Polycystin-1 Induces Resistance to Apoptosis through the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway

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Polycystin-1 (PC-1), the PKD1 gene product, is a large receptor whose expression in renal epithelial cells results in resistance to apoptosis and tubulogenesis, a model consistent with the phenotype observed in patients. This study links PC-1 expression to a signaling pathway that is known to be both antiapoptotic and important for normal tubulogenesis. This study found that PC-1 expression results in phosphorylation of Akt and downstream effectors and that phosphatidylinositol 3-kinase (PI3-K) inhibitors prevent this process. In addition, it is shown that dominant negative Akt can revert PC-1–induced protection from apoptosis. Furthermore, it was observed that increased PI3-K β activity in PC-1–expressing MDCK cells seems to be dependent on both tyrosine-kinase activity and heterotrimeric G proteins. It also was found that PC-1–induced tubulogenesis is inhibited by PI3-K inhibitors. Taken together, these data suggest that the PI3-K/Akt cascade may be a central modulator of PC-1 function and that its deregulation might be important in autosomal dominant polycystic kidney disease.


Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic disorders, affecting 1/1000 (1). The main clinical manifestation is bilateral renal cystic disease, which results in ESRD by age 50 in half of affected individuals. Extrarenal manifestations of the disease include cysts of the biliary tract and pancreas (2) and an assortment of cardiovascular abnormalities (e.g., hypertension, intracranial aneurysms, cardiac valvular defects) (1).

Two genes have been shown definitively to result in ADPKD when mutated: PKD1, which accounts for 85%, and PKD2, which is responsible for the rest (3–6). The 14-kb PKD1 mRNA encodes a 4302–amino acid (aa; 520 kD) protein (polycystin-1 [PC-1]) that is a highly glycosylated plasma membrane receptor (7) with a large (>3000 aa) extracellular N-terminal domain, 11 transmembrane domains (8), and a short intracellular C-terminus of 198 aa (9). The extracellular portion has 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) (10) that we recently showed is functionally active (11). The short intracellular C-terminus of PC-1 was reported to interact with the Gai/Gα0 subunit of heterotrimeric G proteins, suggesting that PC-1 itself might be a GPCR (12–14). PC-1 has also been reported to modulate the activity of protein kinase C and induce activator protein 1 (AP-1), to activate the Wnt signaling pathway, and to modulate mitogen-activates protein kinase activity (15–17). Furthermore, in vitro kinase assays that were performed on the short C-terminus suggest that this portion can be phosphorylated, further suggesting a role for this receptor in cell signaling (18,19). The ligands for PC-1 still remain elusive, although recent work suggests that PC-1 might be able to homodimerize through its PKD (IgG-like) repeats (20). A number of groups have localized the protein to cell–cell junctions and postulated that self-association might be sufficient for its activation (7,20,21).

We previously reported that expression of full-length polycystin-1 in MDCK cells results in reduced growth rates, resistance to apoptosis, and spontaneous tubulogenesis when cells are grown in collagen gels (22). We subsequently found that the Janus activated kinase (JAK)/STAT signaling pathway mediates the growth-suppressive effects in this system (23). In this work, we show that PC-1 expression also induces activation of the phosphatidylinositol 3-kinase (PI3-K) signaling pathway, a system that was implicated previously in regulating these other properties.

Materials and Methods

Antibodies and Inhibitors

Anti–PC-1 antibodies were described previously (7). Anti–P-Ser-Akt, Anti–P-Thr-Akt, Akt, FKHR, PKHR, P-PTEN, and PTEN were from Cell Signaling Technologies (Danvers, MA). The anti–p110α, anti–p110β, anti–p110γ, and anti-phosphotyrosine (PY20) antibodies were from Santa Cruz Biotechnology (cat. nos. sc-7174, sc-602, sc-7177, and sc-508, respectively; Santa Cruz, CA). Two independent anti-pan-p85 polyclonal antibodies were from Upstate Biotechnology (cat. nos. 06-195
and 06-496; Charlottesville, VA). High-affinity anti hemagglutinin (HA) antibody was from Roche (186742; Monza, Italy).

LY294002 (LY) was purchased from Cell Signaling Technology. For studies using the LY inhibitor, the cells were initially grown in 10% FCS/DMEM, then switched to DMEM with 0.5% FCS with LY (FCS-/-LY+) for 3 to 5 h to reduce background. Medium then was replaced with either FCS-/-LY- or FCS+/LY+.

Wortmannin was from Sigma (cat. no. W1628; St. Louis, MO); genistein, herbimycin, and pertussis toxin (PTX) were from Calbiochem (cat. nos. 345834 and 375670, respectively; San Diego, CA); and rapamycin was from Cell Signaling Technologies. All inhibitors were prepared as directed and used at the indicated final concentrations.

**Transient and Stable Transfections, Immunoprecipitations, and Immunoblots**

Full-length and truncated forms of human PKD1 were described previously (7,11). HepG2 and MDCK cells were transiently transfected using Lipofectamine 2000 (Invitrogen, San Giuliano Milanese, Italy). MDCK cells were co-transfected with green fluorescence protein and sorted using a flow cytometer before analysis. HepG2 stable transfectants were generated as described previously (22) with the pCI-β-PKD1-Flag vector previously described (7) and using Zeocin as a selectable marker. Screening for positive clones was carried out by reverse transcription–PCR using the following primers: Forward gdst5'−CTGCTCACCCAGTTTGAC-3', reverse gdst5'−CGTCGTCCTGTAGTCGAG-3'. For immunoprecipitation (IP) studies, cells were lysed (250 mM sucrose, 20 mM imidazole, and 1 mM EDTA [pH 7.4], and 0.5% Triton-X 100) supplemented with Protease Inhibitors Cocktail (Amersham, Cologno Monzese, Italy) and phosphatase inhibitors (1 mM final of glycerophosphate, sodium orthovanadate, and sodium fluoride). A total of 1 μg/μl antibody was added to the supernatants, and the mix was rocked at 4°C for 2 h. Prewashed G-Sepharose beads were added, and the samples were rocked for an additional 2 h, centrifuged, and washed extensively. Laemmli buffer at 1× final was added for SDS-PAGE and immunoblot studies.

**PI3-K Assays**

After IP, samples were washed three times in lithium buffer (LiB; 0.5 M LiCl and 100 mM Tris-HCl [pH 7.4]), three times in EDTA buffer (EB; 1 mM EDTA, 100 mM NaCl, and 10 mM Tris-HCl [pH 7.4]), and three times in kinase buffer (KB; 40 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 20 mM MgCl). Beads then were resuspended in KB + 1 mM dithiothreitol in the presence or absence of Wortmannin at a final concentration of 25 mM. γ,δ-Phosphatidylinositol was used as substrate (Sigma; cat. no. 8443) and resuspended in HEPES (pH 7.6), 1 mM EDTA, and 0.5% deoxycholate at a concentration of 1 μg/μl. A total of 2 μg of lipids was added to each reaction along with 20 mM cold ATP and 20 μCi of 32P-ATP with high specific activity (2000 Ci/mmol; Amersham cat. no. AA0018). The reaction was carried out in a final volume of 100 μl, at room temperature for 10 min, then stopped using 25 μl of 4 N HCl. The lipids were extracted using 200 μl of chloroform/methanol (1:1) and washed with 100 μl of chloroform:1 N HCl (1:1). The organic phase then was dried, resuspended in chloroform, and spotted on a silicagel-60 plate. The samples were resolved in a thin-layer chromatography chamber using chloroform:methanol:water:hydroxylamine (9:7:2:1). The plate was dried, and then the signal was captured using a PhosphoImager SI (Molecular Dynamics, Eugene, OR). ImageQuant was used for quantification of the reactions.

**Apoptosis Assays**

Cells were transfected using Lipofectamine 2000 (Invitrogen). The vectors that encode for the different forms of Akt were previously described (24,25).

Cells were transfected, cultured in complete medium for 24 h, and then treated overnight with recombinant hTNF-α/TNFSF1A (210-TA) 25 nM and Cycloheximide 35 μM or using ultraviolet (UV) light at 30 or 60 J/cm². Cells then were fixed, permeabilized with 0.5% Triton X-100, blocked, and incubated with primary followed by secondary antibodies. Cells then were processed for transferrin-mediated dUTP nick-end labeling (TUNEL) using the Apoptosis Detection System (Promega, Madison, WI) following the manufacturer’s instructions. Hoechst 33342 (bisbenzimidze) was purchased from Sigma (bb2261) and used at a final concentration of 1 μg/ml. All samples were mounted using the Prolong Antifade Kit (Invitrogen) and observed with an Axiohot, Zeiss microscope. Statistical analysis was performed using unpaired t test.

**Tubulogenesis Assays**

For the tubulogenesis assays, cells were grown to confluence, trypsinized, and then cultured in a collagen type I mixture as described previously (22). Where specified, cultures were treated with 15 μM of LY294002 or 25 nM Wortmannin, replaced daily. Quantitative assays were performed as described previously (22).

**Results**

**PC-1 Induces Activation of Akt**

We previously reported that stable expression of PC-1 in MDCK type II cells (MDCKPKD1Zeo) protects them from apoptosis that is induced by serum starvation (22). We also observed resistance to apoptosis induced by UV light or TNF-α but not from anoikis (Supplementary Figures 1, 3, and 4). Because numerous previous studies have shown that Akt can mediate these effects in other cell culture systems (24,26), we queried whether the same pathway might be involved in our system. Akt is activated by phosphorylation on two residues (Ser473 and Thr308) by two distinct kinases: PDK1, which phosphorylates at Thr308 (27), and the recently identified rapamycin-insensitive complex mammalian target of rapamycin (mTOR)/Rictor (28), which phosphorylates Ser473. Both phosphorylation events are necessary to achieve complete activation and can be detected using epitope-specific antibodies (24,26). Therefore, we tested for the presence of phosphorylated Akt in cell lysates that were prepared from three independent MDCK control cell lines and three PKD1+ MDCK cell lines (MDCKPKD1Zeo+) after a short serum starvation pulse to reduce basal levels of serum-induced phosphorylation. As shown in Figure 1a, increased levels of phospho-Akt (both Ser473 and Thr308) were present in each of the three MDCKPKD1Zeo+ cell lines compared with the controls (Figure 1a). Stripping and reprobing the same membrane with an anti-total Akt antibody revealed that almost an equal level of protein was present in each sample, excluding the possibility that differences in loading or Akt expression could account for such an effect (Figure 1a). We then tested whether PC-1 was able to activate Akt in MDCK cells without the possible confounding effect of clonal selection. We expressed by transient transfection wild-type (wt) as well as a series of truncation mutants of PC-1 (Figure 1, b and c) in
MDCK cells and found that wt but not mutant PC-1 was able to induce phosphorylation of Akt (Figure 1c). In agreement with Akt’s being active in MDCK\(^{\text{PKD1Zeo}}\) cells, we found increased phosphorylation levels of the Forkhead transcription factor FKHR-1, a downstream target of activated Akt, in each of the three MDCK\(^{\text{PKD1Zeo}}\) cell lines (Figure 1d, top). Consistent with this, FKHR translocation into the nucleus upon serum starvation is greatly reduced in MDCK\(^{\text{PKD1Zeo}}\) as compared with control cell lines (Figure 1d, bottom) (25).

**Akt Is Necessary for PC-1–Mediated Resistance to Apoptosis**

Expression of a constitutively active form of Akt in MDCK cells is sufficient to induce resistance to apoptosis (29). To test whether Akt is mediating the antiapoptotic effects of PC-1 in our MDCK cell culture system, we transiently transfected HA-
tagged forms of a kinase-dead Akt (DN-Akt, K179M) and HA-tagged wt Akt (WT-Akt) (25) into MDCK controls (MDCKZeo) and in MDCK^{PKD1Zeo}. Cells then were treated with TNF-α, and the apoptotic rates of mock, WT-Akt, and DN-Akt transfected cells were evaluated using a TUNEL assay as described previously (22). As shown in Figure 2a, higher apoptotic rates were observed in MDCK control cells (F6 mock, 19.66 ± 4.00%) as compared with MDCK^{PKD1Zeo} (C8/68 mock, 4.33 ± 2.88%), confirming that these cells are resistant to apoptosis (P = 0.0059). Expression of WT-Akt in C8/68 did not impair the capability of these cells to survive in the presence of an apoptotic stimulus (Figure 2, a and c), whereas transfection with DN-Akt dramatically increased the apoptotic rates in these cells (Figure 2, a and c) despite that equal expression levels for both forms are achieved as revealed by Western blot analysis (Figure 2b). Quantification of the apoptotic rates (Figure 2c) confirmed

Figure 2. Akt is necessary for PC-1–mediated resistance to apoptosis. (a) MDCK control (F6) and MDCK^{PKD1Zeo} (C8/68) were transiently transfected using a mock, a WT (WT-Akt), or a dominant negative (DN_Akt) Akt construct. Apoptosis was induced using TNF-α (see Materials and Methods), and cells were stained with an anti-hemagglutinin (HA) antibody (in red) to identify transfected cells, with a transferase-mediated dUTP nick-end labeling (TUNEL) assay (in green) to visualize apoptotic cells and counterstained with Hoechst 33342 (blue nuclei) to visualize all of the cells that were present in the field. Very few apoptotic cells are visible in mock-transfected C8/68 cells, whereas a considerable number are visible in F6 cells that are treated under the same conditions. Similar results were observed when the WT-Akt construct was transfected in these two cell lines, whereas higher rates of HA-positive/TUNEL-positive cells are visible in C8/68 when DN-Akt is transfected in these cells. (b) Same experimental design as in a except that cell lysates were prepared from transiently transfected F6 and C8/68 cells and analyzed by Western blot using a mixture of anti-HA and anti-actin antibodies as a loading control. Equal expression levels were achieved with all constructs in all cell lines. (c) Quantification of the results visualized in a. In mock-transfected cells, the percentage of apoptosis is calculated as the number of TUNEL-positive cells per the number of total cells present in the field. In WT- and DN-Akt-transfected cells, the percentage of apoptosis is expressed as the number of TUNEL-positive cells over the number of HA-positive cells. The ANOVA test was used to perform statistical analysis. The results for each pairwise comparison reached statistical significance (see text for details). (d) Same experiment as in a through c performed in HepG2 cells. Both the percentage of apoptosis and the statistical analysis were performed as in c.
that significantly higher rates of apoptosis were observed in C8/68 when transfected with DN-Akt (14.66 ± 1.15%) as compared with mock-transfected (4.33 ± 2.88%;  \( P = 0.0045 \)) or WT-transfected (4.66 ± 4.16%;  \( P = 0.016 \)) cells. The results are representative of three different experiments performed in triplicate. Similar results were observed using two different clones F2 and G7/36 (data not shown). In a similar way, we found that higher apoptotic rates are observed in HepG2Zeo (E19) as compared with HepG2PKD1Zeo (A15) stable transfectants when apoptosis was induced by either TNF or UV light (Supplementary Figure 4b). Quantification of the apoptotic rates (Figure 2d) confirmed that significantly higher rates of apoptosis were observed in E19 as compared with A15 when apoptosis was induced using UV light (20.3 ± 3.9  \( \text{versus} \) 5.2 ± 1.5%;  \( P = 0.0001; \) Figure 2d). Furthermore, a significant increase in apoptotic rates was observed in A15 when transfected with DN-Akt (35.6 ± 7.8%) as compared with mock-transfected (5.2 ± 1.5%;  \( P < 0.0001 \)) or WT-transfected (7.1 ± 2.00%;  \( P < 0.0001 \)) cells. The results shown are representative of three different experiments performed in triplicate. We thus conclude from these data that active Akt is a necessary component for PC-1–mediated resistance to apoptosis.

**PC-1–Mediated Activation of Akt Requires Active PI3-K**

We next sought to determine the mechanism by which PC-1 results in activation of Akt. Given that Akt is a well-established target of the PI3-K pathway, we repeated the serum starvation experiment using the three MDCK control and MDCKPKD1Zeo cell lines in the presence or absence of the highly specific PI3-K inhibitor LY294002 (15 \( \mu \text{M} \)). We found that this inhibitor was able to prevent Akt phosphorylation in both MDCK (Figure 3a) and HepG2 (Figure 3b) cells that express full-length PC-1. Because LY294002 was reported to inhibit also the kinase activity of the Ser/Thr kinase mTOR, we treated the cells in the presence of another PI3-K inhibitor (Wortmannin, 12 nM) and of a potent mTOR inhibitor (rapamycin, 25 nM). We found that the former but not the latter was able to prevent Akt phosphorylation in our system (data not shown), demonstrating that PI3-K is a necessary component in this pathway.

The apparent increase in PI3-K/Akt activity observed in association with PKD1 expression could result from either decreased activity of phosphatases that are known to degrade phosphatidylinositol-3,4,5-triphosphate (PIP3), an effector molecule that is involved in Akt activation, or to enhanced activation of PI3-K. PTEN is the primary phosphatase that is known to dephosphorylate the critical D3 position of PIP3, so we examined how PKD1 expression altered PTEN’s properties (30). We found no differences in the level of either total PTEN or its phosphorylated form in MDCKPKD1Zeo cell lines as compared with controls (Figure 3c). These data suggest that Akt activation that was observed with PC-1 expression results from enhanced PI3-K activity.

**PC-1 Induces Activation of PI3-Kβ (p85/p110β)**

Three classes of PI3-K have been described. Class I PI3-K are major producers of PIP3 and thus primarily implicated in Akt activation. Members of this class of enzymes are heterodimers that are composed of a regulatory subunit and a catalytic subunit that are constitutively associated and inhibited by low doses of inhibitors such as Wortmannin and LY294002 (31,32). The larger subclass (class IA) is formed by various combinations of distinct regulatory (p85α, β and p55α, γ) and catalytic (p110α, β, δ) subunits. Class IB PI3-K has only a single member that is composed of a 110-kD catalytic subunit (p110y) and a 101-kD regulatory subunit (p101). Most class IA PI3-K are activated by binding to tyrosine phosphorylated receptors (usually tyrosine kinase receptors) (31,32). Exceptions include p110δ, which can also be activated by GPiCR, and p110α, which can be activated by cytokine receptors via Janus activated kinase 2 (JAK2) (31–34). Class IB PI3-K is activated exclusively by GPiCR, via direct binding of the Gβγ subunits (31,32).

We tested for class I kinase activity in lysates of PC-1–expressing versus control cell lines. We immunoprecipitated the p110α, β, γ catalytic subunits from the MDCK control and MDCKPKD1Zeo cell lines and performed in vitro kinase assays. As shown in Figure 3d, only the p110β IP products of cells that expressed PC-1 catalyzed an increased quantity of 32P-labeled PIP versus controls. Quantification of several independent experiments revealed a low but consistent increase in activity associated with p110β immunoprecipitates (1.29 ± 0.07;  \( n = 6 \)) from MDCKPKD1Zeo versus controls but not with either p110α or p110γ (Figure 3e). These data suggest that p110β catalytic activity is increased by PC-1 expression. Consistent with this interpretation, neither the total level of p110β present in lysates as measured by immunoblot nor the amount of IP product used for the kinase reactions differed between the two cell lines (Figure 3f).

**Activation of PI3-K by PC-1 Requires Tyrosine-Kinase Activity and Heterotrimeric G Proteins**

Class I PI3-K are usually activated by recruitment of the regulatory subunit p85 to tyrosine-phosphorylated receptors or adaptors through its SH2 domain (31,32). We tested for this property by comparing the in vitro kinase activity of anti-phospho-tyrosine immunoprecipitates from MDCKPKD1Zeo versus control cell lysates. Surprisingly, no differences were observed (Figure 4a). In control studies, we found that both sets of cell lines responded normally to treatment with hepatocyte growth factor (HGF) and EGF, with markedly increased kinase activity in anti-phosphotyrosine immunoprecipitates (Figure 4, a and b). These results exclude the presence of a general inhibitory factor in PKD1-expressing cells or a technical problem as the likely explanations for our findings.

Previous studies suggested that p110β can be activated by both tyrosine kinase– and heterotrimeric G-protein–dependent pathways (33,34). We therefore tested whether heterotrimeric G proteins could be involved in this process using PTX, a highly specific inhibitor of heterotrimeric G proteins of the Gi subfamily. Indeed, we observed a dose-dependent inhibition of phospho-Akt in response to PTX (Figure 4c) in the C8/68 subclone and minimal basal inhibition in the control F6. These data suggest that the heterotrimeric G proteins play a role in activation of PI3-Kβ that results from PC-1 expression. Given that Kurosu et al. (33) previously reported that p110β activation by
Gβγ was markedly enhanced by addition of a phosphotyrosyl peptide, we queried whether the process that we observed was completely independent of tyrosine phosphorylation. We addressed this question by examining the effect of generic inhibitors of tyrosine phosphorylation on Akt activation by PC-1. As shown in Figure 4d, we found that both genistein and herbimycin (data not shown) greatly reduced the level of Akt activation that was induced by PC-1. Similar results were obtained for the other PKD1+ cell lines (data not shown). These results suggest that a dual mechanism of activation is occurring in our system, although at too low a level to be detected by kinase assays of anti-phospho-tyrosine immunoprecipitates.

**PI3-K Mediates PC-1–Induced Morphogenesis**

PI3-K also has been implicated in the motogenic and tubulogenic properties in the HGF/SF-MDCK model (35); we therefore tested whether LY294002 or Wortmannin could prevent the morphogenic properties of the MDCKPKD1Zeo cells. As shown in Figure 5, both LY294002 and Wortmannin prevented PC-1–induced tube initiation in a three-dimensional collagen gel assay, strongly suggesting that PI3-K plays a central role in polycystin-1–mediated morphogenesis. Removal of the inhibitor at different time points resulted in further growth of the structures, indicating that treatment with the inhibitors for such short periods did not result in cell death (data not shown).
Discussion

Increased proliferation has been reported in cystic kidneys of individuals with ADPKD (36,37). The disease, however, progresses very slowly throughout an affected individual’s lifetime, a characteristic that is difficult to explain if increased proliferation is the sole or primary defect (1). Increased rates of apoptosis also have been reported in human cystic kidneys, and it therefore has been proposed that the balance between cell proliferation and death is perturbed in the epithelia lining the cysts (37). We previously showed that expression of PC-1 in renal epithelial cells results in reduced growth rates, resistance to apoptosis, and conversion of a cystic phenotype to one of the branching tubules (22). We then showed that activation of the JAK-STAT signaling system by PC-1/PC-2 is responsible for the growth effects seen in this model (23). In this work, we link PC-1 to a signaling pathway that is known to be both antiapoptotic and protubulogenic (24,31,32,35). We have shown that PC-1 expression results in activation of PI3-K/Akt, and effector molecules such as FKHR and that these are inhibited by very low doses of highly specific PI3-K inhibitors. Importantly, in this study, we show that activation of Akt is necessary for PC-1–induced resistance to apoptosis in vitro, because MDCKPKD1Zeo as well as HepG2PKD1Zeo that express a dominant negative form of this kinase are no longer resistant to apoptosis.

We note that our results are consistent with recent findings of Yamaguchi et al. (17). These authors and others had previously reported that cAMP had a mitogenic effect on epithelial cells that were derived from ADPKD cysts but inhibited the proliferation of normal renal epithelial cells (38,39). In seeking to determine the mechanism underlying this phenomenon, they found that they could reproduce the phenotype in normal cells by altering intracellular calcium levels and by inhibiting the PI3-K/Akt pathway, which in turn resulted in activation of extracellular signal–regulated kinase (ERK). They also found that disruption of endogenous PC-1 function by expression of its short C-terminus in the M1 renal collecting duct cell line resulted in a similar upregulation of the ERK pathway and a mitogenic response to cAMP. The authors suggested a model whereby loss of PC-1 function may result in changes in intracellular calcium, downregulation of PI3-K–Akt activity, and activation of ERK in ADPKD cysts.

It is interesting that a recent report by Ma et al. (40) showed that polycystin-2 (PC-2) channel activity can be enhanced by EGF in a process that requires activity of PI3-K. One might hypothesize that PC-1–induced PI3-K activity might be relevant to regulate the PKD2 channel as well, although direct studies would be needed to test this hypothesis.

Several unusual aspects of our findings require comment. The first surprising observation is that we did not find PI3-K activity associated with phospho-tyroisine and is inhibited by pertussis toxin (PTX). (a) Analysis of PI3-K activity of anti-phospho-tyrosine (PY20) immunoprecipitates from MDCK control or MDCKPKD1Zeo cells either in the absence (−) or the presence (+) of Wortmannin (25 nM final) using the same protocol as in Figure 3. Some cultures were treated with hepatocyte growth factor (HGF) or EGF for 5 min before cell harvesting, as indicated, as positive controls. Pip identifies the 32P-labeled phosphatidylinositols. (b) An aliquot of each of the immunoprecipitates used in a was subjected to immunoblot analysis using an anti-p85 antibody (see Materials and Methods). Increased levels of p85 were immunoprecipitated by anti-phosphotyrosine antibodies upon treatment with HGF or EGF but not in untreated cells. (c) The control cell line F6 and the PC-1–expressing clone C8/68 were serum starved for 24 h in the presence or absence of 0, 50, 100, or 500 ng/ml PTX. Cell lysates were immunoblotted using the anti-phospho-Ser473-Akt antibody. Membranes were stripped and reprobed using an anti-Akt antibody. (d) Experimental design similar to that used in c except that genistein was used at the doses (mm) shown.

**Figure 4.** Activated PI3-Kβ is not associated with phospho-tyrosine and is inhibited by pertussis toxin (PTX). (a) Analysis of PI3-K activity of anti-phospho-tyrosine (PY20) immunoprecipitates from MDCK control or MDCKPKD1Zeo cells either in the absence (−) or the presence (+) of Wortmannin (25 nM final) using the same protocol as in Figure 3. Some cultures were treated with hepatocyte growth factor (HGF) or EGF for 5 min before cell harvesting, as indicated, as positive controls. Pip identifies the 32P-labeled phosphatidylinositols. (b) An aliquot of each of the immunoprecipitates used in a was subjected to immunoblot analysis using an anti-p85 antibody (see Materials and Methods). Increased levels of p85 were immunoprecipitated by anti-phosphotyrosine antibodies upon treatment with HGF or EGF but not in untreated cells. (c) The control cell line F6 and the PC-1–expressing clone C8/68 were serum starved for 24 h in the presence or absence of 0, 50, 100, or 500 ng/ml PTX. Cell lysates were immunoblotted using the anti-phospho-Ser473-Akt antibody. Membranes were stripped and reprobed using an anti-Akt antibody. (d) Experimental design similar to that used in c except that genistein was used at the doses (mm) shown.
A mechanism might be responsible for the results that we observed (12–14). In contrast to our study, however, Murga et al. (41) reported that pretreatment with genistein failed to block Akt activation by GPCR agonists in their cell culture system. Our tyrosine kinase inhibitor studies suggest that activation of Akt may result from synergistic cooperation between two path-

Figure 5. PI3-K inhibition prevents PC-1–induced tubulogenesis. (a through q) PC-1–induced tubulogenesis is inhibited by PI3-K inhibition. MDCK and MDCKPKD1Zeo cell lines were grown suspended in a collagen gel as described previously (22) either in the absence (−) or the presence (+) of 15 μM LY294002 or 25 nM Wortmannin for 2 d (a through d and i through m), 4 d (e through h), or 8 d (n through q). Representative examples of the control cell lines (a, b, e, f, i, l, n, and o) and MDCKPKD1Zeo cell lines (c, d, g, h, k, m, p, and q) are shown. Photographs were taken at ×40 magnification; a through m were magnified further ×4.

Quantification of the experiments described in a was performed as described previously (22). The structures observed in three to five wells for each clone were counted. Treatment in the presence of 15 μM LY294002 or 25 nM Wortmannin dramatically reduced, although not completely suppressed, the total number of tubules observed in MDCKPKD1Zeo cultures.
ways, consistent with kinetic studies performed in vitro on p110β (33,34).

How do we reconcile these findings with our inability to show PI3-K activity associated with anti-phosphotyrosine immunoprecipitates? We suggest that it is most likely due to the nature of the model system that we have used. The tyrosine-dependent activation of PI3-K of class IA normally is an extremely rapid on-off process, with complete loss of phosphotyrosine-associated PI3-K activity within 5 to 10 min after exposure to an agonist (42). The lack of a known ligand for PC-1 renders it impossible to study the early phases of activation of PI3-K in our system. Thus, we might miss an initial peak of activation that is dependent on tyrosine phosphorylation. It is also possible that there is a low but continuous level of tyrosine phosphorylation that is below the threshold of detection.

From our data, we could not determine whether PC-1 is directly involved in activation of PI3-K. Because no YXXM sequence is found in the C-terminus or putative intracellular loops of PC-1, it seems that activation is unlikely to occur via direct binding of p85 SH2 domain to PC-1 but rather through an intermediary molecule. One such molecule could be JAK2, because we previously showed it to be activated and bound to PC-1 (23,43,44). Another possibility is that a 14-3-3 adapter protein may mediate the interaction as has been reported for PC-1 (23,43,44). Another possibility is that a 14-3-3 adapter protein, and the latter mediates binding and pendent phosphorylation site in the IL-3 receptor binds to a protein kinase A–dependent activation of PI3-K as a central molecule that is capable of inducing both resistance to apoptosis and tubulogenesis. Future study will define the precise mechanism by which PC-1 activates this process as well as the physiologic relevance of this pathway in PC-1 function.

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