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

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Abstract

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 (AD- PKD) 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 Gαi/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

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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, P-Thr-Akt, Akt, P-FKHR, FKHR, 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 (1867423; 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 cotransfected 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 5'-CTGCTCACCCAGTTTGAC-3', reverse gdst3 5'-CGTCGTCCTTG- TAGTCAG-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 nM. L-α-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 γ32ATP with high specific activity (2000 Ci/mmol; Amersham cat. no. AA0018). The reaction was carried out in a final volume of 100μl, 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 PhosphorImager 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/cm^2. 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 transferase-mediated dUTP nick-end labeling (TUNEL) using the Apoptosis Detection System (Promega, Madison, WI) following the manufacturer's instructions. Hoechst 33342 (bisbenzimide) 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 Axiophot, 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 (MDCK^{PKD1Zeo}) 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 (MDCK^{PKD1Zeo}) after a short serum starvation pulse to reduce basal levels of serum-induced phosphorylation. As shown in Figure 1a, increased levels of phospo-Akt (both Ser 473 and Thr 308) were present in each of the three MDCK^{PKD1Zeo} 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^{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^{PKD1Zeo} cell lines (Figure 1d, top). Consistent with this, FKHR translocation into the nucleus upon serum starvation is greatly reduced in MDCK^{PKD1Zeo} as compared with negative controls (Figure 1d, bottom) (25). We then tested whether PC-1 expression results in activation of Akt in other cell types. We expressed by transient transfection full-length normal and mutant *PKD1* cDNA (Figure 1, b and e) in the hepatic cell line, HepG2, and found that, again, only the wt gene resulted in phosphorylation of Akt (Figure 1f).

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 (MDCK^{Zeo}) 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, $19.66 \pm 4.00\%$) as compared with MDCK^{PKD1Zeo} (C8/68 mock, $4.33 \pm 2.88\%$), 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 that significantly higher rates of apoptosis were observed in C8/68 when transfected with DN-Akt ($14.66 \pm 1.15\%$) as compared with mock-transfected ($4.33 \pm 2.88\%$; $P = 0.0045$) or WT-transfected ($4.66 \pm 4.16\%$; $P = 0.016$) 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 HepG2^{Zeo} (E19) as compared with HepG2^{PKD1Zeo} (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 *versus* $5.2 \pm 1.5\%$; $P = 0.0001$; Figure 2d). Furthermore, a significant increase in apoptotic rates was observed in A15 when transfected with DN- Akt ($35.6 \pm 7.8\%$) as compared with mock-transfected ($5.2 \pm 1.5\%$; $P < 0.0001$) or WT-transfected ($7.1 \pm 2.00\%$; $P < 0.0001$) 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 MDCK^{PKD1Zeo} cell lines in the presence or absence of the highly specific PI3-K inhibitor LY294002 (15 μM). 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 MDCK^{PKD1Zeo} 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 (p110γ) 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 p110β, which can also be activated by GPCR, 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 GPCR, *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 MDCK^{PKD1Zeo} 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 ^{32}P-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 MDCK^{PKD1Zeo} *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 MDCK^{PKD1Zeo} *versus* control 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 G$\beta\gamma$ 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 there-fore tested whether LY294002 or Wortmannin could prevent the morphogenic properties of the MDCK^{PKD1Zeo} 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-Kβ (p85/p110β), 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 MDCK^{PKD1Zeo} as well as HepG2^{PKD1Zeo} 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 the tyrosine phosphorylated pool of proteins. This is in contrast to what is reported for most receptors that induce activation of class IA PI3-K. In most known examples, the SH2 domains of the p85 regulatory subunit are recruited to a phosphorylated tyrosine lying within a signature sequence YXXM, on the receptor itself, or on adaptor molecules. Therefore, PI3-K activity is typically associated with phosphotyrosine pulldowns. It should be noted that there is a precedent for having phosphotyrosine-independent activation of PI3-K when p110β is activated in response to GPCR (41). Because PC-1 has been reported to associate and activate a subset of heterotrimeric G proteins, we speculate that a similar 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-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 phospho-tyrosine-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 the IL-3 receptor (45). In this example, a protein kinase A- dependent phosphorylation site in the IL-3 receptor binds to a 14-3-3 adapter protein, and the latter mediates binding and activation of p85. A similar mechanism might be occurring in our system as a potential protein kinase A phosphorylation site has been identified in the PC-1 C-terminus (18,19).

How the various *in vitro* study results relate to PC-1 function *in vivo* remains to be determined. Nishio *et al.* (46) recently reported a mouse model mosaic for *Pkd1*+/+ and *Pkd1*-/- cells that develops renal cystic disease. In that model system, Akt phosphorylation seems to be increased in both normal and cystic epithelia but does not correlate with the apoptotic rate in either. These results seem to be contrary to the well-established antiapoptotic role of this molecule in most systems studied to date (24). However, the authors do not specify which phosphorylation site they have studied, and, most important, they do not show increased phosphorylation of any downstream effector of Akt. Because phosphorylation of both Ser473 and Thr308 is necessary for Akt to be activated (24,25,27,28), it is impossible on the basis of the currently available

data to know whether Akt is also catalytically active in their system. A direct correlation between our findings and theirs therefore is impossible at the moment. However, one could speculate that phosphorylation of Akt in that context might represent an attempt (possibly failed) of the cells to counteract a strong apoptotic stimulus or could be due simply to upregulation of the EGF receptor signaling (46). In addition, Nishio *et al.* (46) demonstrated that cyst formation *in vivo* seems to require a number of "transforming" events that occur in a step-wise manner. It is possible that careful examination of the phosphorylation state of Akt at the different stages of cyst formation would indeed reveal reduced activation in the absence of *Pkd1* in some but not other of these stages. Systematic evaluation of Akt status at various times after inactivation of *Pkd1* will be required to resolve this issue.

One final possible explanation for the seeming discrepancy between the *in vitro* and *in vivo* findings is that the down regulation of the PI3-K/Akt pathway that results from loss of PC-1 *in vivo* is not responsible for increased apoptosis in cystic epithelia but rather might be responsible for some of the other phenotypes observed in *Pkd1*-/- mice. This hypothesis is consistent with both our findings that specific inhibitors for PI3-K are able to block branching tubulogenesis induced by PC-1 and a growing body of literature that describes a central role for this kinase in the regulation of HGF-induced tubulogenesis in MDCK cells (35,47). We therefore propose a model in which PC-1 activates 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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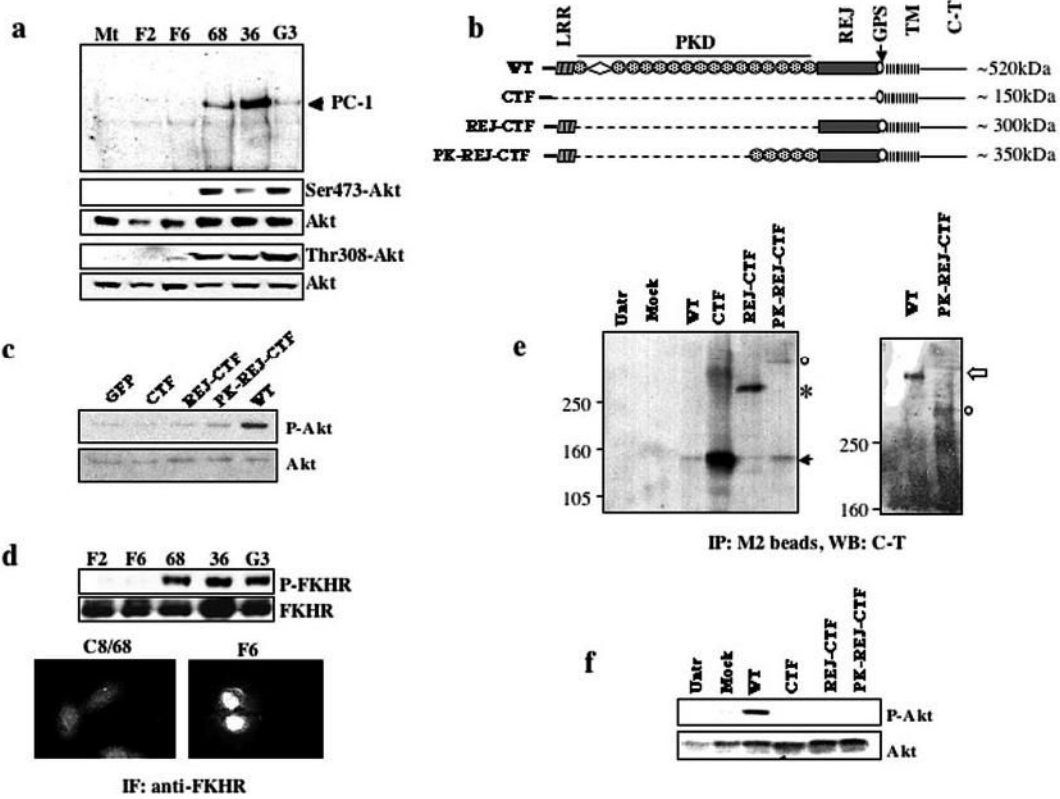
Figure 1.
Polycystin-1 (PC-1) expression results in activation of Akt. (a) Immunoblot (IB) analysis of PC-1 expression in immunoprecipitation (IP) products of three MDCK^{PKD1Zeo} (C8/68 [68], G7/36 [36], and G3) and control cell lines (MDCKtTA [Mt], F6, and F2). An anti-PC-1 C-term (α-CT) antibody was used for both IP and IB (7). Open arrows indicate PC-1 (top panel). (Bottom) Cell lysates were tested for Akt activation with either anti-phospho-Ser473-Akt or anti-Thr-308 antibodies. Membranes were stripped and reprobed for total Akt to control for loading. (b) Several truncation constructs (top) described previously (11) were expressed by transient transfection in HepG2 cells. Wild-type (WT): pCI-β-PKD1Flag (7); C-terminal cleavage products (CTF): AF66; REJ-CTF: HBF160; PK-REJ-CTF: BF110 (11). (c) MDCK cells were transiently co-transfected with the constructs shown in b along with green fluorescence protein, sorted using a flow cytometer, and assayed using an anti-phospho-Ser473-Akt antibody. The same membrane then was stripped and reprobed using an anti-Akt antibody. (d) Total lysates from two control cell lines (F6 and F2) and three PC-1-expressing cell lines (C8/68, G7/36, and G3) cultured as in a were immunoblotted using an anti-phospho-Ser256-FKHR antibody. The same gel was stripped and reprobed with an anti-FKHR antibody to control for loading. (Bottom) Immunofluorescence staining performed using an anti-FKHR antibody after 24 h of serum starvation revealed relocation of FKHR in the nucleus in F6 as compared to C8/68. (e) Immunoblot (IB) analysis of PC-1 expression in HepG2 cells transfected with the various *PKD1* constructs presented in b. M2 beads were used to purify the FLAG-tagged PC-1 variants from an equal quantity of total cell lysate, and the affinity-purified products were analyzed using α-CT in a 5% polyacrylamide gel. The black arrow identifies the CTF that result from cleavage of WT and mutant PC-1 at the GPS site (11). REJ-CTF (*uncleaved form) is cleaved at a markedly reduced rate, explaining the relatively small amount of CTF present in the corresponding lane (11). In contrast, IgG-REJ-CTF (°uncleaved form) and WT are cleaved in a higher proportion. To better visualize the full-length PC-1 (open

arrow), two lanes were loaded with samples from IgG-REJ-PKD1 (°right blot) and wt-PKD1 (open arrow, right blot) in a 4% polyacrylamide gel and analyzed using α-CT. (f) HepG2 cells were transiently transfected, serum starved, and total lysates were assayed using an anti-phospho-Ser473-Akt antibody. The same membrane then was stripped and reprobed using an anti-Akt antibody.
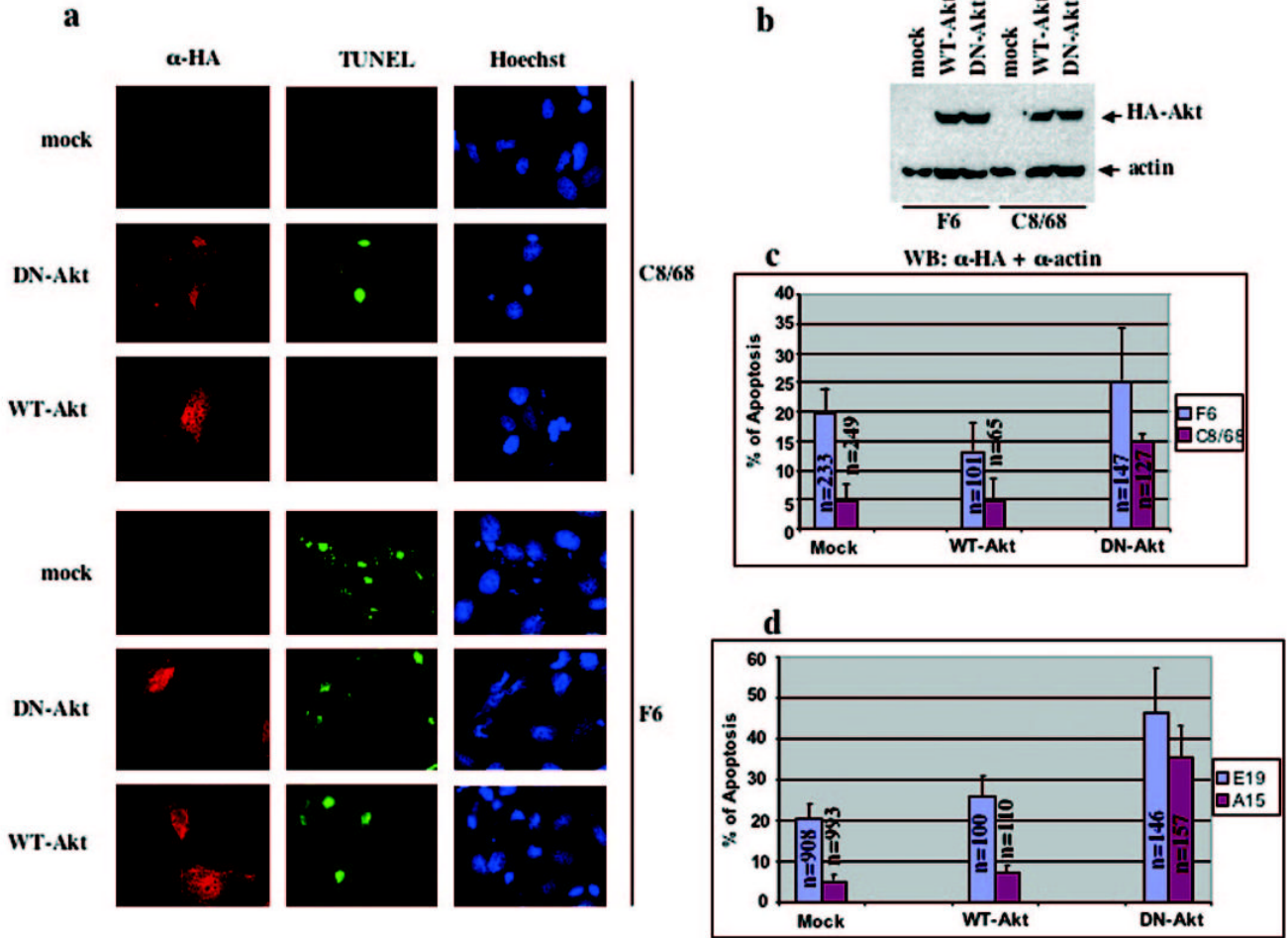
Figure 2.
Akt is necessary for PC-1-mediated resistance to apoptosis. (a) MDCK control (F6) and MDCK^{PKD1Zeo} (C8/68) were transiently transfected using a mock, a WT (WT-Akt), or a dominant negative (DN_Akt) Akt construct. Apoptosis was induced using TNF-α (see Materials and Methods), and cells were stained with an anti-hemagglutin (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 over 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.
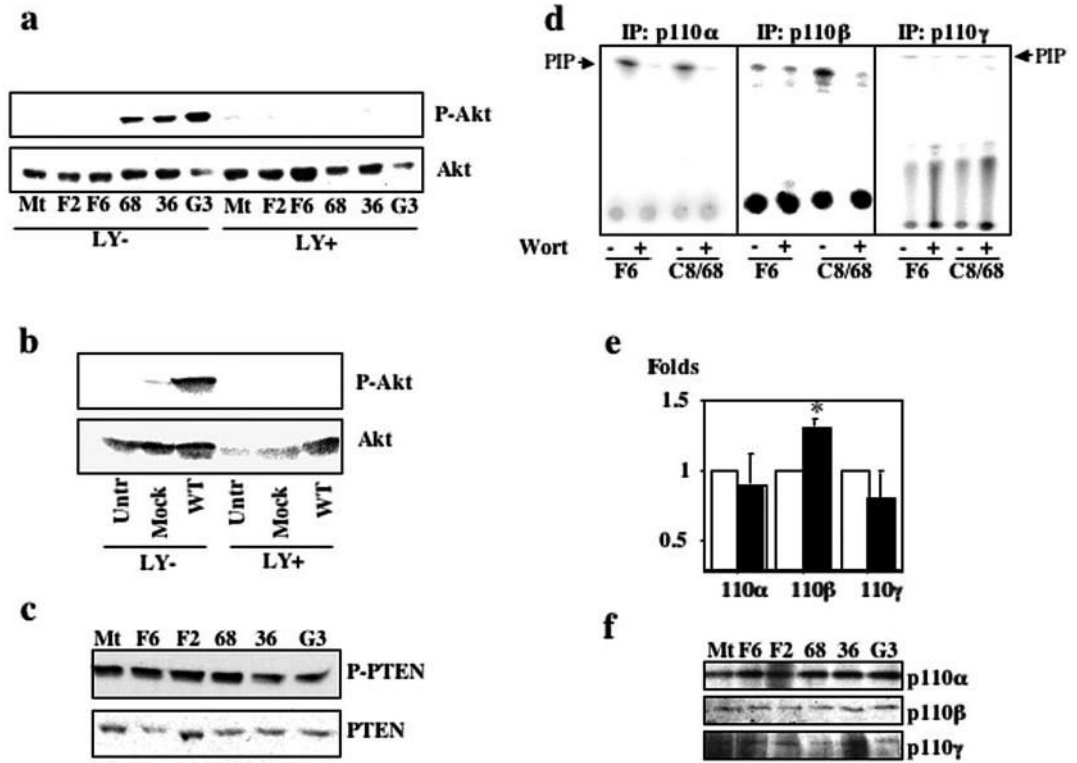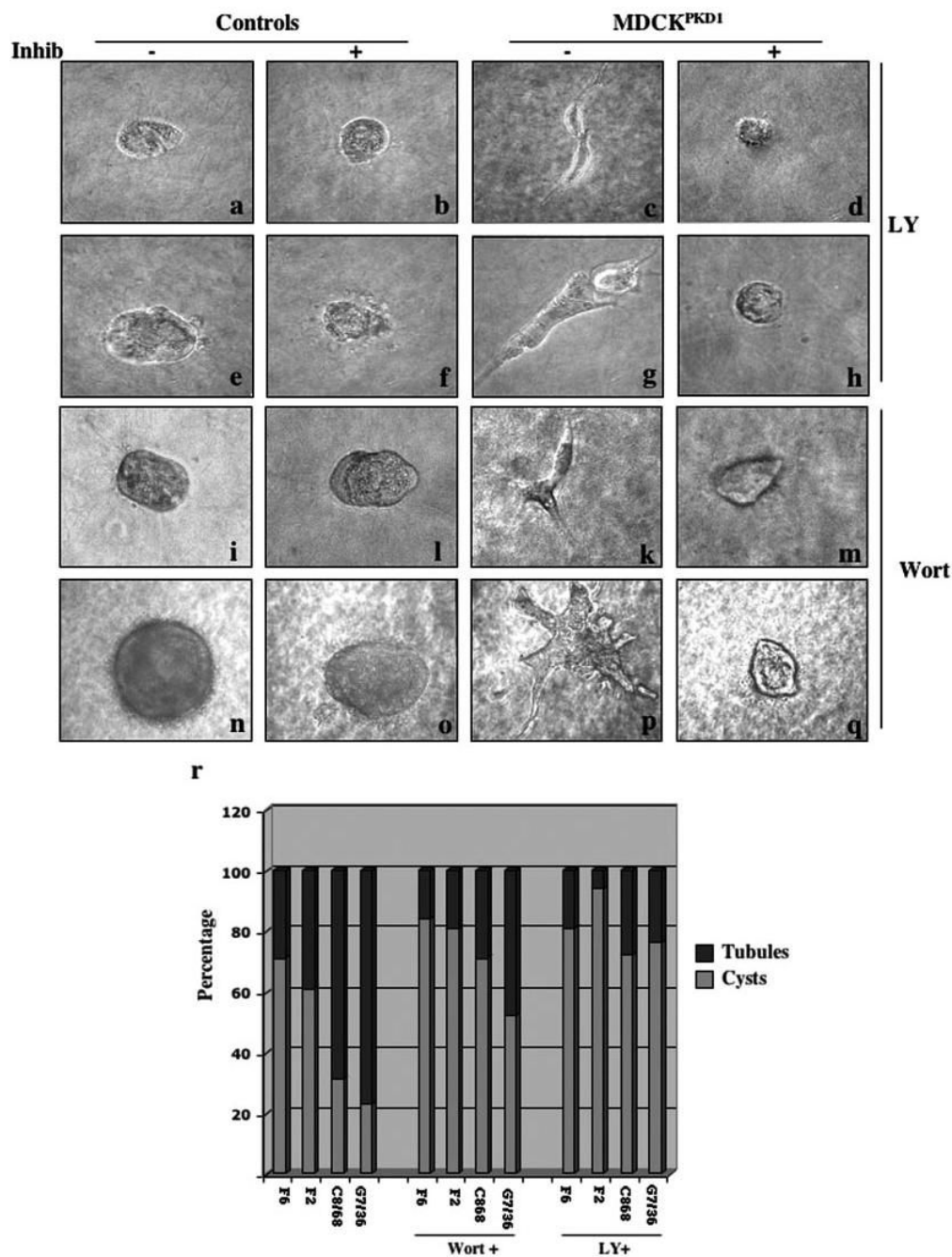
Figure 3.
PC-1 associated activation of Akt is phosphatidylinositol 3-kinase (PI3-K) dependent, and PI3-Kβ has increased activity in MDCK^{PKD1Zeo} cell lines. (a) Lysates from MDCK control (1, MDCKtTA; 2, F6; 3, F2) and MDCK^{PKD1Zeo} cell lines (4, C8/68; 5, G7/36; 6, G3), treated with serum starvation in the presence (LY+) or absence (LY-) of LY29400 (15 μM final), were immunoblotted using an antibody specific for phospho-Ser473-Akt (top). The same membrane was stripped and reprobed using an anti-Akt antibody (bottom). (b) Same experimental design as in a except that the lysates were prepared from HepG2 cells that were transfected with *PKD1* (WT) and transfected with vector (Mock) and from untransfected controls. (c) The same MDCK control and MDCK^{PKD1Zeo} cell lines described in a were serum starved for 24 h, and then cell lysates were assayed using an anti-phospho-Ser380-PTEN antibody and an anti-PTEN antibody. (d) Analysis of PI3-K activity. MDCK control F6 and MDCK^{PKD1Zeo} C8/68 cell lines were serum-deprived for 36 h and then lysed. Each of the class I p110 subunits was immunoprecipitated with a suitable antibody, and then its PI3-K activity was measured in either the absence (-) or the presence (+) of Wortmannin (25 nM final) as described in Materials and Methods. Only the p110β IP product had increased activity. Similar results were obtained using the other negative control MDCK (Mt and F2) and MDCK^{PKD1Zeo} (G3 and G7/36) cell lines (data not shown). (e) Quantification of *in vitro* PI3-K assays performed using lysates of all three negative control MDCK (□) and three MDCK^{PKD1Zeo} cell lines (■). The level of activity observed in controls was set at a value of 1, whereas the activity that was observed in MDCK^{PKD1Zeo} cells was expressed as a ratio over that of the controls. The assay for p110β activity was performed in six independent experiments, whereas that for p110α and p110γ was repeated three times. Results are expressed as the average ± SEM. [*]Statistically significant differences ($P = 0.0019$; *t* test unpaired). (f) Expression level of PI3-K p110 subunits in the same MDCK control (Mt, F6, and F2) and MDCK^{PKD1Zeo} (C8/68, G7/36, G3) cell lines used in b. The products were immunoprecipitated and subjected to immunoblot analysis using the same subunit-specific antibody.
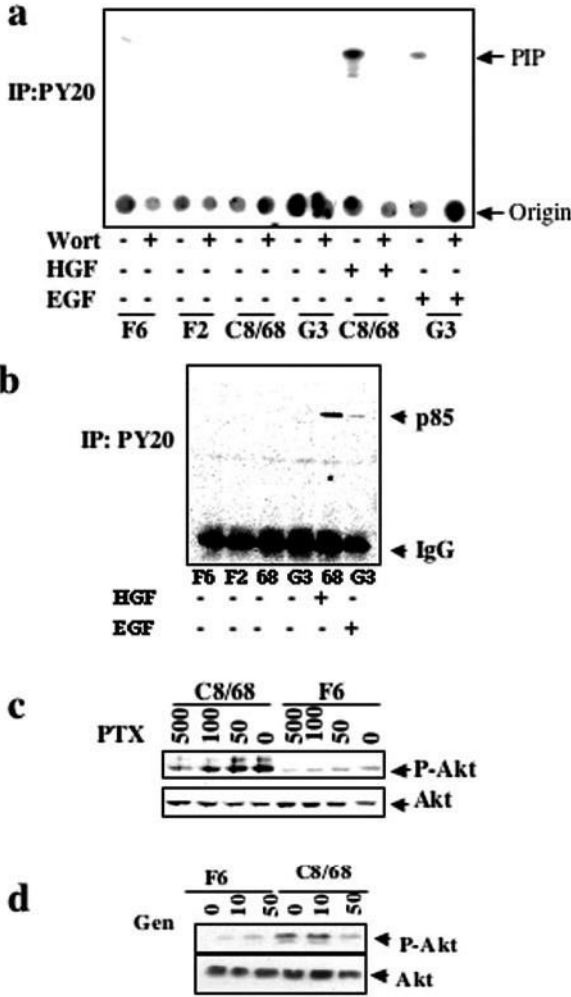
Figure 4.
Activated PI3-Kβ is not associated with phospho-tyrosine and is inhibited by pertussis toxin (PTX). (a) Analysis of PI3-K activity of anti-phospho-tyrosine (PY20) immunoprecipitates from MDCK control or MDCK^{PKD1Zeo} cells either in the absence (-) or the presence (+) of Wortmannin (25 nM 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 ^{32}P-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 5.
PI3-K inhibition prevents PC-1-induced tubulogenesis. (a through q) PC-1-induced tubulogenesis is inhibited by PI3-K inhibition. MDCK and MDCK^{PKD1Zeo} 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 MDCK^{PKD1Zeo} 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. (r) 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 MDCK^{PKD1Zeo} cultures.