition (EMT) that favors a rapid closure of the wound and repolarization. Finally, we show that PC-1 is able to control the turnover of cytoskeletal-associated β -catenin through activation of GSK3 β . Expression of a nondegradable form of β -catenin in PC-1 MDCK cells restores strong cell–cell mechanical adhesion. We propose that PC-1 might be a central regulator of epithelial plasticity and its loss results in impaired normal epithelial homeostasis.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases affecting humans (Gabow, 1993). The hallmark of the disease is the formation of cysts in both kidneys of affected individuals. Cyst formation is observed in the bile ducts and pancreas as well, suggesting that the genes involved in the disease might be key players in the regulation of epithelia homeostasis (Gabow, 1993).

Two genes have been shown definitively to result in ADPKD when mutated: *PKD1*, which accounts for 85%, and *PKD2*, which is responsible for the remaining 15% (Boletta and Germino, 2003). The 14-kb *PKD1* mRNA encodes a 4303-amino acid (aa; 520 kDa) protein (polycystin-1 [PC-1]) that is a highly glycosylated plasma membrane receptor with a large (~3000 aa) extracellular N-terminal domain, 11 transmembrane domains (Nims *et al.*, 2003), and a short intracellular C-terminus of 198 aa (Boletta and Germino, 2003). The extracellular portion has an assortment of do-

main involved in protein–protein interactions (Hughes *et al.*, 1995) including two leucine-rich repeats, a C-type lectin domain, 16 PKD (IgG-like) repeats, an REJ (receptor for egg jelly) domain, and a proteolytic GPS domain (G-protein-coupled receptor [GPCR] proteolytic site) recently shown to be functionally active (Qian *et al.*, 2002). Cleavage of PC-1 at its GPS site results in a C-terminal fragment (CTF) of approximately 150 kDa and a N-terminal fragment (NTF) of ~400 kDa, which remains tethered to the CTF portion (Qian *et al.*, 2002). The subcellular localization of this receptor is highly controversial, in part because of problems of antibody specificity. However, evidence from several different studies seem to reach a consensus on its localization to cell–cell contacts, focal adhesions, and the primary cilium, a thin microtubule-based organelle lying on the apical side of all epithelial cells (Huan and van Adelsberg, 1999; Scheffers *et al.*, 2000; Wilson *et al.*, 1999; Boletta and Germino, 2003; Roitbak *et al.*, 2004). The precise function of this receptor is largely elusive, although PC-1 has been implicated in regulating several biological processes in vitro, including proliferation (Bhunja *et al.*, 2002; Li *et al.*, 2005), apoptosis (Boletta *et al.*, 2000; Boca *et al.*, 2006) and tubulogenesis (Boletta *et al.*, 2000; Nickel *et al.*, 2002).

The intracellular C-terminus of PC-1 contains a coiled-coil domain for interaction with the *PKD2* gene product, polycystin-2 (PC-2), and possibly other molecules (Qian *et al.*, 1997). Heterotrimeric G-proteins were also shown to interact with the PC-1 C-tail through a different motif (Parnell *et al.*, 1998). This interaction was shown to be important for reg-

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ulation of AP-1 (Parnell *et al.*, 2002). These data along with the presence of a GPS site in the extracellular portion of PC-1 (Qian *et al.*, 2002) prompted several investigators to suggest that PC-1 might be acting as an atypical GPCR.

One important signaling pathway that was shown to be activated by PC-1 is the JAK/STAT pathway for regulation of the cell cycle (Bhunia *et al.*, 2002). PC-1 was shown to directly bind JAK2 in a yet unidentified region that does not require the last 76 amino acids for interaction, but it requires them for activation of the system. Finally, several early studies performed using expression of the sole ~200 amino acids corresponding to the intracellular C-terminal domain of the receptor anchored to a membrane have implicated regulation of the Wnt signaling pathway by PC-1, although the biological function regulated by PC-1 through this pathway was never explored (Kim *et al.*, 1999). Recent evidence suggests that expression of constructs coding only for the intracellular C-tail of PC-1 may be functioning in a dominant-negative (Low *et al.*, 2006) or in a dominant-positive manner, depending on the cell type (Basavanna *et al.*, 2007), limiting interpretation of studies based on its use (Kim *et al.*, 1999; Nickel *et al.*, 2002; Parnell *et al.*, 2002; Joly *et al.*, 2006).

We have previously shown that expression of full-length PC-1 in renal epithelial cells (Madin-Darby canine kidney [MDCK]) results in reduced proliferation, in resistance to apoptosis requiring the activity of the phosphatidylinositol 3-kinase (PI3-K/Akt signaling pathway, and in spontaneous tubulogenesis when cells are grown suspended in collagen gels (Boletta *et al.*, 2000; Boca *et al.*, 2006). In the current work we demonstrate that PC-1 induces cell migration in MDCK cells and that it does so by acting on two distinct aspects of epithelial cell morphology and regulation: it induces actin cytoskeleton rearrangements and actin-based cell migration on the one hand and it regulates the mechanical strength of adhesion between cells by controlling the formation of stabilized, actin-associated, adherens junctions on the other. We further show that the first effect is mediated by PI3-K, whereas the second is independent of PI3-K but it requires activation of GSK3 β .

MATERIALS AND METHODS

Antibodies and Inhibitors

The antibodies used in this study are described below. All of them were used following the manufacturer's instructions for dilutions. Tetramethylrhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated phalloidin and anti-actin antibodies were obtained from Sigma (St. Louis, MO; 77418-1EA and a5441, respectively). Anti-E-cadherin and anti- β -catenin (mouse) antibodies were obtained from BD Biosciences (San Jose, CA; cat. nos. 610182 and 610153, respectively). Anti- β -catenin (rabbit), Ser9-GSK3 β , and total GSK3 β antibodies were obtained from Cell Signaling Technology (Beverly, MA; cat. nos. 9562, 93365, and 9315, respectively). Anti-ZO-1 FITC-conjugated was from Zymed (South San Francisco, CA; cat. no. 41091150). Anti-c-myc and -GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA; sc257778 and sc5574, respectively). Anti-PC-1 (C-20, sc-10372, lot K2800) was obtained from Santa Cruz. Anti-hemagglutinin (HA) antibodies were obtained from Roche (Indianapolis, IN; rat monoclonal cat. no. 1867423), anti-flag from Covance Laboratories (Madison, WI; cat. no. PRB-132C), and anti-GFP from Invitrogen (Carlsbad, CA; cat. no. A11122). Inhibitors were obtained as follows: LY294002 (Promega, Madison, WI), pertussis toxin (PTX; Calbiochem, San Diego, CA), and AG 1517 (Biosource International, Camarillo, CA).

Migration Assays

For wound-healing assays cells were seeded in 35-mm plates, and 24 h later they were scratched with a 200- μ l pipette tip. For time-lapse video microscopy analysis, cells were placed over a metal ring stage kept at 37°C in KRH media containing 10% fetal bovine serum (FBS) supplemented with 100 U penicillin/streptomycin (Invitrogen), and migration was recorded using a Zeiss Axiovert 135 TV microscope (Göttingen, Germany) equipped with an Orca II CCD camera (Hamamatsu, Bridgewater, NJ). Digital images were collected every 3 or 2 min over time (as specified in the movies). The area covered

by the monolayer was traced and measured using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). The average rate of cell sheet migration was calculated from at least three separate experiments in each case.

For Boyden chambers studies, PVP-free polycarbonate filters with 8- μ m pores (Costar, Acton, MA) were coated with fibronectin, 50 μ g/ml. Serum-free DMEM was placed in the lower chambers, and the filters were interposed between lower and upper wells. MDCK^{PKD1Zeo} and MDCK^{Zeo} cells were grown in DMEM plus 10% FBS (control), DMEM plus 10% FBS containing 15 μ M of LY294002 or 25 nM of wortmannin overnight at 70% confluence. The day after the cells were washed twice with phosphate-buffered saline (PBS) to eliminate any floating cells and harvested with trypsin. Cells ($n = 50,000$) resuspended in 50 μ l serum-free DMEM were placed in the upper chambers and incubated at 37°C in 5% CO₂ overnight. Cells remaining on the upper surface of the filters were mechanically removed, and those that had migrated to the lower surface were fixed with ethanol, stained with Giemsa stain (Sigma-Aldrich), and counted at 20 $\times$ in 10 random fields per filter. Assays were performed in triplicate and repeated three times in independent experiments.

For transient transfections with PC-1, MDCK cells were transiently transfected with 14 μ g of HA-tagged wild-type (WT) PC-1 and 5 μ g of HA-CTF mutant (plus empty vector to reach the same amount of total DNA used) in combination with a green fluorescent protein (GFP) expression vector. Lipofectamine 2000 (Invitrogen) was used following the manufacturer's directions. As a control a GFP expression plasmid was used. After Boyden chamber migration assay, cells were counterstained with DAPI and counted. All migration assays are representative of at least three independent experiments.

Cell-Cell Adhesion Assays

The different MDCK cell lines were grown as monolayers in six-well plates (Costar) for 24 h followed by treatment in the absence or in the presence of 15 μ M LY294002, 25 nM wortmannin, 20 μ M lithium chloride, or 20 μ M SB415286, for an additional 24 h. After 48 h the confluent monolayers were washed with 1 ml PBS and mechanically dissociated by pipetting 30 times as previously described (Hordijk *et al.*, 1997; Sander *et al.*, 1998). The cells were photographed, and the number of aggregates was quantified (Np). An aliquot of the same cell suspension was trypsinized, and the number of single cells was quantified (Nc). Finally, the dissociation index was expressed as number of particles (cell clusters) per total number of cells (Np/Nc).

For transient transfections, MDCK cells were transiently transfected with the β -catenin-GFP construct, the β -catenin S33Y or β -catenin Δ N47 mutant, by Lipofectamine 2000 (Invitrogen). As control, empty vector and GFP expression plasmid were used. All dissociation assays are representative of at least three independent experiments.

Statistical Analysis

For migration and cell-cell adhesion studies one-way analysis of variance (ANOVA) was applied to establish differences between means. Multiple comparisons were carried out using Fisher's PLSD parameters. The p values obtained are indicated in the figure legends.

Immunofluorescence Studies

For immunofluorescence studies in wound-healing assays, MDCK cells grown to 100% density on coverslips were scratched and incubated for 4 h in growth medium. Paraformaldehyde (PFA)-fixed MDCK cells were washed twice with PBS, permeabilized in PBS/0.5% Triton X-100 (Tx-100), and blocked in blocking buffer (PBS, 3% bovine serum albumin). Primary antibody was applied (all antibodies were diluted 1:100 in blocking and incubated for 1 h at 37°C). Bound antibody was visualized using Alexa Fluor 488- or 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR). The distribution of F-actin was visualized using TRITC-conjugated phalloidin (Sigma). The ProLong Antifade kit (Molecular Probes) was used for mounting the samples. Digitized images of representative fields were captured using a Bio-Rad MRC 1024 confocal microscope (Richmond, CA).

For immunofluorescence studies on polarized cells, the different MDCK cell lines were grown as monolayers on Transwells (Corning Glass Works, Corning, NY) and used for experiments after 5 d in culture.

Samples were fixed with 4% paraformaldehyde at room temperature. Cells were washed with PBS, and nonmatched paraformaldehyde was quenched 10 min at room temperature with 75 mM NH₄Cl, 20 mM glycine (both dissolved in PBS). After washes with PBS, nonspecific sites were blocked with PBS, 0.7% fish skin gelatin (FSG), and 0.025% saponin (Sigma).

Cells were incubated with the appropriate primary antibodies, diluted in PBS-FSG-saponin, for 1 h at 37°C in a humid chamber, followed by washes with PBS-FSG-saponin. Subsequently, the cells were incubated 45 min at 37°C with the appropriate combination of fluorescently labeled secondary antibodies diluted in PBS-FSG-saponin.

The cells were washed with PBS-FSG-saponin, five times with 0.1% Tx-100, dissolved in PBS, followed by a single wash in PBS alone. Cells were postfixed in 4% paraformaldehyde for 15 min at room temperature, and washed with PBS, and the ProLong Antifade kit (Molecular Probes) was used for mounting the samples. Fluorescence images were obtained using a confocal laser scanning microscope TCS SP2 from Leica Microsystem (Deerfield, IL).

Immunoblotting

Cells were washed twice with ice-cold PBS containing 1 mM orthovanadate and lysed at 4°C in Tx-100 lysis buffer (150 mM NaCl, 20 mM NaP buffer, 10% glycerol, 1% Tx-100) and supplemented with cocktail of protease (Complete, Amersham Pharmacia Biosciences, Piscataway, NJ) and phosphatase inhibitors. The cells were scraped from the dishes and lysed for 30 min on ice. Nuclei were discarded after centrifugation at $10,000 \times g$ for 10 min. After quantification, the lysates were analyzed on SDS-PAGE gels. Equal amounts of protein lysates (the concentration was determined using Bio-Rad Laboratories' protein assay) were loaded on reducing SDS-PAGE gels. After transferring onto PVDF membranes, immunoblotting was performed followed by the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biosciences) and detected using the ECL system (Roche).

For Tx-100-insoluble fractions, cells were grown to 100% density for 24 h, rinsed twice in PBS, and solubilized in 50 μ l of Tx-100 lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors. Cells were lysed for 30 min on ice and sedimented in a 4°C centrifuge for 5 min at 12,000 rpm. The soluble supernatant, defined as the Tx-100-soluble fraction, was collected, and the cell pellet was extensively washed in Tx-100 lysis buffer and finally trituated in Tx-100 lysis buffer supplemented with 0.1% SDS. After centrifugation in a 4°C centrifuge for 5 min at 12,000 rpm, the supernatant was defined as the Tx-100-insoluble fraction. Protein concentrations were established using Bio-Rad's Bradford assay, loaded on polyacrylamide gels, and processed for immunoblotting as described above.

Pulse-Chase Experiments

Cells were seeded at 90% density in a 50-mm dish (Costar). The following day, cells were rinsed twice in PBS, starved in 2 ml/dish of pulse medium (DMEM cat. no. BE12-734F; Biowhitaker-Cambrex, Walkersville, MD; w/o L-cysteine and L-methionine supplemented with 1% dialyzed fetal calf serum [FCS] and 100 $\times$ Pen/Strep 15140-122, L-glutamine cat. no. 25030-024, sodium pyruvate 11360-039; Invitrogen) for 30 min, and then the cells were added to a 1 ml/dish Pulse Medium containing 10 μ Ci/ml 35 S-labeled promix in vitro cell labeling (cat no. SJQ0079-2.5 MCi; GE Healthcare, Milano, Italy) and pulsed-labeled for 15 min, rinsed twice with 1 $\times$ PBS, and chased by adding 2 ml/dish of growth medium for 0, 3, 6, and 9 h. At each time point, Tx-100-soluble and -insoluble fractions were generated as described above, and 500 μ g of total proteins were rocked overnight at 4°C in the presence of anti- β -catenin antibody, and then 50 μ l of g-Sepharose beads were added, incubated for 2 h at room temperature (RT), and washed three times with lysis buffer. Immunoprecipitates were separated by 10% SDS-PAGE, then electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA), dried and exposed 48 h at RT with a storage phosphor screen (GE Healthcare) cassette. The signal was acquired using phosphorimager Typhoon 9000 series (GE Healthcare), and the signals were quantified by Image Quant software

(Molecular Dynamics, Sunnyvale, CA). Subsequently, Western blot against β -catenin was performed as described above.

Fluorescence Recovery After Photobleaching

Time-lapse images were obtained using a Zeiss LSM510 confocal microscope system (Carl Zeiss). MDCK clones expressing enhanced GFP (EGFP) fused with β -catenin were observed at 37°C in 20 mM HEPES-buffered DMEM during the course of observation. Temperature was controlled with a Nevekt air stream stage incubator (Burnsville, VA). EGFP molecules were excited with the 488-nm line of a krypton-argon laser and imaged with a 505–530-nm bandpass filter. Confocal digital images were collected at 5-s intervals using a Zeiss Plan-Neofluor 40 $\times$ dry objective (NA 0.8) with open pinhole in order to maintain the entire cell within the center of the focal depth and thus to minimize changes in fluorescence. Selective photobleaching in regions of interest within the cell was carried out on the Zeiss LSM510 using 100 consecutive scans with a 488-nm laser line at full power. Average fluorescence intensities within regions of interests were quantified using LSM 3.2 software. A minimum of five different cells in each condition were analyzed and quantified.

Electron Microscopy Analysis

Monolayers of MDCK cells were fixed with 1% glutaraldehyde, postfixed in 1% OsO_4 , processed through ethanol of increasing concentrations, and embedded in Epon-812. Thin sections were prepared at Leica Ultracut microtome. Electron microscopy images were acquired from thin sections under a FEI Philips Tecnai-12 electron microscope (Philips, Eindhoven, The Netherlands) with the use of an Ultra View CCD digital camera.

RESULTS

PC-1 Induces Cell Scattering, Cytoskeletal Rearrangements, and Cell Migration

We have previously described a set of MDCK type II cell lines stably transfected either with a full-length human *PKD1* cDNA construct (MDCK^{PKD1Zeo}) or with an empty vector (MDCK^{Zeo}) selected in identical conditions in the presence of zeocin (Boletta *et al.*, 2000). During routine culturing of MDCK^{PKD1Zeo} cells, we noticed that the cells have a scattered phenotype when plated at low density as compared with controls growing in clusters (Figure 1A). Staining of the actin cytoskeleton using FITC-labeled phalloidin revealed that MDCK^{PKD1Zeo} have profound cytoskeletal rear-

Figure 1. Expression of full-length PC-1 in MDCK cells results in cell scattering and cytoskeletal rearrangements. (A) Two MDCK^{Zeo} controls (F6 and F2) and three independently-derived MDCK^{PKD1Zeo} cell lines [G7/36 (36); C8/68 (68), and G3] were subject to Western blot analysis using an anti-PC-1 antibody. PC-1 expression was visible only in cells stably transfected with the PKD1 gene (top) and all of the three clones had a scattered phenotype when grown on plastic compared with controls as revealed by bright field images (bottom). (B) Incubation of the cells with a FITC-labeled phalloidin (green) and anti-paxillin antibodies (red) revealed that MDCK^{PKD1Zeo} cell lines have profound morphological changes as well as actin cytoskeletal rearrangements (G7/36 and G3) compared with the controls in which actin bundles are localized at cell-cell junctions (F6). Bars, 20 μ m. (C) MDCK cells were transiently transfected with HA-tagged WT PC-1 or the HA-CTF mutant constructs (Qian *et al.*, 2002) and subject to immunofluorescence. Anti-HA antibodies allowed identification of the transfected cells, whereas FITC-labeled phalloidin was used to determine changes in the actin cytoskeleton. WT PC-1 induces cell scattering and stress fibers as shown in inset high magnification photomicrograph on the left, whereas mutant PC-1 does not. Bars, 20 μ m.

rangements, with stress fibers and lamellipodia observed (Figure 1B). This phenotype was even more visible when costaining with the focal adhesion marker paxillin was performed (Figure 1B). Transient transfection in MDCK cells revealed that the effect of cytoskeletal rearrangements is specific to WT PC-1, because it was not observed in cells transfected with a mutant construct encoding for the CTF fragment corresponding to a molecule entirely lacking the N-terminal portion, but including the 11 transmembrane domains and the intracellular C-terminus (Figure 1C; Qian *et al.*, 2002). This phenotype resembles the one observed in MDCK cells after treatment with HGF/SF (Zegers *et al.*, 2003). In the latter model, this is the result of the motogenic properties induced by HGF, which result in tubule formation, a phenotype similar to the one we have observed in our MDCK^{PKD1Zeo} cells as well (Boletta *et al.*, 2000; Boca *et al.*, 2006). We therefore decided to test if PC-1 was able to induce cell migration in a manner similar to what has previously been described for the Met receptor.

To assess the migratory properties of PC-1-expressing cells, we performed time-lapse videomicroscopy of wound-healing assays. As previously described for MDCK cells the control cells move forward as a compact unit, the edge remaining attached to adjacent cells and not pulling away from the monolayer (Supplementary Movie 1; F6, Figure 2A and see Figure 4). By contrast, PC-1-expressing cells start pulling away from the edge as individual cells and acquire a polarized migratory phenotype (Supplementary Movie 2; C8/68, Figures 2A and 4). In addition, PKD1-expressing cells (C8/68) move forward at a faster rate and are able to close the wound in a shorter time (Supplementary Movie 1, Figure 2A and data not shown). These observations were

corroborated by measuring the speed of migration of single cells monitored over time. This analysis revealed that MDCK^{PKD1Zeo} cells migrate at an average rate of $14.62 \pm 2.38 \mu\text{m/h}$ compared with MDCK^{Zeo} controls that move at an average speed of $9.25 \pm 2.71 \mu\text{m/h}$ (Figure 2B), in line with other previous studies (Santy and Casanova, 2001). To further confirm that PC-1 was able to induce cell migration we used a Boyden chamber test and observed that indeed all three PC-1-expressing cell lines migrate at a much faster rate than the controls (Figure 2C). We have previously reported that PC-1 induces cell cycle arrest in the G0/G1 phase of the cell cycle in several cell types (Bhunia *et al.*, 2002). This property would be expected to slow the rate of migration of PKD1-expressing cells if in fact differences in proliferation rates were responsible for the observed difference in migration rates. Nonetheless, to exclude differences in proliferation rates as a trivial explanation for our findings, we repeated the same assays in the presence of mitomycin to block cell proliferation during the migration assays and we found that both wound-healing and Boyden chamber assays showed similar results as in the absence of this drug (data not shown).

To test the effect of mutant PC-1 on cell migration, we have expressed the same mutant PC-1 used in Figure 1C and found that although WT PC-1 is able to enhance cell migration in Boyden chamber assays, the CTF-truncated mutant is not able to do so (Figure 2D).

Finally to determine whether or not absence of PKD1 correlates with a reduced capability of the cells to migrate we used a set of MEFs isolated from Pkd1^{+/+} or ^{-/-} mice that we have recently isolated and characterized (Distefano and Boletta, unpublished data) and tested for their capability to migrate. As shown in Figure 2, E and F, Pkd1^{+/+}

Figure 2. PC-1 induces cell migration. (A) Monolayers of controls (F6) or MDCK^{PKD1Zeo} cell lines (C8/68) were subject to wound-healing assays as described in *Materials and Methods*. Photomicrographs taken at different time points revealed that MDCK^{PKD1Zeo} cell lines tend to close the wound faster than controls (See also Supplementary Movies 1 and 2). (B) Quantification of the speed of migration of cells in several different fields in at least three independent experiments confirmed that MDCK^{PKD1Zeo} cell lines migrate at a faster rate than their counterpart controls. (C) Boyden chamber assays performed on two MDCK^{Zeo} controls (F6 and F2) and three MDCK^{PKD1Zeo} cell lines [G7/36 (36), C8/68 (68), and G3] over a period of 12 h confirmed that stable expression of PC-1 results in enhanced cell migration. ****p* < 0.0001. (D) MDCK cells were transiently transfected with a GFP encoding construct alone or in combination with the HA-tagged WT PC-1 or the HA-CTF mutant constructs. Western blot analysis revealed that both constructs are expressed (top). The cells were subject to Boyden chambers assays as in C. WT PC-1 induces cell migration in these cells, whereas the mutant only has a minor effect. n.s., differences are not statistically significant; **p* < 0.005. (E) Mouse embryonic fibroblasts derived from Pkd1^{+/+} or ^{-/-} embryos were subject to wound-healing assays, and photomicrographs were taken at different time points. Times 0 and 10 h are shown. Ten hours after wounding although controls (Pkd1^{+/+}) have moved forward considerably, Pkd1^{-/-} cells are moving in a slower manner toward the wound. (F) Boyden chamber assays performed over a period of 3 h on the same MEFs confirmed that Pkd1^{-/-} cells have lost their capability to migrate. All migration assays are representative of at least three independent experiments. ***p* < 0.001.

Figure 3. PC-1-mediated cell scattering, cytoskeletal rearrangements, and migration depend on PI3-K. (A) Two MDCK^{PKD1Zeo} (F2 and F6) and two MDCK^{PKD1Zeo} cells [C8/68 (68), G7/36 (36)] were plated at high density. Total lysates of cells before (0 h) or 1 h after wounding were collected and analyzed using anti-phospho-Ser473-Akt. Increased levels of phospho-Akt are observed in MDCK^{PKD1Zeo} cells, but not in controls. (B) The same experiment described in A was performed on the C8/68 and F6 cell lines in the presence or absence of the PI3-K inhibitor LY294002 (LY). Treatment in the presence of the inhibitor completely reverts the phospho-Akt levels in response to wounding in MDCK^{PKD1Zeo} cells. (C) Actin cytoskeleton rearrangements and cell scattering in MDCK^{PKD1Zeo} cell lines (G7/36 and G3) were reverted upon treatment with the PI3-K inhibitor LY294002 at 15 μ M for 48 h. (D) Wound-healing assays performed on both controls (F6) and MDCK^{PKD1Zeo} cell lines (C8/68) were followed over time. Photomicrographs of three frames taken at different times (0, 4, and 8 h) show that PI3-K inhibitors are able to revert the increased migration rates of PC-1-expressing cells. (E) Measurements of the speed of migration and (F) Boyden chamber assays confirmed that the migration rates are greatly reduced in MDCK^{PKD1Zeo} cell lines upon treatment with the PI3-K inhibitor LY294002 (15 μ M). Interestingly, neither controls nor PC-1-expressing cells were completely arrested by this inhibitor (see Supplementary Movies 3 and 4). *** $p < 0.0001$. (G) Boyden chamber assays in the presence of 500 ng/ml pertussis toxin (PTX) revealed that this inhibitor is able to prevent PC-1 mediated cell migration. *** $p < 0.0001$.

MEFs migrate at a faster rate both in wound-healing assays (Figure 2E and data not shown) and in Boyden chambers assays (Figure 2F). We therefore conclude that PC-1 can mediate cell migration and initiated a series of studies to better characterize the model in MDCK cells.

PC-1-induced Cell Migration and Cytoskeletal Rearrangements Are Mediated by PI3k

We have previously reported that PC-1 expression induces activation of the PI3-K/Akt signaling pathway responsible for PC-1-induced resistance to apoptosis (Boca *et al.*, 2006). For this reason, while searching for the molecules that could mediate cell migration in our model system, we therefore studied if the PC-1-mediated cytoskeleton rearrangements and motility response are dependent on PI3-K activity. First, we performed Western blot analysis on the phosphorylation status of Akt in response to wounding. We show in Figure 3, A and B, that Akt phosphorylation in Ser 473 is enhanced 1 h after wounding in two independently derived MDCK^{PKD1Zeo} cell lines (C8/68 and G7/36), but not in the controls F6 and F2. Treatment in the presence of the specific PI3-K inhibitor LY294002 (15 μ M) inhibits Akt phosphorylation in the PKD1-expressing cell lines (Figure 3B) and prevents cell scattering and cytoskeletal rearrangements (Figure 3C).

Furthermore, upon inhibition of PI3-K we found that PC-1-expressing cells (C8/68) did not pull away from the leading edge, but the cells moved forward as a compact sheet

similar to that of control cells (F6; Supplementary Movie 3, Figures 3D and 5). In addition, the rates of migration were comparable to controls after treatment in the presence of LY294002 in the wound-healing assays (Figure 3E). It was surprising and interesting to note that the normal sheet-like migration of control cells does not induce Akt phosphorylation (Figure 3, A and B) and consistent with this it was not influenced by inhibition of PI3-K, suggesting that this molecule is not required for this type of migration (Supplementary Movie 3 and Figure 3, A, B, and E). In addition, the migration of cells of all three MDCK^{PKD1Zeo} [C8/68 (68), G7/36 (36), G3] cell lines was inhibited by both LY294002 and wortmannin in Boyden chamber assays (Figure 3F and data not shown). We conclude from these data that cell scattering, actin cytoskeleton rearrangements, and cell migration induced by PC-1 all depend on PI3-K activity. Finally, because we have previously reported that PC-1 is activating the PI3-K/Akt pathway in a heterotrimeric G-protein *Gai*-dependent manner (Boca *et al.*, 2006), we tested the effect of the PTX in PC-1-induced cell migration, and we found that this inhibitor prevents migration in Boyden chamber assays (Figure 3G), consistent with our previous findings (Boca *et al.*, 2006).

PC-1 Induces Adherens and Tight Junctions Molecules Reabsorption during Cell Migration in Epithelial Cells

Besides the difference in migration rates we noticed that MDCK^{PKD1Zeo} cells have a different morphology in the

wound-healing migration assays compared with the controls, resembling the scattered phenotype shown in Figure 1. One major difference between the wound-healing and Boyden chamber migration assays is that the latter measures the capability of single cells to move across the filter, whereas the former measures the capability of a monolayer with adherens and tight junctions already formed to respond to an external stimulus. Under these conditions epithelial cells typically move as a compact unit to fill the gap, as observed in our control cells and as widely reported in the literature (Santy and Casanova, 2001; Matsubayashi *et al.*, 2004). The fact that PC-1-expressing cells tend to pull away from the monolayer as single cells prompted us to evaluate the status of the actin cytoskeleton and the distribution of adherens and tight junctions in wound-healing assays. Immunofluorescence studies performed in control and MDCK^{PKD1Zeo} cells using rhodamine-labeled phalloidin after wound-heal-

ing assays revealed that PC-1-expressing cells have cytoskeletal rearrangements with abundant stress fibers and lamellipodia (Figure 4). Furthermore, E-cadherin, β -catenin, and ZO-1 staining was strong and clearly visible until the first line of the leading edge in MDCK^{Zeo} (F6) controls, as previously reported in the literature for WT MDCK cells (Figure 4; Santy and Casanova, 2001; Matsubayashi *et al.*, 2004). In contrast, in PC-1-expressing cells (C8/68) the distribution of all three markers appeared weaker and punctate at cell-cell contacts in the first line of the leading edge and appeared to be reabsorbed quickly within the first few hours of migration (Figure 4 and data not shown).

As stated above, the morphology of MDCK^{PKD1Zeo} cells is profoundly altered in the presence of LY294002 inhibitors, resulting in a compact unit moving toward the wound, similarly to what is observed in control cells (Figure 3D). We looked at the status of actin and ZO-1 at the leading edge of MDCK^{PKD1Zeo} when cells are treated in the presence or absence of LY294002 inhibitors and found a pattern similar to controls, with the protein localized at the cell-cell boundaries up to the leading edge of the wound (Figure 5). We therefore conclude that in wound-healing assays both cytoskeletal rearrangements and the reabsorption of cell-cell adhesion molecules require the activity of PI3-K.

MDCK^{PKD1Zeo} Cells Polarize Normally, But Their Mechanical Strength of Cell-Cell Adhesion Is Reduced

The data shown above seem to suggest that PC-1 is able to influence the reabsorption of both adherens and tight junctions upon a wound-healing stimulus. However, the cells at the back of the wound do not show signs of altered

Figure 4. PC-1 induces cytoskeletal rearrangements and reabsorption of adhesion molecules at the leading edge of migrating cells. During wound-healing, MDCK^{PKD1Zeo} cells (C8/68) acquire an elongated morphology compared with controls (F6) that migrate as a compact unit (top, left panel bright field). See also Supplementary Movies 1 and 2. Staining of the actin cytoskeleton using TRITC-phalloidin (bottom, left panels) show that C8/68 has profound cytoskeletal rearrangements with several lamellipodia and stress fibers, whereas in controls actin forms bundles at the cell-cell junctions. Immunofluorescence studies show that E-cadherin (top, middle panel), β -catenin (bottom, middle panel), and ZO-1 (bottom, right panel) at the leading edge are reabsorbed in MDCK^{PKD1Zeo} cells 4 h after wounding, whereas in controls (F6) all molecules are still at cell-cell contacts up to the leading edge. By contrast, no major differences are observed for all three adhesion molecules in both cell lines at the back where cells still form a monolayer (top, right panel and data not shown).

Figure 5. PI3-K inhibitors prevent the phenotype at the leading edge of migrating cells. Wound-healing assays performed on both MDCK^{PKD1Zeo} cells (C8/68) and controls (F6) in the presence of the PI3-K inhibitor LY294002 (15 μ M) show that the elongated morphology (bottom panels) as well as the actin cytoskeleton rearrangements (top panels) and adhesion molecules reabsorption (ZO-1, middle panels; E-cadherin and β -catenin, not shown) of cells at the leading edge is restored by treatment in the presence of the inhibitor.

0.8 ± 0.2; G7/36, 0.7 ± 0.15; G3, 0.8 ± 0.18) than in controls (F6, 0.22 ± 0.06; F2, 0.26 ± 0.1). ****p* < 0.0001. (E) Treatment of all cell lines in the presence of two different PI3-K inhibitors LY294002 and wortmannin had no effect on the capability of these cells to mechanically adhere to each other, suggesting that regulation of cell-cell mechanical adhesion by PC-1 is independent of PI3-K. n.s., differences are not statistically significant.

adherens (Figure 4) or tight (not shown) junctions, suggesting that no major differences are present in the absence of a stimulus and that cells in a monolayer appear normally polarized. To formally test this, we grew the cells polarized on filters and observed the morphology and localization of cell-cell junction molecules. We found that PKD1-expressing cells are able to properly polarize and localize β-catenin, E-cadherin (not shown), and ZO-1 correctly at adherens and tight junctions, respectively (Figure 6A, top panels and Z-sections). Western blot analysis of total amounts of E-cadherin, β-catenin, and c-myc in Tx-100-soluble fractions revealed no differences in the expression levels of these molecules in MDCK^{PKD1Zeo} [C8/68 (68), G7/36 (36), G3] compared with control cells [MDCKtTA (M), F6 and F2] (Figure 6B). Furthermore, coimmunoprecipitation studies in MDCK^{PKD1Zeo} (C8/68) and MDCK^{Zeo} (F6) cells of E-cadherin and β-catenin in the Tx-100-soluble fraction revealed that equal amounts of β-catenin are bound to E-cadherin at 50, 80, or 100% density in MDCK^{PKD1Zeo} cells and controls, suggesting that no major differences in the composition of adherens junctions are present (Figure 6C and Supplementary Figure 1B). However, during routine culturing of the cells we noticed that they were dissociating faster one from the other. We therefore tested if mechanical adhesion was comparable to controls. To assess if PC-1 regulates MDCK cell-cell adhesion, confluent monolayers of transfectants

Figure 6. PC-1-expressing cells polarize normally, but have a reduced cell-cell adhesion. (A) Cells were grown polarized on filters for 5 d. Immunofluorescence studies using antibodies against ZO-1 (top) or β-catenin (middle) revealed that in both MDCK^{PKD1Zeo} cells and in MDCK^{Zeo} controls these molecules localize normally. Z-sections projections (bottom panels) show that both tight and adherens junctions can assemble normally. Nuclei are counterstained with DAPI. Bars, 23.8 μm. (B) Western blot analysis of Tx-100-soluble supernatants using anti-E-cadherin, anti-β-catenin, and anti-c-myc antibodies show no differences in the total amounts of these molecules in MDCK^{PKD1Zeo} cells lines (4, C8/68; 5, G7/36; 6, G3) compared with controls (1, MDCKtTA; 2, F6; 3, F2). Actin staining was used as a loading control. (C) Immunoprecipitation of E-cadherin at different density states (50, 80, 100%) followed by immunoblots against E-cadherin (top) and β-catenin (bottom) revealed no differences in the amount of β-catenin bound to E-cadherin in MDCK^{PKD1Zeo} cells (C8/68) compared with controls (F6) in all cell density states analyzed. See Supplementary Figure 1B for quantification of the Western blots. (D) Dissociation assays were performed as previously described (Hordijk *et al.*, 1997) by applying mechanical force to the cells. Photomicrographs of the multicellular complexes observed after applying mechanical force on MDCK^{PKD1Zeo} (C8/68) cells and on MDCK^{Zeo} (F6) controls revealed that larger complexes are recovered from controls, suggesting that these cells have stronger adherens junctions. Quantification of the assays, as previously described, showed that the dissociation index in MDCK^{PKD1Zeo} cells is much higher (C8/68, 0.8 ± 0.2; G7/36, 0.7 ± 0.15; G3, 0.8 ± 0.18) than in controls (F6, 0.22 ± 0.06; F2, 0.26 ± 0.1). ****p* < 0.0001. (E) Treatment of all cell lines in the presence of two different PI3-K inhibitors LY294002 and wortmannin had no effect on the capability of these cells to mechanically adhere to each other, suggesting that regulation of cell-cell mechanical adhesion by PC-1 is independent of PI3-K. n.s., differences are not statistically significant.

[C8/68 (68), G7/36 (36), and G3] and controls (F6 and F2) were mechanically dissociated as previously described (Hordijk *et al.*, 1997; Sander *et al.*, 1998). As shown in the photomicrographs in Figure 6D (top) when applying mechanical force to these cells, smaller clusters were observed in MDCK^{PKD1Zeo} cells (C8/68) compared with MDCK^{Zeo} controls (F6). To quantify this effect, we used a previously described technique by counting the number of particles (Np) observed and the total number of cells (Nc; Hordijk *et al.*, 1997; Sander *et al.*, 1998). The ratio of Np/Nc represents a dissociation index of these cells included between 0 and 1, where 1 is a complete dissociation of the cells (Np = Nc, i.e., particles composed of 1 cell). As shown in Figure 6D, bottom, adhesion between PC-1-expressing cells was much weaker than adhesion between control cells. To test if PI3-K was contributing to the cell-cell adhesion effects as well, we performed the same dissociation assays as shown in Figure 6D, in the presence or absence of both LY294002 and wortmannin. We found that neither of the two inhibitors was able to restore normal mechanical force of cell-cell adhesion, (Figure 6E), despite their capability to restore normal localization of adherens and tight junctions up to the leading edge in wound-healing experiments (Figure 5), suggesting that PI3-K is not involved in regulating mechanical strength of adhesion in these cells.

Figure 7. Enhanced turnover of β -catenin at sites of cell–cell junctions in PC-1–expressing MDCK cells. (A) Immunoblots performed on Tx-100–soluble (TnX100-S) or –insoluble (TnX100-ins) fractions using antibodies against E-cadherin and β -catenin revealed that reduced amounts of both molecules are recovered in the Tx-100–insoluble (TnX100-ins) fraction in MDCK^{PKD1Zeo} cells [G7/36 (36); C8/68 (68) and G3] compared with controls (F6 and F2), whereas the amount of both molecules recovered in the Tx-100–soluble (TnX100-S) fractions of the same preparation are identical and served as loading controls (bottom blots). See Supplementary Figure 1C for quantification of the Western blots. (B) Pulse-chase experiments were performed as described in *Materials and Methods* on Tx-100–soluble (top, TnX100-S) or –insoluble (bottom, TnX100-ins) fractions. Cells were pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine for 15 min (0h) and then chased for 3, 6 or 9 h. β -catenin was immunoprecipitated from Tx-100–soluble or –insoluble fractions, and the total amount of incorporated radiolabeled amino acids ([³⁵S] [aa]) was revealed by autoradiography. The same membranes were incubated using anti- β -catenin antibodies. Radioactive intensity was measured and expressed as ratio over total β -catenin for each sample. Time 0 h was considered as 100%. The graphs on the right represent average quantification of two independent experiments. (C) Time-lapse images of MDCK clones transiently transfected with EGFP-fused β -catenin. The area indicated by the arrows was bleached using 100 consecutive scans with a 488-nm line of a krypton-argon laser. Recovery of the signal (FRAP) was imaged with a 505–530-nm bandpass filter at 5-s intervals. The images shown indicate 1, 2, 3, and 4 min after bleaching. (D) Quantification of the FRAP assays shown in C and in Supplementary Movies 5–8, performed on two controls (F6 and F2) and two MDCK^{PKD1Zeo} cells (C8/68 and G7/36) clones, was carried out by average fluorescence intensities within regions of interests in at least five different cells per clone and revealed a much faster recovery of the signal in MDCK^{PKD1Zeo} cells compared with controls.

PC-1 Induces Enhanced Turnover of β -Catenin at Adherens Junctions

The major cell structures involved in regulating epithelial cell–cell mechanical adhesion are the adherens junctions. We therefore hypothesized that perhaps mature, cytoskeleton-associated adherens junctions might be altered in cells expressing PC-1. To test this hypothesis, we investigated the amount of E-cadherin and β -catenin that could be retrieved associated with the Tx-100–insoluble fraction in three different MDCK^{PKD1Zeo} cells compared with controls, and we found that indeed the amount of both molecules associated with the cytoskeletal fraction was reduced upon expression of PC-1 (Figure 7A). These results suggest that adherens junctions might be less stabilized and therefore more dynamic in MDCK^{PKD1Zeo} cells, and this could favor the quick reabsorption observed during wound-healing assays. To test this hypothesis, we have used two complementary systems. First, we performed pulse-chase experiments on controls and MDCK^{PKD1Zeo} cells to assess the time course of degradation of β -catenin in the two cell systems. Cells were grown to confluence, starved in methionine-free medium for 12 h, and pulsed using a [³⁵S]methionine–[³⁵S]cysteine mix for 15 min. After extensive washing cell lysates from Tx-100–soluble or –insoluble fractions were collected at different time points (0, 3, 6, and 9 h) and immunoprecipitated using an anti- β -catenin antibody, and radioactivity was examined as described in *Materials and Methods*. As shown in Figure 7B,

we found no difference in the degradation rates of β -catenin in the Tx-100–soluble fraction (top radiography, Western blot, and histogram). By contrast, we found a considerable difference in the degradation rates of β -catenin associated with the Tx-100–insoluble fraction (bottom radiography, Western blot, and histogram). In addition, it is interesting to note that the amount of radiolabeled cytoskeleton-associated β -catenin increases in controls of up to 20% at the 3-h time, whereas in C8/68 is not. This suggests that both the incorporation and degradation rates in PC-1–expressing cells are increased. To further examine this aspect and to formally prove that this enhanced turnover is taking place at sites of cell–cell junctions, we performed FRAP experiments using a previously described GFP-fused β -catenin construct (Sharma and Henderson, 2007). As shown in Figure 7, C and D, and in the Supplementary Movies 5–8, the FRAP is greatly enhanced in both C8/68 and G7/36 (Figure 7, C and D, and Supplementary Movies 7 and 8) compared with F6 and F2 controls (Figure 7, C and D, and Supplementary Movies 5 and 6). All of these data taken together demonstrate that PC-1 enhances the turnover of β -catenin at sites of adherens junctions. It is interesting to note that in other model systems of cell migration in epithelial cells, reduced mechanical cell–cell adhesion strength was associated with increased rates of migration (Hordijk *et al.*, 1997; Sander *et al.*, 1998; Zegers *et al.*, 2003).

cell lines. Stripping and reprobing the membrane with an anti-actin antibody revealed that differences in loading cannot account for the differences observed and that the effect is specific for β -catenin. (D) Overexpression of two different nondegradable mutant forms of β -catenin in MDCK^{PC1Zeo} cell lines restores their cell–cell adhesion. A Flag-tagged S33Y- β -catenin or GFP-tagged Δ N- β -catenin were expressed in the C8/68 cell line. The expression of the constructs was checked by Western blot analysis (left), and cell–cell adhesion was assessed as in B. Both mutants are able to completely restore cell–cell adhesion in these cells. *** $p < 0.0001$.

PC-1 Induces GSK3 β Activation to Regulate the Cytoskeleton-associated Adherens Junctions and Cell–Cell Mechanical Adhesion

Given the fact that GSK3 β is one of the major regulators of β -catenin turnover, we tested if GSK3 β activity could be implicated in our model system. Glycogen synthase kinase is a serine threonine kinase that can be inhibited by several stimuli inducing its phosphorylation in Ser9. The generally accepted model for its regulation is that the kinase is constitutively active within cells, but it is inhibited upon phosphorylation by specific kinases in response to different extracellular stimuli. Ser-9, its major regulatory site, can be phosphorylated by several different kinases including Akt, PKA, PKC, p90RSK, and S6K1 (Cohen and Frame, 2001). We therefore tested the phosphorylation levels of this kinase in our system and found that GSK3 β phosphorylation is reduced in three independently derived MDCK^{PC1Zeo} [C8/68 (68), G7/36 (36), and G3] cell lines compared with MDCK^{Zeo} controls grown as confluent monolayers (F6 and F2; Figure 8A). A similar difference was observed in cells plated at low density and in wound-healing experiments (data not shown). We then tested if GSK3 β activity was required for PC-1-induced effects on cell–cell adhesion. We found that both LiCl and the specific inhibitor SB415286 were able to restore normal mechanical adhesion between cells (Figure 8B). Furthermore, treatment in the presence of LiCl and SB415286 completely restored the amount of β -catenin associated with the cytoskeletal fraction to levels comparable to those of controls (Figure 8C). Therefore, we conclude that PC-1 is able to decrease the inhibitory phosphorylation of GSK3 β in Ser9, most likely resulting in enhancement of its catalytic activity responsible for the stabilization of mature, actin-associated β -catenin and that this results in regulation of cell–cell mechanical adhesion. We reasoned that if this model is correct, expression of a mutant form of β -catenin that cannot be degraded should restore normal cell–cell adhesion. This experiment was therefore performed using two different forms of nondegradable

Figure 8. Enhanced activity of GSK3 β is responsible for reduced stabilization of adherens junctions and reduced cell–cell mechanical adhesion in PC-1–expressing MDCK cells. (A) Western blot analysis using anti-phospho-GSK3 β antibodies showed reduced phosphorylation in Serine 9 of this kinase in all three MDCK^{PC1Zeo} cell lines analyzed [G7/36 (36); C8/68 (68) and G3] compared with MDCK^{Zeo} controls (F6 and F2) despite equal loading of total protein in each lane (total GSK3 β and GAPDH applied after stripping the same membranes), suggesting enhanced activity of GSK3 β in response to PC-1 expression. (B) Cell–cell dissociation assays performed in the presence of two different inhibitors of GSK (LiCl and SB415286, both at 20 μ M) revealed that inhibition of GSK3 β can restore cell–cell adhesion mechanical force in MDCK^{PC1Zeo} cell lines. *** $p < 0.0001$, * $p < 0.005$. (C) Western blot analysis shows that both GSK3 β inhibitors LiCl and SB415286 (20 μ M) are able to restore the amount of β -catenin present in the Tx-100-insoluble fraction in MDCK^{PC1Zeo}

β -catenin previously reported (Kolligs *et al.*, 1999). As can be seen in Figure 8D, the expression of both constructs are able to enhance cell–cell mechanical adhesion of PC-1–expressing cells. Finally, because PC-1 has previously been reported to localize to desmosomes (Scheffers *et al.*, 2000), additional structures able to regulate the mechanical strength of adhesion in epithelial cells, we tested the contribution of these structures in our model system. We have found that expression of PC-1 is able to reduce the amount of expression of desmoplakin (Supplementary Figure 2), although desmosomes are properly formed in these cells as assessed by both immunofluorescence and EM studies (Supplementary Figure 2, B and C). Because plakoglobin, the adaptor molecule corresponding to β -catenin in desmosomal junctions, was reported to interact with axin and to be regulated through a mechanism similar to that of β -catenin (Kodama *et al.*, 1999), involving phosphorylation by GSK3 β , it was important to establish whether or not the reduction in mechanical adhesion was dependent on desmosomal plaques as well. Using the two different inhibitors LiCl and SB415286, we found that neither of the two was able to restore normal desmoplakin expression (Supplementary Figure 2A and not shown). Because these inhibitors are able to completely restore normal β -catenin accumulation into the cytoskeletal fraction (Figure 8C) and cell–cell adhesion (Figure 8B) and because a nondegradable mutant form of β -catenin completely restores normal cell–cell adhesion (Figure 8D), we conclude that regulation of cell–cell mechanical adhesion in response to PC-1 expression is mainly mediated by adherens junctions, although the desmosomes might as well contribute to a minimal extent.

DISCUSSION

PC-1 is a plasma membrane receptor involved in several biological functions, including proliferation, morphogenesis, and antiapoptotic processes (Boletta and Germino, 2003). The structure of the protein points to a receptor involved in

cell-cell/matrix interactions or mechanosensation. The sub-cellular localization of PC-1 is highly controversial. Nevertheless there is consensus on the fact that PC-1 can localize to both cell-cell adhesions and primary cilia in epithelia monolayers, whereas it seems to localize to focal adhesions in other cell types or in nonconfluent epithelial cells (Huan and van Adelsberg, 1999; Wilson *et al.*, 1999; Boletta *et al.*, 2001; Yoder *et al.*, 2002; Boletta and Germino, 2003; Nauli *et al.*, 2003; Joly *et al.*, 2006).

In this study we have demonstrated for the first time that PC-1 is able to control the actin cytoskeleton and cell migration in renal epithelial cells and that it might therefore function by translating an extracellular stimulus into a dynamic change in the actin cytoskeleton and the shape of the cell. We have shown that PC-1 enables confluent epithelia to "sense" the presence of a wound and to induce a transient EMT, resulting in cytoskeletal rearrangements and adherens- and tight-junction reabsorption that allows active migration of the cells to close the wound. We further show that PC-1-induced actin reorganization depends on PI3-K activity because inhibitors of this kinase are able to revert the cytoskeletal rearrangements, cell scattering, and cell migration.

We have previously shown that PC-1 induces spontaneous tubulogenesis in MDCK cells grown suspended in three-dimensional collagen gels in a system that resembles the model of hepatocyte growth factor (HGF)-induced tubulogenesis, although it differs in the antimetastatic effects induced by PC-1 (Boletta *et al.*, 2000). In the current study we show an additional similar phenotype of PC-1 and the HGF/Met system: both are able to induce cell scattering, cytoskeletal rearrangements, and cell migration in a PI3-K-dependent manner (Derman *et al.*, 1995). It is interesting to note that in the HGF morphogenesis system a similar transient EMT has been proposed as a central event in allowing further elongation of the tubules and full differentiation in collagen gels (Zegers *et al.*, 2003). In a similar way, we propose that PC-1 allows epithelial cells to transiently dedifferentiate in order to reorganize in a more efficient way to migrate in response to a wound and/or to elongate and branch in response to a three-dimensional collagen gel.

The physiological implications of our findings might be at several levels. This function of PC-1 might be important during development when renal tubules are forming, elongating, and branching. The capability of PC-1 to regulate the actin cytoskeleton might be important in tissue regeneration or maintenance of a normal epithelium in the adult tubule, a process that is compromised by acquired inactivation of the WT allele of individual cells with germline PKD1 mutations (Qian *et al.*, 1996). Finally, we show that *Pkd1*^{-/-} cells lose their capability to migrate. The cystic epithelia show some features of benign tumors as evidenced by increased rates of proliferation and occasional formation of micropolyps (Evan *et al.*, 1979; Grantham *et al.*, 1987). The increased rates of apoptosis observed in the same epithelia are believed to be important to avoid malignant transformation of these cells (Woo, 1995). A reduced capability of migration might also contribute to avoid malignant transformation of the cystic epithelia.

In the current study we have found that during the formation of fully established adherens junctions, PC-1-expressing cells have a decreased stabilization of the adherens junctions that seems to be mediated by increased activity of GSK3 β . A previous study showed that expression of the C-terminal domain of PC-1 induces down-regulation of GSK3 β activity and increased β -catenin (Kim *et al.*, 1999). The authors proposed that the role of

PC-1 is to up-regulate the Wnt signaling pathway and to stabilize β -catenin. In this report we show that full-length PC-1 behaves in the opposite manner, i.e., it induces up-regulation of GSK3 β activity. These discrepancies might be reconciled in the light of several more recent lines of evidence suggesting that the C-tail of PC-1 expressed alone in a number of different systems seems to interfere with PC-1 normal function. In line with this, expression of the short C-tail of PC-1 in zebrafish results in cyst formation, a phenotype similar to what is observed upon down-regulation of the *Pkd1* gene using morpholino in this model system (Low *et al.*, 2006). In addition a recent report has highlighted a role for inversin, another molecule generating renal cyst formation when mutated in mice and men, in inhibiting the Wnt pathway (Simons *et al.*, 2005). Furthermore, transgenic mice overexpressing an oncogenic form of β -catenin were reported to develop massive PKD (Saadi-Kheddouci *et al.*, 2001). Increased stabilization of β -catenin has thus been proposed as a general mechanism of cystogenesis, and our data are consistent with this model. However, our results show that the pool of β -catenin in the Tx-100-insoluble fraction, the one in stabilized, actin-associated adherens junctions, but not the one in the soluble fractions, is affected by PC-1 and that both its degradation and its turnover at sites of adherens junctions are regulated by PC-1. Recent studies have shown that the regulation of β -catenin is more complex than anticipated and that cells contain pools of β -catenin differently capable of mediating cell-cell adhesion or transcription (Harris and Peifer, 2005). GSK3 β was described as the kinase that phosphorylates soluble β -catenin in the cytosol and targets it for degradation (Cohen and Frame, 2001). Our data suggest that this kinase might be able to control specifically the pool of β -catenin associated with stable adherens junctions as well.

One puzzling aspect of our results is the fact that GSK3 β Serine-9 is one of the substrates of Akt. In our model system we find activation of the PI3-K/Akt signaling pathway (Boca *et al.*, 2006 and current work), but Akt does not phosphorylate GSK3 β Serine-9. Several explanations can be provided to address this point. First, GSK3 β can be phosphorylated by a number of different kinases including Akt, but also PKC, p90RSK, and S6K1 (Cohen and Frame, 2001). Both p90RSK and S6K1 are down-regulated in the MDCK^{PKDZeo} model system (Distefano and Boletta, unpublished data). These molecules might be responsible for the decreased phosphorylation levels of GSK3 β . It is also possible that the pool of Akt active in our system is not the one responsible for phosphorylation of GSK3 β . Several recent reports have suggested that the specificity of substrates phosphorylated by Akt might change depending on the mechanism of activation of the complex responsible for phosphorylating Akt in Ser473, the mTORC2 complex (Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Shiota *et al.*, 2006). Examples were provided showing that lack of Akt phosphorylation in Ser473 results in impaired phosphorylation of the forkhead transcription factor FOXO (or FKHR1), but not of GSK3 β (Guertin *et al.*, 2006; Jacinto *et al.*, 2006; Shiota *et al.*, 2006). Similarly, in our model system active Akt phosphorylates the forkhead transcription factor FKHR (or FOXO) (Boca *et al.*, 2006), but not GSK3 β (current work). The precise mechanism of activation of Akt in response to PC-1 and the selection of substrates by this kinase must be the focus of future investigations.

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