Polycystin-1 Protein Level Determines Activity of the Gα_{12}/JNK Apoptosis Pathway

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Mutations in PKD1 are the most common cause of autosomal dominant polycystic kidney disease (ADPKD). The protein product of PKD1 (polycystin-1 (PC1)) is a large transmembrane protein with a short intracellular C terminus that interacts with numerous signaling molecules, including Gα_{12}. Cyst formation in ADPKD results from numerous cellular defects, including abnormal cilia, changes in polarity, and dysregulated apoptosis and proliferation. Recently, we reported increased apoptosis in Madin-Darby canine kidney (MDCK) cells through Gα_{12} stimulation of JNK and degradation of the anti-apoptotic protein Bcl-2 (Yanamadala, V., Negoro, H., Gunaratnam, L., Kong, T., and Denker, B. M. (2007) J. Biol. Chem. 282, 24352–24363). Herein, we confirm this pathway in Gα_{12}-silenced MDCK cells and utilize MDCK cell lines harboring either overexpressed or silenced PC1 to demonstrate that PC1 expression levels determine activity of the JNK/Bcl-2 apoptosis pathway. PC1-overexpressing MDCK cells were resistant to thrombin/Gα_{12}-stimulated apoptosis, JNK activation, and Bcl-2 degradation. In contrast, PC1-silenced MDCK cells displayed enhanced thrombin-induced apoptosis, JNK activity, and Bcl-2 degradation. In pulldown experiments, PC1 bound to Gα_{12}, but not the related Gα_{13} subunit, and thrombin-stimulated MDCK cells led to increased interaction of Gα_{12} with the PC1 C terminus. In transient transfection assays, a PC1 C-terminal mutant lacking the G protein-binding domain was uncoupled from PC1-inhibited apoptosis. PC1 expression levels may be increased or decreased in ADPKD, and these findings suggest a mechanism in which levels of PC1 expression modulate Gα_{12}/JNK-stimulated apoptosis. Taken together, these findings are consistent with a set point model in which PC1 expression levels regulate specific G protein signaling pathways important to cyst development.

Inherited cystic kidney diseases are a heterogeneous group of disorders caused by mutations in several protein families. The most common form of these diseases, autosomal dominant polycystic kidney disease (ADPKD),^3^ develops as the result of mutations in the PKD1 (∼70–85%) or PKD2 (∼15–30%) gene, which encodes the protein product polycystin-1 (PC1) or polycystin-2, respectively. PC1 is a large 11-transmembrane protein containing an extracellular domain with Ig repeats and a short cytoplasmic domain. PC1 is localized in cilia and at sites of cell-matrix and cell-cell interactions. Mutations in PKD1 lead to defects in cilial function and changes in epithelial cell growth/apoptosis and cell-cell and cell-matrix interactions. Many mutations have been identified in PKD1, but the mechanisms leading to cyst enlargement and renal failure remain incompletely understood. Mutations in PKD1 are most often deletion-insertion, frameshift, or nonsense mutations and lead to inactivation of one allele. However, disease development requires an additional somatic mutation or other injury leading to the focal development of cysts that is characteristic of the disease (reviewed in Ref. 1). In normal adults, PKD1 RNA and protein are expressed at moderate-to-low levels in collecting ducts and distal tubules, and with the development of ADPKD, PC1 protein levels are increased by ∼2-fold (2, 3). Numerous animal models have been established, and homozygous loss of PKD1 results in lethality with diffuse cystic disease (reviewed in Ref. 1). Conditional knock-out of PKD1 reveals important roles during development and has led to new insights into the mechanisms necessary for cyst development and progression in vivo (see Ref. 1). Although loss of PC1 clearly leads to cyst development in vivo, there is also evidence that PC1 overexpression results in PKD. In patients with ADPKD, PC1 expression persists and is even enhanced in most but not all cysts (4). In addition, transgenic mice overexpressing PC1 develop PKD with renal failure, suggesting that, in some cases, a gain of function may be a pathogenic mechanism (5). Taken together, these observations have led to the suggestion that there is a normal set point for PC1 levels important for epithelial cell function and renal development (6). Cell culture studies with overexpressed and silenced PC1 also support the concept that decreased or increased levels of PC1 lead to abnormal signaling. For example, overexpression of PC1 in Madin-Darby canine kidney (MDCK) cells leads to decreased apoptosis (7), and silencing of PC1 leads to increased apoptosis due to reduced cell adhesion (anoikis) (8).

With receptor-activated G protein signaling, ligand binding results in conformational changes in Go that lead to dissociation

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^3^ The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; PC1, polycystin-1; MDCK, Madin-Darby canine kidney; GPCR, G protein-coupled receptor; JNK, c-Jun N-terminal kinase; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
tion of GDP and separation from Gβγ. GTP binds to Ga, and
signal transduction occurs through Ga and Gβγ subunits until
the intrinsic GTPase activity of Ga hydrolyzes GTP to GDP.
More recent studies have revealed additional complexity; G
proteins are found in subcellular microdomains and interact
with numerous regulatory and scaffolding proteins (including
PC1). PC1 functions as an atypical G protein-coupled receptor
(GPCR), binds Ga12/13, and regulates calcium flux through poly-
cystin-2 (a member of the transient receptor potential family of
calcium channels) by release of GTP to GDP (9, 10). Several
heterotrimeric Ga subunits (11–13) and at least one RGS (reg-
ulator of G protein signaling) protein (14) interact with PC1.
Furthermore, we reported binding of wild-type and activated
(Q229L point mutant) Ga12 to the PC1 C terminus (13). In
transient overexpression systems, both Ga12 and Ga13 regulate
AP1 transcription factor activity in a PC1-dependent manner,
although Ga13 is significantly more effective (12). In addition,
there are other potential overlapping pathways between Ga12
and PC1. For example, PC1 regulates junction assembly in
MDCK cells (15), and Ga12 interacts with ZO-1 in the tight
junction and with E-cadherin to regulate tight junctions and
cell adhesion (16, 17). Finally, the Ga12 family regulates stress
fiber formation and numerous other fundamental cellular pro-
cesses, including proliferation, transformation, and cell migra-
tion (reviewed in Ref. 18).

G proteins are important regulators of apoptosis (reviewed in
Ref. 19), and we identified Ga12-regulated apoptosis in MDCK
cells through a mechanism involving JNK activation and Bcl-2
degradation (20). Dysregulated apoptosis is an important fea-
ture of ADPKD; for instance, increased apoptosis was detected in
polycystic kidneys from patients with and without renal fail-
ure, but not in controls (21). In human ADPKD, steady-state
Bcl-2 mRNA was increased 20-fold with markedly elevated pro-
tein levels, yet apoptosis was increased in ADPKD kidneys com-
pared with normal controls despite the increase in Bcl-2 pro-
tein levels (2). Animal models of PKD have also revealed
important roles for apoptosis in cyst development in combina-
tion with changes in proliferation (22). To test the hypothesis
that PC1 expression levels regulate specific signaling pathways
and to further define the link between PC1 and Ga12, we used
established MDCK cell culture models with overexpressed and
silenced PC1 to examine Ga12/JNK-stimulated apoptosis. Exam-
nation of apoptosis, JNK activation, and Bcl-2 expression in these
cell lines revealed that PC1 expression levels determined activa-
tion of the Ga12/JNK pathway and the degree of apoptosis. We
found specificity of the interaction of PC1 with Ga12, but not the
related Ga13 subunit. Ga12 binding to PC1 could be increased by
thrombin stimulation of MDCK cells, and the inhibition of throm-
bin/Ga12-stimulated apoptosis by the PC1 C terminus was lost
using a PC1 C-terminal mutant lacking the G protein-binding
domain. Taken together, these findings are consistent with a
model in which PC1 protein levels determine the activity of activ-
ated Ga12 available for downstream signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—PC1-overexpressing MDCK cells and controls
were provided by Gregory Germino (National Institutes of
Health) and have been described previously (7). Cells were
maintained in Dulbecco’s modified Eagle’s medium (Invitro-
gen) containing 5% fetal bovine serum (Invitrogen), 50 µg/ml
Zeocin, and 100 µg/ml G418 (Cellgro). PC1-silenced MDCK
cells and controls were provided by G. Luca Gusella (Mount
Sinai School of Medicine, New York). Cells were maintained
in Dulbecco’s modified Eagle’s medium supplemented with 5%
fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomyc-
in (Invitrogen), 2 mM glutamine, 250 ng/ml amphotericin
as described previously (8). Ga12-silenced MDCK cells were
established by stable expression of short hairpin RNA, com-
pared with green fluorescent protein short hairpin RNA control
cells established in parallel (described in Ref. 23), and main-
tained in Dulbecco’s modified Eagle’s medium containing 5%
fetal bovine serum, 100 µg/ml G418, and 100 units/ml penicil-
lin/streptomycin. MDCK cells with Tet-Off inducible Ga12
expression (Ga12-MDCK cells) (17, 24, 25) were maintained
in Dulbecco’s modified Eagle’s medium containing 5% fetal
bovine serum, 100 µg/ml G418, 100 µg/ml hygromycin, and 40
mg/ml doxycycline. Ga12 expression was induced by removal
of doxycycline, and cells were analyzed at 48 h.

Flow Cytometry—Ga12-silenced, PC1-silenced, and PC1-
overexpressing MDCK cells and transfected HEK293 cells were
grown to confluence and serum-starved for 24 h before treat-
ment with thrombin (2 units/ml; Enzyme Research Laborato-
ries) or bradykinin (100 µM; Sigma). Adherent cells were col-
lected by trypsinization, pooled with floating cells by low speed
centrifugation, washed with phosphate-buffered saline (PBS),
and fixed with 70% ethanol in PBS at −20 °C for 30 min. Sub-
sequently, cells were incubated with 1 µg/ml RNase A and 50
µg/ml propidium iodide (Invitrogen) in PBS at 37 °C for 30 min
and analyzed by flow cytometry (propidium iodide/phycoc-
erythrin-Texas Red channel). To confirm that there was no
increase in cell necrosis in these MDCK cell lines, trypan blue
staining and quantification of dead cells were performed. There
were no significant differences in necrotic cells for any of the
cell lines under these conditions.

In Vitro Translation and Glutathione S-Transferase (GST)
Pulldown—Mouse Ga12 and Ga13 cDNAs in pBS or pcDNA3
were translated in vitro using 0.5–1 µg of plasmid, the appro-
priate RNA polymerase in a coupled rabbit reticulocyte trans-
lation system (TNT system, Promega, Madison WI), and
[35S]methionine (PerkinElmer Life Sciences) as described pre-
viously (26). Protein expression was analyzed by SDS-PAGE
and autoradiography. GST and GST-PC1 C-terminal fusion
proteins were described previously (13). Approximately 1 µg of
GST fusion protein was incubated with 10–20 µl of [35S]me-
thionine-labeled Ga12 or Ga13 overnight at 4 °C. Glutathione-
agarose beads (Amersham Biosciences) were added for 2 h, and
samples were centrifuged, washed, and eluted in SDS sample
buffer, followed by SDS-PAGE and autoradiography. For
thrombin-stimulated Ga12 binding to the PC1 C terminus, Tet-
Off Ga12-MDCK cells (described previously in detail (17, 24,
25)) were treated with thrombin (2 units/ml) for the indicated
times after overnight serum starvation. Cells were lysed on ice
and incubated with GST fusion proteins (GST alone or with the
PC1 C terminus) immobilized on glutathione-Sepharose beads
at 4 °C for 2 h. Beads were centrifuged and washed five times
with 1 ml of ice-cold PBS containing 0.5% Triton X-100, and
samples were eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blotting with anti-rabbit Ga12 antibody (1:500 dilution; Santa Cruz Biotechnology). Blots were stripped and reprobed with anti-rabbit Ga13 antibody (1:500 dilution; Santa Cruz Biotechnology).

**JNK Activity Assay**—JNK activity was determined with a CASE™ kit for JNK Thr183/Tyr185 (SABiosciences). Briefly, cells were cultured to 60–80% confluence and treated with thrombin for 30 min in a 96-well plate. Subsequently, cells were fixed with 4% cell-fixing buffer (1.3 ml of 37% formaldehyde in 10.7 ml of PBS) for 20 min at room temperature. The plate was washed for 5 min, antigen retrieval buffer (all buffers/solutions were provided by the manufacturer) was added, and the plate was microwaved for 3 min. After cooling to room temperature, blocking buffer was added for 1 h. After washing, primary antibody (phosphoprotein or total JNK) was added to the appropriate wells and incubated for 1 h. The plate was washed twice, incubated with secondary antibody for 1 h, washed, and developed. After 5 min, stop solution was added, and absorbance at 450 nm was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader.

**Western Blot Analysis**—Cells were cultured to confluence in 6-well plates. After overnight serum starvation, cells were incubated with 2 units/ml thrombin for 30 min at 37 °C. Cells were lysed with JNK extraction buffer (BioVision). Lysates were analyzed according to the manufacturer’s instructions (KinaseSTAR JNK activity screening kit, BioVision). Briefly, lysates were incubated with GST-c-Jun on glutathione-Sepharose, centrifuged, and resuspended with 200 μM ATP at 30 °C for 30 min. Samples were eluted in SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting for phospho-c-Jun.

**RESULTS**

We recently identified a novel Ga12-regulated apoptosis pathway in epithelial cells that requires JNK activation and degradation of the anti-apoptotic protein Bcl-2 (20). Activating endogenous Ga12 through thrombin receptor stimulation or through the use of inducible, constitutively active Ga12 (QLG12) expression led to increased apoptosis in MDCK and HEK293 cells. To extend these observations, we examined this pathway in Ga12-silenced MDCK cells. Ga12 was silenced using stable expression of short hairpin RNA as described (23) and was compared with control cell lines expressing short hairpin RNA for green fluorescent protein. Fig. 1 shows that thrombin stimulated apoptosis by 2–3-fold in control cells but had no effect in Ga12-silenced MDCK cells. As expected, the increased apoptosis in thrombin-stimulated control cells was mediated by increased JNK activity and degradation of Bcl-2 (Fig. 1, B and C). However, in Ga12-silenced cells stimulated with thrombin, there was no significant change in JNK activity, and no changes in Bcl-2 protein levels were detected. Taken together, these results confirm our previous findings that Ga12 modulates apoptosis in MDCK cells through JNK and regulation of Bcl-2 (20).

To examine the role of PC1 in regulating Ga12 signaling pathways, we utilized previously characterized MDCK cells with overexpression of full-length PC1 (7, 28, 29). PC1-overexpressing MDCK cells undergo spontaneous tubulogenesis and display resistance to apoptosis through a mechanism that involves activation of the phosphatidylinositol 3-kinase pathway (29). Fig. 2A shows that apoptosis in serum-starved cells was significantly lower in PC1-overexpressing (G7/68) MDCK cells than in control cells (F67). Initial observations were confirmed with two independent clones, and increased PC1 protein was confirmed by Western blotting (supplemental Fig. 1). Subsequent experiments were performed on G7 (PC1-overexpressing) and F67 (control) cell lines. Thrombin stimulated a 2-fold increase in apoptosis in control cells, whereas thrombin had no effect on apoptosis in PC1-overexpressing cells. Thrombin activates several Gα subunits, including Ga12, Ga13, and Gaq. To control for potential stimulation of apoptosis through Ga13 or Gaq pathways, we tested bradykinin-stimulated (activates Ga13 and Gaq) apoptosis in control and PC1-overexpressing cells (Fig. 2B). There was no significant difference in apoptosis with bradykinin stimulation of the control and PC1-overexpressing cells, indicating no significant activation of apoptosis through these other G protein pathways. The absolute degree of apoptosis was higher in the PC1-overexpressing and matched control cell lines than in the Ga12-silenced MDCK cells (Fig. 1). This difference in the degree of apoptosis is likely the result of the selection process and clonal variability used to establish these cell lines. Nevertheless, the degree of thrombin-
PC1 Regulates Gα_{12}

**Figure 1.** Thrombin-stimulated apoptosis in MDCK cells requires Gα_{12}.A, apoptosis. Gα_{12}-silenced MDCK and control cells with and without thrombin (2 units/ml) were serum-starved overnight, fixed and stained with propidium iodide, and analyzed by flow cytometry as described under “Experimental Procedures.” The percentages of apoptotic cells were plotted using GraphPad Prism. Results are the mean ± S.E. of two independent experiments with n = 3 at each condition. *, significance at p = 0.001. B, relative JNK activity. Gα_{12}-silenced MDCK (Gα_{12}-Sil) and control cells with and without thrombin treatment for 30 min were analyzed using the CASE kit for JNK Thr^{183}/Tyr^{185} as described under “Experimental Procedures.” Phosphorylated JNK (pJnk) was normalized to total JNK and expressed relative to base-line activity in the control cells. Results are the mean ± S.E. of two independent experiments with n = 3 at each condition. *, significance at p = 0.01. C, thrombin-stimulated Bcl-2 degradation is inhibited in Gα_{12}-silenced MDCK cells. Western blots for Bcl-2 are shown at base line and at 8 and 24 h of thrombin stimulation and normalized to the GAPDH loading control. Densitometry was done using ImageJ, and the relative Bcl-2 levels are shown in the bar graph. Results are expressed as the range of two independent experiments relative to the base-line level.

stimulated apoptosis was similar in the two control cell lines (~2-fold). PC1-overexpressing MDCK cells were resistant to apoptosis through activation of the phosphatidylinositol 3-kinase pathway. This pathway is partially regulated by pertussis toxin-sensitive G proteins (29), and thus, Gα_{12} would not be expected to regulate this response. To confirm that thrombin was not inhibiting apoptosis through indirect effects on the phosphatidylinositol 3-kinase pathway, we examined Akt phosphorylation with and without thrombin stimulation of these cells. Fig. 2 (C and D) shows that, at base line, Akt phosphorylation was similar in the PC1-overexpressing cells compared with the control cells and that the addition of thrombin had no significant effect on phospho-Akt levels in either cell line.

We next examined JNK activity and Bcl-2 expression in these cells with and without thrombin stimulation. Fig. 2 (E–G) shows that thrombin stimulation did not activate JNK in PC1-overexpressing cells as determined by two different methods. Fig. 2E shows that precipitating active JNK from untreated or thrombin-treated cells led to significantly increased phosphorylation of c-Jun in control cells but not in PC1-overexpressing cells (quantification in Fig. 2F). Similar results were found in direct JNK activity assays using ELISA (Fig. 2G). There was a significant increase in JNK activity as determine by ELISA after thrombin stimulation of control cells. However, there was no increase in JNK activity after thrombin stimulation of PC1-overexpressing cells. Analysis of the base-line JNK activity in PC1 and control cells revealed a small but non-significant decrease in the basal activity in PC1-overexpressing cells (time = 0) (Fig. 2, F and G).

We next examined Bcl-2 levels in PC1-overexpressing and control cells with and without thrombin stimulation (Fig. 2H). There was nearly complete degradation of Bcl-2 in control cells after 24 h of thrombin stimulation, as expected from activation of the Gα_{12}/JNK pathway. However, PC1-overexpressing cells revealed no degradation of Bcl-2, indicating that thrombin stimulation of the Gα_{12}/JNK pathway was inhibited in these cells. Bcl-xL and NF-κB levels were not different between these cell lines and were unaffected by thrombin stimulation. We noticed that baseline Bcl-2 protein levels were higher in PC1-overexpressing cells compared with control cells. Quantification of six experiments revealed an ~3-fold increase (Fig. 2I), and this is consistent with findings from ADPKD kidneys (2). The increased Bcl-2 levels seen in PC1-overexpressing MDCK cells may play an additional role in their resistance to apoptosis, but there were no changes in Bcl-2 levels seen with thrombin stimulation (Fig. 2H). Taken together, these findings suggest that PC1 overexpression in MDCK cells inhibits Gα_{12}-stimulated apoptosis that is mediated by JNK activation and Bcl-2 degradation.

Because PC1 overexpression inhibited Gα_{12}-stimulated apoptosis, we next examined whether PC1-silenced MDCK cells would exhibit the opposite phenotype. MDCK cells with silenced PC1 (confirmed by reverse transcription-PCR) (supplemental Fig. 1) show a small increase in apoptosis when grown on plastic (8). Fig. 3A shows that, at base line, there was no detectable difference in apoptosis of PC1-silenced and control cells with serum starvation for 24 h. The addition of thrombin led to a 2-fold increase in apoptosis in control cells and a nearly 4-fold increase in apoptosis in PC1-silenced MDCK cells. Changes in JNK activity and Bcl-2 expression levels were consistent with a mechanism by which PC1 expression levels regulate this pathway. Thrombin-stimulated JNK activity was enhanced in PC1-silenced MDCK cells compared with control cells (Fig. 3, B and C). The increase in JNK activity was demonstrated by increased c-Jun phosphorylation (Fig. 3B) and increased activity by ELISA (Fig. 3C). In both assays, base-line JNK activity was slightly higher in PC1-silenced cells, and the relative stimulation by thrombin was similar with the two methods. Examination of Bcl-2 protein levels showed a decrease of Bcl-2 to ~60% at 24 h in control cells and ~20% in PC1-silenced cells (Fig. 3D).

Next, we re-examined the interaction of Gα_{12} and tested for binding of Gα_{13} with the PC1 C terminus. Using GST pulldown assays, we previously showed binding of Gα_{12} and constitu-

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**Note:** The image contains figures and graphs that represent data and experimental results. The text describes experiments and findings related to PC1 regulation of Gα_{12} and apoptosis in MDCK cells. The figures illustrate the effects of thrombin and PC1 overexpression on apoptosis, JNK activity, Bcl-2 expression, and JNK/JNK signaling pathways. The text provides a detailed explanation of these findings and their implications.
FIGURE 2. PC1 overexpression in MDCK cells inhibits G\textsubscript{\alpha_{12}}-stimulated apoptosis. A, apoptosis. PC1-overexpressing (G7/68) and control (F67) MDCK cells with and without thrombin (2 units/ml) were serum-starved overnight, fixed and stained with propidium iodide, and analyzed by flow cytometry as described under “Experimental Procedures.” The percentages of apoptotic cells were plotted using GraphPad Prism. Results are the mean ± S.E. of three independent experiments with \( n = 3 \) under each condition. *, significance for base-line apoptosis at \( p = 0.004 \); #, significance for thrombin-stimulated apoptosis at \( p = 0.005 \). B, apoptosis in PC1-overexpressing and control cells stimulated with bradykinin (100 nM) overnight after serum starvation as described above. C, thrombin stimulation does not affect phosphatidylinositol 3-kinase activity. PC1-overexpressing (G7) and control (F67) MDCK cells were stimulated with thrombin for 24 h, and lysates were analyzed by Western blotting for phospho-Akt Ser473. The blot was stripped and reprobed for total Akt and GAPDH. D, summary of the results of two experiments \( ± \) range. E, JNK activity in PC1-overexpressing and control MDCK cells. Cells were stimulated with and without thrombin for 30 min, lysed, and incubated with GST-c-Jun beads and 200 mM ATP, followed by Western blotting for phospho-c-Jun (P-c-Jun). F, summary of the results of two experiments \( ± \) range. G, JNK activity by ELISA. PC1-overexpressing and control MDCK cells with and without thrombin (2 units/ml) were analyzed at 30 min using the CASE kit for JNK Thr\textsuperscript{183}/Tyr\textsuperscript{185} as described under “Experimental Procedures.” Results are the mean ± S.E. of two independent experiments with \( n = 3 \) under each condition. *, significance at \( p < 0.001 \). Bcl-2 expression was preserved in thrombin-stimulated PC1-overexpressing MDCK cells. H, Western blots for Bcl-2, Bcl-x\textsubscript{L}, NF-\kappaB, and GAPDH in thrombin-stimulated PC1-overexpressing and control (Cont) cells. I, quantification of the base line of Bcl-2 expression in PC1-overexpressing and control cells after normalization to GAPDH from six independent experiments using ImageJ. *, significance at \( p = 0.04 \).
PC1 Regulates $\alpha_{12}$

**FIGURE 3. Silencing PC1 in MDCK cells enhances $\alpha_{12}$-stimulated apoptosis.** A, apoptosis. PC1-silenced and control cells with and without thrombin stimulation for 24 h were analyzed by fluorescence-activated cell sorting as described under “Experimental Procedures.” The percentages of apoptotic cells were plotted using GraphPad Prism. Results are the mean ± S.E. of two independent experiments with $n = 3$ under each condition. The increase in apoptosis of control cells was significant at $p = 0.03$ (*). The difference in apoptosis with thrombin stimulation (#) was significant at $p = 0.04$. B, JNK activity in PC1-silenced (PC1 Sil) and control MDCK cells. Cells were stimulated with and without thrombin for 30 min, lysed, and incubated with GST-c-Jun beads and 200 mM ATP, followed by Western blotting for phospho-c-Jun (p-c-Jun). The relative amounts of phospho-c-Jun from two independent experiments are shown in the bar graph. C, JNK activity by ELISA. PC1-silenced and control MDCK cells with and without thrombin (2 units/ml) were analyzed at 30 min using the CASE kit for JNK Thr$^{183}$/Tyr$^{185}$ as described under “Experimental Procedures.” Results are the mean ± S.E. of two independent experiments with $n = 3$ under each condition. *, significance at $p = 0.02$ for thrombin-stimulated JNK activity. D, Western blot of Bcl-2 in PC1-silenced and control (Con) cells with and without thrombin stimulation for 24 h. The bar graph summarizes the relative expression of two independent experiments ± range.

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...actively $\alpha_{12}$ ($\alpha_{12}$) to the PC1 C terminus (13). To examine whether the PC1 C terminus can distinguish between the two family members $\alpha_{12}$ and $\alpha_{12}$ (67% identical), we used Tet-Off inducible MDCK cells expressing $\alpha_{12}$ and $\alpha_{12}$, that have been characterized previously (17, 24, 25). Endogenous $\alpha_{12}$ is below the detection limit in most cell types, whereas $\alpha_{12}$ is readily detectable. Fig. 4A (Lysate row) shows the induced levels of $\alpha_{12}$ and $\alpha_{12}$ expression in these cells 48 h after the removal of doxycycline. Lysates were incubated with GST-PC1 C terminus, and the interaction was examined by Western blotting for $\alpha_{12}$. As we reported previously (13), both $\alpha_{12}$ and $\alpha_{12}$ interacted similarly with PC1. The blot was stripped and reprobed for $\alpha_{12}$ and $\alpha_{12}$, which is readily detectable in the lysates from both MDCK cell lines. However, we could detect no interaction of $\alpha_{12}$ with the PC1 C terminus in the pulldown lanes. To confirm this observation, we expressed [35S]methionine-labeled $\alpha_{12}$ and $\alpha_{12}$ by in vitro translation in rabbit reticulocyte lysate and tested for binding to PC1. We have shown previously that $\alpha$ subunits expressed in this system are folded correctly (26). Fig. 4B confirms the interaction of [35S]methionine-labeled $\alpha_{12}$, but not $\alpha_{12}$, with the PC1 C terminus. Taken together, these findings suggest a direct interaction of $\alpha_{12}$ with PC1, and although PC1 binds multiple $\alpha$ subunits, it discriminates between the two family members $\alpha_{12}$ and $\alpha_{12}$.

The observation that both wild-type $\alpha_{12}$ and GTPase-deficient $\alpha_{12}$ (activated $\alpha_{12}$, $\alpha_{12}$) interact similarly with the PC1 C terminus (Fig. 4A) (13) suggests that the binding of $\alpha_{12}$ to the PC1 C terminus is constitutive. However, classical GPCRs show regulated interactions with G proteins. To determine whether there is regulated binding of $\alpha_{12}$ to PC1, we examined the interaction of wild-type $\alpha_{12}$ after thrombin stimulation of MDCK cells with GST-PC1 C terminus in pulldown assays. 48 h after the removal of doxycycline, Tet-inducible $\alpha_{12}$-MDCK cells were stimulated with thrombin for 2 or 10 min, rapidly lysed, and incubated with GST-PC1 C-terminal fusion protein. Fig. 4C shows that, compared with non-silenced cells, there was increased binding of $\alpha_{12}$ to the PC1 C terminus with thrombin stimulation. The upper panel shows a Western blot indicating the amount of $\alpha_{12}$ precipitated with the PC1 C terminus (pulldown). There was no detectable $\alpha_{12}$ binding to the GST control, and as shown in Fig. 4A, $\alpha_{12}$ from non-stimulated cells (time = 0) interacted with GST-PC1 C terminus. With thrombin stimulation for 2 min, there was a significant increase (~2-fold) in the fraction of $\alpha_{12}$ binding to the PC1 C terminus. Reprobing the blot for $\alpha_{12}$ failed to detect any interaction (data not shown). The results of three separate experiments are quantified in Fig. 4D. The amount of $\alpha_{12}$ in the lysate for each experimental condition is shown beneath the pulldown, and the amount of GST fusion protein used for each condition is shown in the Ponceau-stained nitrocellulose membrane (Fig. 4C). This finding suggests that the interaction of $\alpha_{12}$ with PC1 can be regulated. To confirm that $\alpha_{12}$-stimulated apoptosis is directly regulated by its interaction with PC1, we examined apoptosis with and without thrombin stimulation of HEK293 cells transiently transfected with $\alpha_{12}$ and previously characterized PC1 C-terminal deletion mutations (kindly provided by Thomas Weimbs) (27). Thrombin stimulation of $\alpha_{12}$-transfected HEK293 cells led to a significant increase in apoptosis as pre-
dicted (Fig. 5). When $\alpha_{12}$ was cotransfected with the full-length PC1 C terminus (FLCT, amino acids 4077–4302) expressed as a CD16-CD7-PC1 C-terminal fusion protein, there was complete inhibition of thrombin-stimulated apoptosis. This is consistent with the results in the MDCK cells with overexpressed PC1 (Fig. 2). We next examined two PC1 C-terminal deletion mutations, CTM and NTM (Fig. 5). NTM contains PC1 C-terminal amino acids 4077–4168 and contains the 20-amino acid G protein activation peptide (amino acids 4134–4153) and most of the previously identified G protein-binding site (11). NTM effectively inhibited thrombin-stimulated apoptosis, similar to the findings with FLCT. In contrast, CTM (amino acids 4191–4302) is missing the entire G protein-binding sequence (amino acids 4110–4183), and when CTM was cotransfected with $\alpha_{12}$, thrombin-stimulated apoptosis was indistinguishable from that with $\alpha_{12}$ alone. These findings indicate that PC1 inhibition of $\alpha_{12}$-stimulated apoptosis requires the G protein-binding domain of PC1.

DISCUSSION

The findings reported in this work are important in two distinct areas. First, to our knowledge, this is the only study to examine a single G protein-coupled pathway in the context of PC1 expression levels, and second, this study reveals new insights into the potential regulation of apoptosis in ADPKD, a poorly understood component of the disease. Our results are consistent with a mechanism in which the steady-state PC1 protein expression levels regulate the activation state of intracellular signaling pathways. The inhibition of $\alpha_{12}$/JNK/Bcl-2-mediated apoptosis in PC1-overexpressing cells is consistent
with a dominant-negative function for PC1, as is our finding that lowering PC1 protein levels removes this inhibition and facilitates enhanced signaling through this apoptosis pathway. Because ADPKD is characterized by both loss and gain of PC1 protein at various stages of disease and within different nephron regions, both scenarios are biologically plausible and important to understanding the natural history of ADPKD.

ADPKD is characterized by both overexpression and loss of PC1 depending upon the stage of disease and the cystic region studied. Animal models also reveal cystic disease when PC1 is overexpressed (5) or deleted (reviewed in Ref. 1). Differences in PC1 protein levels, Bcl-2 expression, and apoptosis in ADPKD have been identified in numerous studies. However, the role of apoptosis in ADPKD remains poorly understood in part because the long time course of disease progression and the focal nature of cyst development have made in vivo analysis very difficult. We identified higher base-line Bcl-2 levels in PC1-overexpressing cells (Fig. 2), and this is similar to earlier observations in ADPKD kidneys (2). Regulating Bcl-2 expression and apoptosis in epithelia is an important component of cystogenesis. Bcl-2 knock-out mice develop renal failure from severe PKD characterized by dilated proximal and distal tubular segments (31). When Bcl-2 was overexpressed in MDCK cells, there was resistance to apoptosis that prevented cyst cavitation, and these cells spontaneously developed branching structures (32). The cell culture studies reported here are consistent with a variable effect on epithelial apoptosis that depends on the level of PC1 expression. This may be an important component of cyst development at various stages of ADPKD progression.

PC1 is a large protein with numerous cell functions and signals as an atypical GPCR (reviewed in Ref. 1). The cytoplasmic tail of PC1 binds numerous signaling molecules and has been implicated in several signaling pathways. Multiple Go subunits interact with PC1, and to date, there has been little selectivity identified. Early studies with purified Go subunits showed binding of PC1 to Goαq and Goαi (11), and we identified interactions with Goαq, Goα12/13, and Goα12 (13). Additional studies showed that PC1 regulates JNK activity in a Goα12- and Gβγ-dependent manner and that PC1 regulates AP1 transcription factor activity by Goαi11, Goα12, Goα13, Goαq, Goα12, and Goα13 when transiently expressed in HEK293 cells (12).GPCRs can couple to multiple Go subunits in the same cell (33), but specificity in signaling is achieved through multiple mechanisms, including localization, relative affinity, and association with distinct Gβγ subunits (34). How specificity in PC1-regulated G protein signaling is achieved is not known. We confirmed the earlier observation that Goα12 bound to the PC1 C terminus in pull-down assays in Goα12-expressing cells. However, we were surprised to find that Goα13 did not interact with the PC1 C terminus, and this was confirmed using a different technique (in vitro translation) (Fig. 4). There are several potential explanations for the lack of Goα13 binding seen in our studies. One possibility is that, unlike Goα12, Goα13 interacts with PC1 only in the activated conformation. However, we did not detect binding of endogenous Goα13 to the PC1 C terminus in thrombin-stimulated MDCK cells (Fig. 4C). Another potential explanation could be the differences in conformation of the C terminus in the full-length protein versus the GST fusion protein. Finally, the in vitro design of our approach could lead to the loss of an important adaptor or linker protein necessary for the interaction of Goα13. Bradykinin had no effect on apoptosis in either the control or PC1-overexpressing cells (Fig. 2B), and this is consistent with the lack of a role for PC1 and Goα13 in apoptosis. However, these findings are in contrast to those reported by Parnell et al. (12), who showed that both Goα12 and Goα13 stimulated AP1 activity in a PC1-dependent manner. These studies were done in transiently transfected HEK293 cells, and Goα12 was more effective than Goα13 in stimulating AP1 activity. The different experimental conditions and readouts are likely to account for these different conclusions, and perhaps when Goα13 is expressed at high enough levels, it can bind PC1 and regulate downstream signaling. Additional studies will be necessary to define PC1/Goα13 signaling.

The finding that thrombin-activated Goα12 preferentially bound to the PC1 C terminus (Fig. 4) is consistent with regulated binding of G proteins to PC1 in vivo and would be expected with PC1 functioning as a GPCR. However, this finding does not distinguish between Goα12 interacting with PC1 in the GTP- or GDP-liganded conformation. When expressed in MDCK cells, wild-type Goα12 and the constitutively active QLα12 mutant interact with the PC1 C terminus with similar relative affinities. This lack of activation dependence for binding Goα12 could indicate a scaffolding function for the PC1 C terminus, permitting the interaction of several signaling molecules that remain tethered to the complex even after activation. However, the lack of known PC1 ligands and difficulties in expressing the full-length protein make direct examination of these questions difficult at this time. It is also possible that G protein activation through canonical GPCRs regulates PC1 function(s). There are numerous examples of G proteins regulating transmembrane proteins, especially ion channels (reviewed in Ref. 35), and PC1 was recently shown to function as an adhesion molecule (15). We recently identified Goα12 regulation of integrins through an inside-out signaling mechanism (23). Therefore, it is possible that PC1-regulated adhesive properties are regulated through an analogous mechanism in which a traditional GPCR (such as PAR1) activates Goα12, leading to a change in PC1 function.

In summary, we have demonstrated that levels of PC1 expression directly affect the activation of Goα12/JNK-stimulated apoptosis pathways. The PC1/Goα12 interaction is necessary for the regulation of Goα12-stimulated apoptosis. These findings are consistent with a model in which PC1 protein levels titrate the Goα12 available for signaling. We speculate that PC1 expression levels regulate multiple signaling pathways through this titration mechanism. Identifying the signaling pathways regulated through this mechanism will lead to a new understanding of PC1 function that will permit the development of novel treatment strategies for ADPKD.

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