A mutation affecting polycystin-1 mediated heterotrimeric G-protein signaling causes PKD

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the growth of renal cysts that ultimately destroy kidney function. Mutations in the PKD1 and PKD2 genes cause ADPKD. Their protein products, polycystin-1 (PC1) and polycystin-2 (PC2) have been proposed to form a calcium-permeable receptor-channel complex; however the mechanisms by which they function are almost completely unknown. Most mutations in PKD1 are truncating loss-of-function mutations or affect protein biogenesis, trafficking or stability and reveal very little about the intrinsic biochemical properties or cellular functions of PC1. An ADPKD patient mutation (L4132D or DL), resulting in a single amino acid deletion in a putative G-protein binding region of the PC1 C-terminal cytosolic tail, was found to significantly decrease PC1-stimulated, G-protein-dependent signaling in transient transfection assays. Pkd1DL/DLmice were embryo-lethal suggesting that DL is a functionally null mutation. Kidney-specific Pkd1DL/Cond mice were born but developed severe, postnatal cystic disease. PC1DL protein expression levels and maturation were comparable to those of wild type PC1, and PC1DL protein showed cell surface localization. Expression of PC1DL and PC2 complexes in transfected CHO cells failed to support PC2 channel activity, suggesting that the role of PC1 is to activate G-protein signaling to regulate the PC1/PC2 calcium channel.

Introduction

ADPKD cyst growth leads to massive kidney enlargement and ultimately to renal failure. Mutations in the PKD1 gene cause ~85% of ADPKD cases and those in the PKD2 gene ~15% of ADPKD cases (1–3). PKD1 and PKD2 encode PC1 and PC2, respectively.
Significance Statement

ADPKD is caused by mutations in either of two genes, PKD1 or PKD2, whose protein products, polycystin-1 (PC1) and polycystin-2 (PC2), are proposed to form a calcium-permeable receptor-channel complex. A single leucine deletion (ΔL) in the cytosolic C-tail of PC1 was found to cause severe ADPKD in a knock-in mouse model. Most mutations in PKD1 affect PC1 protein levels, biogenesis, trafficking, stability or interactions with PC2 and reveal little about the biochemical function of PC1. In contrast, ΔL appears to specifically impair PC1-dependent heterotrimeric G-protein signaling and activation of the PC1/PC2 receptor-channel complex, suggesting that the function of PC1 is to regulate PC2 through heterotrimeric G-proteins.

Results

PKD1 patient mutations within the G-protein binding region affect PC1 signaling

We had previously demonstrated that the C-terminal tail of PC1 binds and activates heterotrimeric G-proteins (16), and that PC1-mediated activation of JNK and AP-1 (12) is mediated by both Go and Gβγ subunits (14). We also identified a polybasic G-protein activation domain (Fig. 1) that promotes nucleotide exchange, located within a highly conserved region of the PC1 C-tail required for stable binding of G-proteins (16). To determine whether mutations within this region affect PC1-mediated G-protein-dependent signaling, we conducted a literature and database search to identify ADPKD patient mutations for further study. Our analysis identified a cluster of in-frame deletion or missense variants within

![Figure 1. PC1 structure and C-tail sequence. The G-protein activation domain (G-activation, black cylinder) is a peptide region capable of activating nucleotide exchange of heterotrimeric G-proteins (Gβγ). The site of ADPKD-associated human mutation L4132Δ (ΔL) is indicated by an arrow in the C-tail cytosolic domain. The coiled-coil (coiled-coil, hatched cylinder) mediates interactions with PC2. PC1 is cleaved at a G-protein coupled receptor proteolytic site (GPS cleavage, star) to produce N- and C-terminal fragments (NTF, CTF; respectively).](image-url)
the G-protein binding domain in human PC1 (Supplementary Material, Fig. S1). To screen for function, corresponding mutations were introduced into a mouse PC1 C-tail fusion construct (slg-PKD222), and their effect on AP-1 activation was determined in transient transfection assays in HEK293T cells (14).

AP-1 activity was significantly stimulated by the WT slg-PKD222 construct but was reduced in cells expressing slg-PKD222 with patient mutations corresponding to L4132A, R4136T, R4136G (24–26) and engineered mutation R4135L (Fig. 2). An alanine substitution (L4132A) at the same site as L4132Δ did not have an effect on AP-1 activity (Supplementary Material, Fig. S2). These results show that mutations L4132A, R4135L, R4136T and R4136G interfere with AP-1 activation by the PC1 C-tail.

Previous work had shown that co-transfection of Gα subunits augmented AP-1 activation by WT slg-PKD222 (14,15). To determine if the patient mutations would have an effect on Gα augmentation of AP-1 activation, HEK293T cells were co-transfected with Gα12 or Gαq and WT or mutant slg-PKD222 expression constructs. As previously observed, PC1-dependent AP-1 activity was augmented in cells co-expressing WT slg-PKD222 and Gα12 (Fig. 2). Gα12 augmented activity was reduced in cells expressing R4136T or R4136G. Activity was further reduced in cells expressing the R4135L mutation, and was eliminated in cells expressing the L4132Δ mutant. Similar results were observed with co-expression of Gαq (Supplementary Material, Fig. S3A). Despite these pronounced effects on AP-1 activation, the mutant fusion proteins were able to interact and co-immunoprecipitate (IP) with co-transfected PC2 (Supplementary Material, Fig. S3B), consistent with these mutations not disrupting the coiled-coil region of PC1. These results suggest that the mutations specifically impair the ability of PC1 to activate heterotrimeric G-protein signaling. Of all the mutations studied, L4132A had the strongest effect on PC1-dependent AP-1 activation and was selected for further analysis.

**Generation of Pkd1<sup>NEO</sup>Δ/Δ and Pkd1<sup>Δ/Δ</sup> mice**

The human mutation L4132A (ΔL) was identified in an ADPKD family. The mutation was found in all affected family members, but not the unaffected parent, suggesting that L4132A was the causative mutation in that family (24). However, it could not be certain if this mutation was pathogenic, as the 5’ coding region of the Pkd1 gene was not examined for mutations in that analysis.

To determine whether the corresponding mouse ΔL mutation (L4122A) is sufficient to cause PKD, a targeting vector was constructed to introduce this mutation into mice using standard ES cell technology. Germline transmission of the Pkd1<sup>NEO</sup>Δ/Δ allele was observed in six founders, which were normal and viable. Progeny integration of the targeting construct was verified by Southern blotting (Supplementary Material, Fig. S4).

To generate the Pkd1<sup>Δ/Δ</sup> allele, heterozygous Pkd1<sup>NEO</sup>Δ/+/ mice were crossed to a Cre-deleter strain (Ella-Cre) to excise the floxed NEO cassette leaving a single, remnant loxp site within intron 45 (27). Excision was verified by genotyping using PCR primers that span the excised region and by sequence analysis (Supplementary Material, Fig. S4).

**Characterization of Pkd1<sup>NEO</sup>Δ/Δ and Pkd1<sup>Δ/Δ</sup> transcripts**

We reasoned that the presence of the floxed NEO cassette within intron 45 would interfere with proper splicing of the Pkd1<sup>NEO</sup>Δ/Δ transcript, effectively generating a null allele, and that Cre-mediated excision would restore splicing and proper transcription of Pkd1<sup>Δ/Δ</sup> message. To test this, RNA was isolated from mouse embryos and used as templates for RT-PCR using primers designed to selectively amplify Pkd1<sup>Δ</sup>, Pkd1<sup>NEO</sup>Δ/Δ and Pkd1<sup>Δ/Δ</sup> transcripts. The results indicated that the Pkd1<sup>NEO</sup>Δ/Δ allele is most likely non-functional due to lack of intron 45 splicing. In contrast, the Pkd1<sup>Δ/Δ</sup> allele is properly spliced. As such, phenotypes arising from this allele are likely to be specific to the ΔL deletion (Supplementary Material, Fig. S5).

**Characterization of Pkd1<sup>NEO</sup>Δ/Δ and Pkd1<sup>Δ/Δ</sup> mice**

Pkd1<sup>NEO</sup>Δ/+/ mice were crossed with mice heterozygous for the Pkd1<sup>Δ/Δ</sup> allele, which is essentially null (28), to determine...
whether the Pkd1<sup>NEO AL</sup> allele behaves as a null. Of eight live births, zero animals were genotyped as Pkd1<sup>NEO AL/m1Bei</sup>. We next cultured E15.5 embryonic kidneys in the presence of 8-Br-cAMP. Pkd1<sup>NEO AL/m1Bei</sup> kidneys developed cyst-like dilations (Supplementary Material, Fig. S6) as previously observed for Pkd1<sup>m1Bei/m1Bei</sup> kidneys (29). These dilations arise from CFTR- and NKCC1-mediated chloride-dependent fluid secretion, indicative of an underlying tubule disorder consistent with a lack of PC1 function. These results indicated that the Pkd1<sup>NEO AL</sup> allele is non-functional.

To determine to what extent the Pkd1<sup>LK</sup> mutation affects PC1 function, Pkd1<sup>LK/+</sup> mice were crossed to generate Pkd1<sup>LK/AL</sup> mice. Of 100 live births, zero animals were genotyped as Pkd1<sup>LK/AL</sup>, consistent with a severe, embryo lethal phenotype. E15.5 Pkd1<sup>LK/AL</sup> embryos were edematous (Fig. 3A). To verify the presence of cysts in vivo, embryonic kidneys were isolated at E15.5 and analyzed microscopically. Early cyst formation, including glomerular cysts, was observed in these kidney sections (Fig. 3B). Kidneys isolated from E15.5 pups and maintained in organ culture in the presence of 8-Br-cAMP developed large cyst-like dilations consistent with a loss of PC1 function (Fig. 3C).

To assess the effect of the AL allele on the growth and development of polycystic kidneys, and to circumvent the embryonic lethality, Hoxb7-Cre:Pkd1<sup>L/cond</sup> mice were generated (30,31). Hoxb7-Cre:Pkd1<sup>L/cond</sup> mice were born and had enlarged kidneys and elevated kidney weight to body weight ratios by P9 (Fig. 3D). Kidneys became even more enlarged by P14, with cyst formation throughout the entire kidney (Fig. 3E), comparable to cystic kidneys obtained from Hoxb7-Cre:Pkd1<sup>L/cond</sup> mice (32). These results strongly suggest that the corresponding L4132A patient mutation is pathogenic.

**Figure 3.** Analysis of Pkd1<sup>L1K22A (LK)</sup> kidneys. (A) E15.5 heterozygous embryos (LK/+; left) are normal. Homozygous embryos (LK/LK, right) are frequently inviable or exhibit edema (white arrow). (B) H&E-stained AL/AL kidneys revealed early cyst formation (arrows). (C) AL/AL kidneys developed severe cystic dilations following 4-day culturing in the presence of cAMP. Dilations of proximal tubular origin (broad arrows) as well as other nephron segments (narrow arrows) were detected by brief incubation with fluorescein, which is taken up by the proximal tubule. (D) Hoxb7-Cre Pkd1<sup>LK/cond</sup> pups exhibited massively enlarged kidneys at P9 and P14. E) H&E-stained kidneys from P14 Hoxb7-Cre Pkd1<sup>LK/cond</sup> pups revealed cyst formation throughout the entire kidney, as compared to a WT kidney (+/+).

### Analysis of mutant PC1 protein

To determine how the AL deletion disrupts PC1 function, we asked whether the mutation affected PC1 expression or cleavage. Full-length WT, AL-mutated or coiled-coil truncating S4213X human PC1 proteins were expressed in HEK293T cells. Western blot analysis confirmed that the AL mutation did not affect PC1 protein expression levels, electrophoretic mobility or the ability of PC1 to undergo N- and C-terminal cleavage events (Fig. 4A). Full-length AL-mutated PC1 retained the ability to interact with PC2 in HEK293T cells in co-IP experiments (Fig. 4B), suggesting that the AL mutation does not disrupt the ability of PC1 to form complexes with PC2.

To determine whether the AL mutation affects cleavage or maturation of endogenous PC1 we examined membrane preparations from mouse embryonic fibroblasts (MEFs) isolated from E14.5 embryos. The preparations were treated with endoglycosidase H (EndoH; cleaves basic N-glycosides on immature proteins in the ER but is inactive toward glycodies on mature proteins processed in the Golgi) or peptide-N-glycosidase F (PNGaseF; cleaves oligosaccharides from both mature and immature proteins). Full-length protein, cleaved NTF and cleaved CTF were detected in Pkd1<sup>LK/AL</sup> MEF membrane preparations, and all glycoforms responded to EndoH and PNGaseF treatment in a manner identical to that of WT PC1 from Pkd1<sup>L+/+</sup> MEF membranes (Fig. 5A and B). In a similar analysis, we observed that the product of the Pkd1<sup>NEO AL</sup> allele was smaller than full-length PC1, and was cleaved to produce a single immature NTF glycoform that was entirely EndoH-sensitive (Fig. 5C). Given that the Pkd1<sup>NEO AL</sup> mRNA fails to splice to exon 46 (Supplementary Material, Fig. S5B), the protein product most likely behaves like a C-terminal truncation.
mutant. These truncation mutants are known to undergo GPS cleavage but fail to mature beyond the ER due to an inability to interact with PC2, which is required for full maturation of PC1 (33,34). This is in marked contrast to the mature, full-length ΔL protein.

To determine whether the ΔL mutation affects PC1 protein levels, we quantified the amount of the PC1 glycoforms (full-length, mature and immature cleaved NTF) in membranes prepared from three independent WT and Pkd1ΔL/ΔL MEF cultures. The ΔL mutation did not affect the ratio of mature to immature NTF or the ratio of full-length PC1 relative to a membrane marker protein, ALIX, and there was only a small non-significant reduction in the amount of mature NTF relative to ALIX (Fig. 5C, and see discussion).

To determine whether the ΔL mutation affects PC1 intracellular membrane localization we analyzed protein from E14.5 whole embryo membrane preparations following fractionation over a heavy water/5–30% sucrose gradient (35) to separate membrane compartments. Immunoblot analysis of the total membrane preparations revealed comparable levels of full-length, and mature and immature NTF glycoforms of PC1 from WT and Pkd1ΔL/ΔL embryos (Fig. 6 and Supplementary Material, Fig. S7A). As observed with MEF cultures, mature NTF could not be detected in the total membrane preparation from Pkd1NEOΔL/NEOΔL embryos. Following centrifugation, gradient fractions were collected and analyzed by immunoblot. Full-length protein, mature NTF and immature NTF from both WT and PC1ΔL protein had the same fractionation pattern across the entire spectrum of the gradient (Fig. 6 and Supplementary Material, Fig. S7A). In contrast, PC1NEOΔL protein had a qualitatively different fractionation pattern marked by decreased levels of mature NTF in peak fractions, increased levels of

Figure 4. Expression of human PC1ΔL and its interaction with PC2. (A) Full-length (FL) 3xHA-tagged (C-terminal) human PC1 proteins were expressed in HEK293T cells and detected with anti-HA (detects FL PC1, cleavage products CTF and p100, but not NTF) and N-terminal PC1 antibody 7E12 (detects FL and cleaved NTF, but not CTF and p100). PC1S4213X is truncated just upstream of the coiled-coil domain (but also contains a C-terminal 3xHA tag), leading to faster migration of its CTF and p100 products. Dashed arrows indicate crop lines where separate portions of the same image are spliced together. (B) 3xHA-tagged PC1 and myc-tagged PC2 were expressed in HEK293T cells. Tagged PC1 was immunoprecipitated (IP’d) with anti-HA antibody and proteins were detected by IB with 7E12 for PC1 and anti-myc for PC2. The blot shows that PC1ΔL is as capable as WT PC1 to interact with and IP PC2.
immature NTF, and the persistent presence of immature NTF toward the bottom of the gradient. These results suggest that the expression and membrane localization of PC1\textsuperscript{DL} is fundamentally identical to that of WT PC1. We also analyzed E14.5 embryonic kidneys for PC1 protein by immunoblotting. Full-length and mature NTF were easily detected in membrane preparations (Fig. 6 and Supplementary Material, Fig. S7A), but not in whole-cell kidney lysates where immature NTF was the only PC1 glycoform detected (Supplementary Material, Fig. S7B). To confirm that PC1\textsuperscript{DL} protein was able to localize to the plasma membrane/cell surface, porcine LLC-PK1 cells were electroporated with constructs encoding GFP-tagged PC2 and either N-terminal mCherry-tagged WT PC1 or PC1\textsuperscript{DL} proteins (Fig. 7A) (33), and protein expression was confirmed in whole-cell lysates by immunoblot (Supplementary Material, Fig. S8A and B). PC1 was detected by immunofluorescence using antibody directed against the mCherry epitope tag under both non-permeabilized and permeabilized conditions (33). Under non-permeabilized...
conditions, both WT and ΔL-mutated PC1 could be detected across the entire plasma membrane (Fig. 7B). Incubation without primary antibody to mCherry showed no PC1 detection (Supplementary Material, Fig. S8C). Under permeabilized conditions, both WT and ΔL-mutated PC1 were readily detected throughout the cell, with reduced immunofluorescence in the nuclear region (Fig. 7B). To determine whether PC1ΔL trafficking to the cell surface (relative to WT PC1), GFP-positive cells in 5–10 random fields (non-permeabilized) were counted, followed by counting of mCherry-positive cells in the same fields. The percentage of all counted cells positive for GFP alone, mCherry alone or both GFP and mCherry is shown in the pie charts. Comparable transfection efficiency of the WT and ΔL constructs was confirmed by counting the number of cells positive for GFP, mCherry and GFP/mCherry signal as detected using permeabilized conditions (data not shown).

Discussion

The majority of PKD1 mutations analyzed has been shown to affect PC1 protein biogenesis, trafficking or stability and have revealed little about the intrinsic biochemical or cellular functions of PC1. In theory, analysis of missense Pkd1 alleles has the potential to identify critical signaling domains and functional properties of PC1. We previously identified a highly conserved region within the C-terminal, cytosolic tail of PC1 that constitutes a minimal binding domain for heterotrimeric G-proteins

Figure 7. Surface expression of PC1ΔL in porcine kidney epithelial cells. (A) LLC-PK1 cells were electroporated with constructs encoding GFPPC2 and mCherryPC1 (WT or ΔL), and analyzed by immunofluorescence. PC2 was detected using the intrinsic GFP fluorescence, and PC1 was detected using mCherry (mCh) antibodies. (B) Non-permeabilized conditions were used to detect surface PC1 expression, and permeabilized conditions were used to detect internal PC1 expression. (C) To determine the efficiency of PC1ΔL trafficking to the cell surface (relative to WT PC1), GFP-positive cells in 5–10 random fields (non-permeabilized) were counted, followed by counting of mCherry-positive cells in the same fields. The percentage of all counted cells positive for GFP alone, mCherry alone or both GFP and mCherry is shown in the pie charts. Comparable transfection efficiency of the WT and ΔL constructs was confirmed by counting the number of cells positive for GFP, mCherry and GFP/mCherry signal as detected using permeabilized conditions (data not shown).
and contains a 20 aa peptide sequence capable of activating heterotrimeric G-protein nucleotide exchange (16). To determine whether disruption of PC1-mediated activation of heterotrimeric G-protein signaling might be relevant to PKD, we analyzed ADPKD-associated missense mutations that lie in and around the G-protein activation domain using AP-1 activation as a surrogate for PC1-mediated heterotrimeric G-protein activity (14).

Human substitution mutations (R4135L, R4136T and R4136G) that removed the positive charge from the first two basic residues of the previously identified, polybasic G-protein activation peptide reduced PC1-mediated basal and Ga-augmented AP-1 activation without affecting the ability of PC1 to co-IP with PC2. Similar results were obtained for the single aa deletion L4132A (AL). Secondary structure algorithms (NPS@ and PORTER) (36,37) predict that L4132 lies within an amphipatic helix contiguous with and including the G-protein activation peptide. As such, deletion of L4132 would likely offset the distribution of multiple side-chain residues that could be important for PC1-mediated activation of heterotrimeric G-proteins (Supplementary Material, Fig. S9). An L4132A substitution at the same location, which we predicted would not alter the overall side-chain charge distribution, had no effect on PC1-mediated AP-1 activity.

We also noted the frequency of ADPKD-associated mutations affecting the G-protein activation domain (Supplementary Material, Fig. S10). While it may appear that there are no strong mutational hotspots when all loss-of-function mutations throughout the PKD1 gene are considered (38), our analysis indicates that within the C-tail region there is a clustering of patient mutations within and around the conserved G-protein activation domain. Of the mutations studied here, human mutation I4132A (24) had the largest impact in an AP-1 transcriptional assay, blocking both basal and Ga-augmented activity. We selected this mutation for further analysis to determine pathogenicity and to gain insight into the importance and relevance of PC1-mediated G-protein signaling to PKD.

In all aspects tested, the Pkd1<sup>AL</sup> allele behaves as a null allele. Pkd1<sup>AL/AL</sup> mice are embryo lethal, and Hoxb7-Cre:Pkd1<sup>AL/m1Bei</sup> mice develop severely cystic kidneys within the first 2 weeks following birth. A striking feature of the Pkd1<sup>AL</sup> allele is that the Pkd1<sup>AL</sup> protein shows normal expression, cleavage and maturation. In this regard, it is helpful to compare the properties of the Pkd1<sup>AL</sup> allele to that of other missense Pkd1 alleles such as Pkd1<sup>RC</sup>, Pkd1<sup>V</sup> and Pkd1<sup>m1Bei</sup>. Among these alleles, Pkd1<sup>RC</sup> has the least severe phenotype. Pkd1<sup>RC/RC</sup> mice have obvious renal defects by 3 months of age, but can survive up to a year of age (23). The Pkd1<sup>RC</sup> protein appears to be a temperature-sensitive folding mutant, with a ~65% reduction in PC1<sup>RC</sup> protein levels relative to WT PC1 (39). Pkd1<sup>V</sup> is more severe, with Pkd1<sup>V/V</sup> mice exhibiting cystic defects by P4 and dying by about 1 month of age. Full-length PC1<sup>V</sup> is expressed, but fails to undergo GPS cleavage to produce NTF and CTF (39). Pkd1<sup>m1Bei</sup> is the most severe, with in utero cyst formation and embryonic lethality in Pkd1<sup>m1Bei/m1Bei</sup> animals (28). Analysis of the PC1<sup>m1Bei</sup> protein revealed that it is expressed and undergoes GPS cleavage. However, the resulting cleavage products are sensitive to EndoH, indicating that it is unable to egress from the ER (34). As such, all three of these missense Pkd1 alleles have PKD phenotypes because of decreased levels of mature, cleaved protein.

While the disease severity of the Pkd1<sup>AL</sup> allele is comparable to that of Pkd1<sup>m1Bei</sup> and to Pkd1<sup>AL</sup> null alleles, our analysis of the Pkd1<sup>AL</sup> protein reveals that there is at most only a small and non-significant decrease in the overall level of mature NTF. While this small reduction in mature NTF could in principle influence cystogenesis, it cannot account for the lethal phenotype. In a comparable experiment (33), MEFs isolated from Pkd1<sup>+/−</sup> embryos, which do not develop cystic disease, exhibit a greater reduction (~50%) in mature NTF, establishing this as a threshold for sufficient PC1 function. MEFs isolated from Pkd1<sup>RC/RC</sup> embryos, which will develop only mild cystic disease as adults, exhibit a ~65% reduction in mature NTF. In contrast, MEFs derived from severely cystic (embryo lethal) Pkd1<sup>AL/AL</sup> embryos have mature NTF levels that are well above these threshold levels, indicating that the embryonic lethality caused by the AL mutation cannot be explained by a reduction in PC1<sup>AL</sup> expression relative to WT PC1.

Further evidence suggesting that PC1<sup>AL</sup> protein expression and localization are comparable to those of WT PC1 was obtained by analyzing native protein in whole embryo... 

Figure 8. The Pkd1<sup>AL</sup> mutation decreases PC1/PC2 channel activity. (A) La<sup>+</sup>-sensitive current density at –80 mV for voltage-clamped CHO cells expressing WT or mutant PC1 together with PC2 (*, P < 0.05, n = 10, 8 and 9, respectively from at least 4 independent transfections). (B) La<sup>+</sup>-sensitive current density at –80 mV for voltage-clamped CHO cells expressing WT or channel-defective (D511V) PC2 together with WT PC1 (*, P < 0.05, n = 5 and 7, respectively). (C) Representative current traces recorded from an individual cell overexpressing WT PC1/PKD before and after superfusion with 0.5 mM La<sup>3+</sup> and from an untransfected cell. (D) Current-voltage relationship in CHO cells expressing WT or AL mutant of PC1 and PC2 (E<sub>m</sub>, voltage; I-density of La<sup>+</sup>-sensitive current)
membrane preparations and in embryonic kidney lysates; and by analyzing proteins transiently expressed in porcine kidney epithelial cells. In all of these assays, the levels of PC1\textsuperscript{AL} expression, its fractionation profile, and its cellular localization were comparable to that of WT PC1. This was in marked contrast to the behavior of the protein produced from the Pkd1\textsuperscript{AL} precursor allele, Pkd1\textsuperscript{HISO}.\textsuperscript{AL}, which does not produce appreciable levels of mature NTF, and has a different membrane fractionation profile. As such, our results indicate that the AL mutation disrupts a critical biochemical function of PC1.

In the current study, we sought to determine whether the AL mutation would affect the channel activity of PC2. Several lines of evidence suggest that PC2 channel activity might be affected by alterations in the ability of PC1 to activate heterotrimeric G-protein signaling \cite{17,40}. CHO cells expressing full-length PC1 and PC2 exhibited significantly higher channel activity than cells expressing PC2 and a C-terminally truncated PC1\textsuperscript{AL} that cannot interact with PC2, or cells expressing PC1 and PC2 channel mutant, D511V \cite{(9)}, indicating that this channel activity requires the PC1/PC2 complex. Cells expressing full-length PC1\textsuperscript{AL} and PC2 had current levels comparable to that of channel-inactive PC1\textsuperscript{D511V/PC2} or PC1/PC2\textsuperscript{D511V} cells. We believe that in these experiments the loss of PC2 channel activity occurs from an inability of the PC1\textsuperscript{AL} mutant to initiate G-protein signaling.

A disruption in PC1-mediated heterotrimeric G-protein signaling could potentially alter PC2 channel activity by several different mechanisms. For instance, PC2 localization and channel activity are regulated by phosphorylation \cite{(7,41,43)}, IP\textsubscript{3}-induced Ca\textsuperscript{2+} release \cite{(44)}, and membrane lipid composition and intracellular Ca\textsuperscript{2+} \cite{(45-47)}. Each of these regulatory mechanisms involves second-messenger-dependent signaling that may be downstream of PC1-mediated G-protein signaling. PC2 could also be regulated through direct interactions with G-protein subunits activated by PC1. There are numerous examples of G-protein subunits directly regulating channel protein activity \cite{(48,49)}, a possibility that remains to be explored for PC2. Given these potential mechanisms, we speculate that the inability of the PC1\textsuperscript{AL} mutant to initiate G-protein signaling may result in an otherwise structurally normal PC1/PC2 complex that is functionally impaired.

Additional questions remain concerning the role of heterotrimeric G-protein signaling in PKD. Wu et al. \cite{9} demonstrated that in mice, Pkd1 knockout knockdown increased G\textsubscript{q}\textsubscript{16} activity, and genetic deletion of Gna12 blocks cystogenesis induced by deletion of Pkd1. In this model, PC1 is hypothesized to antagonize cystogenesis via its ability to sequester G\textsubscript{q} subunits in an inactive state \cite{(50)}. In a more recent study, Zhang et al. \cite{12} demonstrate a PKD phenotype in the Xenopus pronephric kidney following loss of the G-protein \alpha subunit Gnas. However, this phenotype is antagonized by pharmacological and genetic inhibition of G\textsubscript{q} signaling, suggesting that loss of Gnas leads to increased, cystogenic G\textsubscript{q} signaling \cite{(51)}. Both of these results are in contrast with our model of PC1 signaling, which predicts that loss of PC1 signaling results in cystogenesis due to decreased heterotrimeric G-protein signaling. At this point it is difficult to reconcile these seemingly disparate models of PC1 function, but a common theme between Wu et al., Zhang et al. and our present findings is the central importance of the PC1 G-protein binding domain to regulate heterotrimeric G-protein signaling \cite{(14,16)}.

In conclusion, our results have implications for treatment of PKD, as therapies designed to mimic or restore G-protein-mediated activation of PC2 may be sufficient to counteract cystogenesis in cases where PC1 is not expressed or incapable of activating G-proteins. Likewise, signaling mutants such as PC1\textsuperscript{AL} may be exquisitely responsive to small-molecule therapy designed to act specifically on inactive PC1/PC2 receptor-channel complexes. Future efforts should focus on revealing the molecular dynamics of the PC1/PC2 complex and the mechanisms by which PC1-activated G-protein subunits may regulate PC2 channel activity.

**Materials and Methods**

Detailed methods are available as supplementary material.

**Plasmids**

Plasmid slg-PKD\textsubscript{222} and slg-0 were described previously \cite{(14)}. Mutations were introduced as previously described \cite{(52)}. 7xAP-1 Firefly luciferase reporter and pBlueScript were from Stratagene. G\textsubscript{s} expression constructs were from the Missouri S&T cDNA Resource Center (www.cdna.org; date last accessed May 6, 2018). pRL-null was from Promega. PC2 expression plasmid pcDNA3 PKD2\textsuperscript{AL} was provided by Leonidas Tsiokas \cite{(11)}; pTag\textsuperscript{GFP}PC2 was described previously \cite{(33)}. The D511V mutation was introduced via PCR. Human PC1 expression plasmids were derived by subcloning PKD1 sequences from AF20 and AF20 3xHA (provided by the Baltimore Polycystic Kidney Disease Research and Clinical Core Center) into pcDNAsFRT/TO (Invitrogen) or mCherryPC1 \cite{(33)}. The Pkd1 targeting vector was derived from a fragment containing mouse Pkd1 exons 39–46 and a portion of the 3’ end of Tsc2 isolated from a λ phage FIX II (Stratagene) library clone provided by Marianna Rodova \cite{(27)}.

A floxed NEO cassette from pNTL \cite{(53)} was inserted into intron 45, and the AL mutation was introduced with a QuikChangeII Site-Directed Mutagenesis Kit (Stratagene). All cloning manipulations were verified by DNA sequencing.

**Cell culture, AP-1 assays, IP and Western blotting**

HEK293T (ATCC) cells were transfected as described previously \cite{(14,15)}. AP-1 activity was determined with the dual luciferase assay kit (Promega) using an EG\&G Berthold 9507 Luminometer. For co-IP following dual luciferase, slg proteins were precipitated with EZ-View Protein A beads (Sigma) and proteins detected as described previously \cite{(14,52)}. For co-IP of full-length PC1 and PC2, HA-tagged PC1 was precipitated with agarose-conjugated HA-probe (F-7; Santa Cruz Biotech).

**ES cell production**

The targeting vector was linearized and electroporated into 129/Ola parental cells by the University of Kansas Medical Center Transgenic and Gene-Targeting Institutional Facility. Neomycin resistant clones were screened by long-range PCR. Positive clones were expanded and karyotyped, and proper recombination was verified by Southern blotting.

**Southern blotting**

ES cell or mouse tail DNA was isolated with the Wizard Genomic DNA Purification Kit (Promega), digested with BglII, and electrophoresed and detected by Southern blotting. Probe DNA was generated by PCR amplification of WT ES cell DNA. The product was cloned into pGEM-T (Promega), and an ~800 bp
NcoI fragment was labeled using the AlkPhos DIRECT kit (GE Healthcare).

Mice
Super-ovulated C57BL/6 females (The Jackson Laboratory) were mated to C57BL/6 males. Blastocysts were injected with Pkd1-Neo-AL targeted ES cells and transferred to recipient CD-1 females (Charles River Laboratories). Chimeric mice were identified by agouti coloration and mated to C57BL/6 mice to obtain germline transmission. Agouti pups were genotyped by PCR and the presence of the Pkd1-Neo-AL allele was verified by Southern. Pkd1-AL mice were generated by crossing F1 Pkd1-AL heterozygotes to Elia-Cre mice. The rearranged Pkd1-AL allele was detected by PCR and verified by DNA sequencing. Hoxb7-Cre;Pkd1-AL/conf mice were acquired by crossing Pkd1-AL/+ and Hoxb7-Cre;Pkd1-AL/conf (30,31) mice. Phenotypic analysis was performed on mice of both sexes over a range of 2–4 backcrosses to C57BL/6. Total body weight and kidney weight were determined. Kidneys were fixed in formalin, paraffin embedded and stained with hematoxylin and eosin. All animal methods were approved by the University of Kansas Medical Center IACUC.

RNA isolation and RT-PCR
Kidney or brain tissue was homogenized in TRIzol (Invitrogen) and RNA was isolated per manufacturer’s instructions. RNA was reverse transcribed with GoScript (Promega) and PCR was performed with GoTaq (Promega). Restriction digest with EcoRI (New England Biolabs) was used to distinguish WT from Pkd1-Neo-AL or Pkd1-AL transcripts.

Organ culture
Metanephroi were dissected from embryonic mice and lysed in SDS-PAGE sample buffer or PC1 lysis buffer (Supplementary methods) for immunoblot analysis, or cultured ±100 μM 8-BrcAMP and analyzed as described previously (29).

Mouse embryonic fibroblasts (MEFs)
Genotyped embryo carcasses were minced and digested twice with trypsin. Cells were maintained in DMEM (Cellgro) containing additional L-glutamine (2 mM) and 100 000 units penicillin/100 mg streptomycin per liter supplemented with 10% heat-inactivated fetal calf serum. Genotypes were verified after the first passage.

Deglycosylation assays
Total cellular membranes were purified from hyper-confluent MEF cultures as previously described (33) with minor modifications. Membrane fractions were treated with EndoH or PNGaseF (Promega) according to manufacturer’s instructions with minor revisions.

Membrane preparation and sucrose gradient centrifugation
E14.5 embryos in lysis buffer were disrupted by polytron, Dounce homogenization and sonication. Membranes were clarified by gentle centrifugation, equilibrated and applied to a heavy water/5–30% sucrose gradient (35) and centrifuged at ~27 000g for 16 h in a Sorvall TH-641 rotor. Gradient fractions were collected from the top down using a BioComp fraction collector. The refractive index of each fraction was determined, and proteins were detected by immunoblotting.

Immunofluorescence
Porcine LLC-PK1 cells were electroporated (Nucleofector II; Amaza with Basic Nucleofector Kit for Primary Mammalian Epithelial cells; Lonza) with pTAG GFP-PC2 and mCherry-PC1 constructs, and surface and intracellular expression of WT and AL-mutated PC1 was detected by immunofluorescence using antibody directed against the mCherry epitope (33). Images were obtained with an Eclipse TE2000-U microscope (Nikon) using a 60× oil objective and Image-Pro Premier (MediaCybernetics) and Photoshop (Adobe) software. For quantification, images were taken of 5–10 non-overlapping fields (20× objective) that contained GFP-positive cells. The same fields were subsequently imaged for Texas Red, and the number of cells positive for GFP, Texas Red or both was determined. Quantification was from three independent transfection experiments.

Electrophysiology
CHO cells (ATCC) were transfected using Polyfect reagent (Qiagen) as described previously (40,54) with green fluorescent protein (pEGFP-F; Takara Clontech) and full-length PC1/PC2 expression constructs. Whole-cell macroscopic recordings of PC1/PC2 channel activity were made under voltage-clamp conditions on EGFP-positive cells 48–72 h after transfection as previously described (11,55) with an Axopatch 200B amplifier (Molecular Devices) interfaced via a Digidata 1440 (Molecular Devices) to a PC running the pClamp 10.3 software suite (Molecular Devices).

Secondary structure prediction analysis
Predictions were performed using NPS@ (Network Protein Sequence Analysis; https://npsa-prabi.ibcp.fr; date last accessed May 6, 2018) (36) and PORTER (37). Helical wheel projection analysis and hydrophobic moment calculations were performed at http://rrlab.ucr.edu/scripts/wheel/wheel.cgi; date last accessed May 6, 2018 (56).

Statistics
Data are means ± SE. Statistical significance was determined by one-way ANOVA and Student–Newman–Keuls (S–N–K) posttest for multiple comparisons.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
The authors thank Marianna Rodova, Terry Peterson, Joshua Anderson and Lance Brandenburgh for generation of plasmids, Lynn Magenheimer for assistance maintaining mouse colonies, Darren Wallace for assistance with the manuscript, Don Armstrong for assistance with helical wheel predictions, Vladimir Gainullin for advice on PC1 deglycosylation assays, and the Baltimore Polycystic Kidney Disease Research and
Clinical Core Center, P30 DK090868, for plasmids and E8 antibody.

Conflict of Interest statement. None declared.

Funding
The study was supported by National Institutes of Health NS069759 (M.L.H.), 1F31NS076237 (M.A.H.), R35 HL135749 (A.S.), K99/R00 HL116603 (T.S.P.), F50 DK057301 (J.P.C.), P30 DK106912 (J.P.C.), American Heart Association 16EIA26720006 (A.S.) and a University of Kansas Medical Center Jared Grantham Kidney Institute Pilot and Feasibility award (S.C.P.). Funding to pay the Open Access publication charges for this article was provided by The Jared Grantham Kidney Institute PKD Rodent Model and Drug-Testing Core.

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