Progressive development of polycystic kidney disease in the mouse model expressing Pkd1 extracellular domain

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by slow progression of multiple cysts in both kidneys that lead to renal insufficiency in mid-life or later. ADPKD is associated with mutations mainly in the PKD1 gene (encoding polycystin-1 or PC1) and less frequently in the PKD2 gene (encoding polycystin-2 or PC2). To mimic naturally occurring human PKD1 mutations and gain insight into the PC1 extracellular domain function, four transgenic mouse lines were established with exclusively the extracellular domain of the Pkd1 gene (Pkd1extra) under endogenous transcriptional regulation. Expression of the Pkd1extra transgene was 2- to 80-fold above endogenous levels. Strikingly, the Pc1extra protein was more abundant, proportionally to the endogenous levels. All four transgenic mouse lines consistently displayed progressive renal cystic phenotype. Consequently, these transgenic mice reproducibly developed renal functional alterations similar to human ADPKD with proteinuria, renal insufficiency, anemia and died of renal failure late in life. In precystic kidneys, the Pkd1extra transgene modulated Pc2 expression and thereby, uncovered a potential Pc1-mutant/Pc2 pathogenic crosstalk mechanism. Moreover, the pathophysiologic mechanism also implicates c-myc, a major modulator of cystogenesis. Altogether, the novel Pkd1extra mouse model is the first Pc1 extracellular mutant that reproduces human ADPKD clinical progression and physiopathology.

INTRODUCTION

Human autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases. ADPKD is characterized by the presence of many renal epithelial cysts in all segments of the nephron that affect both kidneys. The cystic dilatations of tubules and glomeruli in these kidneys ultimately result in the remodeling of the renal architecture and alter renal physiologic functions. Consequently, ADPKD patients develop renal insufficiency during the fifth to sixth decade of life that accounts for \~10% of all individuals requiring renal replacement therapy.

Mutations in the PKD1 or PKD2 genes are associated with ADPKD. The PKD1 gene, the most frequently mutated, is large and spans 54 kb consisting of 46 exons, generates a 14 kb transcript and encodes a 4302 amino acid protein called polycystin-1 (PC1) (1–4). In silico, PC1 analysis revealed that the amino terminal portion consists of multimodular domains described as: leucine-rich repeats flanked by cysteine-rich structures (LRR), an LDL-A domain, a C-type lectin domain, 16 PKD (Ig-like) tandem repeats, an REJ-like domain (receptor for egg jelly), a G protein-coupled receptor proteolytic site or GPCR-autoproteolysis inducing (GPS or GAIN) (1,3,5,6) just prior to the first transmembrane domain. These motifs are followed by 11 transmembrane domains. The first intracellular/cytoplasmic loop contains a so-called PLAT domain (for PC1, lipoxygenase and alpha toxin) (7–10), whereas the last intracellular loop contains a potential PEST sequence and at the C-terminal end, a coiled-coil domain comprised of five heptad repeats. Based on these predictions, the PC1 protein could act as a cell-surface receptor or participate in a large membrane protein complex that could be involved in cell–cell and/or cell–matrix interactions. However, the role of PC1 and in particular that of the extracellular domain remains elusive.

A wide spectrum of mutations for the PKD1 gene has been reported and most of them belong to a unique family.
Recently, we have produced transgenic mice with specific truncated allele and a wild-type allele developed PKD. Although ADPKD transmission is dominant, the pathogenic mechanism leading to the disease is still not clearly established. Few groups have proposed that a PKD1 gene-dosage or -imbalance-dependent mechanism, based on amorphic/loss-of-gene-function, hypomorphic and hypermorphic/gain-of-gene-function alleles, is responsible for PKD1 pathogenesis. Evidence for recessive inactivation of PKD1 was provided by somatic mutations, in addition to an inherited germ-line mutation, found in a subset of renal cysts (17–19). Hypomorphic alleles have also been identified in some ADPKD patients (20). Evidence also points to a gain-of-function mechanism underlying human ADPKD pathogenesis that is supported by overexpression of the normal PKD1 allele as well as by the majority of cysts staining positively for PC1 (15,21,22).

In mice, cystogenesis can result from various Pkd1 mutations. A loss-of-heterozygosity, hypomorphic allele has been shown to be associated with the formation of renal cysts (23–30). More recently, we have produced transgenic mice with specific renal and systemic Pkd1 overexpression that developed a cystic phenotype (31,32). Altogether, these results indicated that a renal imbalance in Pc1 levels could lead to PKD phenotype and suggest that a similar mechanism may prevail in human ADPKD.

To mimic a human ADPKD mutation in mice and gain insight into the role of PC1 extracellular domain in mice, we have generated a truncated form of the Pkd1 gene based on four described truncated ADPKD mutations (p130/E3020X, WS219, Y2991X, W3001X). These mutations are located between the extracellular region and the membrane junction (11,14), and PC1 lacks specifically both the membrane-spanning and intracellular domains. Using homologous recombination on our characterized Pkd1-BAC, we have engineered a truncated Pkd1 gene (Pkd1extra) that produces only the extracellular Pc1 domain (Pc1extra). Expression of this truncated Pkd1extra transgene caused a slow, progressive, cystic phenotype associated with renal insufficiency recapitulating the development of human ADPKD.

RESULTS

Production of Pkd1extra-BAC and transgenic mice

To investigate the pathogenic role and function of the Pc1 extracellular domain, we mimicked a truncated form of PC1 that was previously documented in ADPKD patients namely, WS219, p130/E3020X, Y2991X, W3001X (11,12,14,33). The Pkd1 gene was truncated to delete the protein region just prior to the first transmembrane and the membrane-spanning and intracellular domains (Fig. 1A), using homologous recombination on the Pkd1-BAC that we have isolated and characterized (31). The Pkd1-BAC was modified with a recombination vector that contained Pkd1 exon23–25 gene till nucleotide 31 129 corresponding to amino acid 3043 just prior to the first transmembrane region, followed by two point mutations to create a stop codon (F3043X) and adjoined directly to the non-coding sequences of Pkd1 exon 46, poly A and flanking sequences for appropriate transcript processing (Supplementary material, Fig. S1A). This vector served to produce the Pkd1extra-BAC that was then analyzed for integrity with four restriction enzymes and seven probes as described for wild-type Pkd1-BAC (31) (Supplementary material Fig. S1B). The Pkd1extra fragment (77 kb) was isolated from the Pkd1extra-BAC (Fig. 1A) that removed BAC vector sequences, Tsc2 regulatory elements and 5′ half of the gene to avoid altered expression and was purified for transgenic mice production.

Four Pkd1extra transgenic mouse lines were generated carrying multiple copies of the transgene (~2, 12, 50, 85). These transgenic lines were analyzed for transgene integrity by genomic DNA analysis using polymorphisms, Southern and PCR (32). All lines showed intact flanking sequences of the 5′ Pkd1extra transgene based on the presence of a polymorphism (~4.5 kb upstream) specific to the 129/Sv strain from which the Pkd1-BAC derives compared with the C57BL/6J and CBA/J strains that served to generate transgenic mice (Fig. 1B). The Pkd1extra transgene integrity was demonstrated by Southern blot analysis using three restriction enzymes and five probes (Fig. 1C and D).

Pkd1extra transgene expression analysis

Transcript analysis was carried out by various complementary means. First, northern blotting using a two-step agarose gel showed that Pkd1extra kidneys from the different transgenic lines expressed a 9.76 kb specific transcript of the expected size, whereas the endogenous transcript was detected at 14.14 kb (Fig. 2A). Among the four different transgenic lines (n = 3–5 mice per line), ~2- to ~80-fold renal expression levels were obtained for the Pkd1extra transgene above the endogenous expression level. Noticeably, the Pkd1extra transgene levels did not appear to alter the endogenous Pkd1 expression levels. Second, semi-quantitative RT–PCR was performed for the four transgenic lines at adult age and was designed to distinguish the transgenic from the endogenous transcript by using primers in exon 24 and 3′ UTR due to the novel 3′ Pkd1extra junction (Fig. 2B). Expression of the truncated Pkd1extra transgene was evaluated relative to the S16 ribosomal protein gene product as internal control. Various quantities of RT aliquots were used to determine the linear range for expression analysis. The Pkd1extra transgene was expressed in all tested tissues (kidneys, brain, liver, lung, spleen) of the four transgenic mouse lines, with the highest expression in brain and lung (Fig. 2B). The transgene expression pattern essentially followed that of the Pkd1 endogenous gene. Third, quantitative expression analysis for total endogenous and transgene expression was carried out by real-time PCR with primers in exon1 and 2, on transcript from numerous tissues including kidneys, brain, heart, lung, spleen, liver and pancreas. As shown in Figure 2C, renal expression of total Pkd1 increased proportionally with transgene copy number. In comparison with the kidneys, total Pkd1 expression was generally higher in brain, heart and lung, whereas levels were lower in spleen, liver and pancreas.
Pc1extra protein expression analysis

We then monitored protein expression in the Pkd1extra transgenic mice. We first verified by using a Tsc2 antibody against the C-terminal domain that the transgene did not alter Tsc2 protein (tuberin) expression levels since it can induce cyst formation (34–36). As shown in Figure 3A, Tsc2 protein levels were similar both in the lowest and highest Pkd1extra transgenic expressors as in the controls. Significantly, no truncated Tsc2 protein was detected in the transgenic kidneys. These findings confirmed that the transgene did not modify Tsc2 expression levels.

Expression of the protein from the Pkd1extra transgene Pc1 extracellular domain (Pc1extra) in comparison with endogenous full-length transgenic Pc1 controls was quantified in adult kidneys (n = 5–6/transgenic line) of the four transgenic lines. Pc1 and Pc1extra were detected by immunoblotting with the monoclonal antibody 7e12 which recognizes the extracellular LRR domain of murine Pc1 gene. The 100 bp band corresponds to the region of Pc1extra transgene. The 9.6 kb band indicates the integrity of this region in Pkd1extra transgenic. M, 100 bp DNA ladder; C, H2O, control for PCR reaction; C57, C57BL/6J mice; CBA, CBA/J mice; 129, 129/Sv mice. (A) Schematic representation of the Pc1 wild-type and Pkd1extra-BAC construct deleted of transmembrane and intracellular domains. The Pkd1extra-BAC is shown in dark gray (exons: boxes; introns: lines), and the deleted Pkd1 domains are indicated as open boxes/lines. The STOP on Pkd1extra-BAC corresponds to the insertion of a termination translation codon in exon 25. Six probes were used for the analysis of genomic integrity that spans exons 1, 7–15, 15–20, 23–25, 36–45, 46 illustrated below the Pkd1extra-BAC construct. Restriction sites are indicated above the construct as follows: E, EcoRI; B, BamHI; H, HindIII; C, ClaI; M, MluI. (B) Genomic analysis of the 5′ regulatory region of transgenic Pkd1extra mice was monitored using a polymorphism at ~4.5 kb upstream of the ATG translation initiation codon of murine Pkd1 gene. The 100 bp band corresponds to the 5′ region of Pkd1 gene of 129/Sv origin that indicates the integrity of this region in Pkd1extra transgenic. M, 100 bp DNA ladder; C, H2O, control for PCR reaction; C57, C57BL/6J mice; CBA, CBA/J mice; 129, 129/Sv mice. (C) Production of Pkd1extra transgenic mice. (D) Analysis of the 3′ exon 25–46 junction of the Pkd1extra transgene by Southern blot using EcoRI digestion with the exon 7–15 probe. The 9.6 kb band indicates the integrity of the Pkd1 gene in this region. M, λ HindIII marker. (E) Analysis of the 3′ exon 25–46 junction of the Pkd1extra transgene by Southern blot using EcoRI digestion with the exon 7–15 probe. The 9.6 kb band indicates the integrity of the Pkd1 gene in this region. M, λ HindIII marker.
Since the Pc1/Pc1extra band appears as a doublet in wild-type and transgenic mice and Pc1 has many glycosylation sites, we examined the glycosylation status of Pc1/Pc1extra. Western analysis of total kidney protein extracts was performed with untreated (non-deglycosylated, ND) samples, treated with PNGase, which removes all N-linked carbohydrate groups, or treated with Endoglycosidase H (Endo H), which cleaves high mannose. To monitor Pc1/Pc1extra within a same set of experiments, significantly less protein (~2.5- to 25-fold) was used for Pkd1extra kidneys relative to controls, thereby resulting mainly in the analysis of the transgenic protein. Pc1extra or native Pc1 in Pkd1extra controls and Pkd1TAG mice are heavily glycosylated as indicated by the major shift in size upon treatment with PNGase (Fig. 3D). Further, Pc1/Pc1extra from Pkd1extra controls and Pkd1TAG mice displayed apparently same molecular weight protein. Of interest, transgenic Pkd1extra and controls treated with Endo H generated both forms: Endo H sensitive Pc1/Pc1extra, which is presumably localized in the ER, and Endo H resistant, which has reached the medial-Golgi and potentially the plasma membrane. Notably, the Pc1extra appears to have lower proportion of Endo H resistant to Endo H sensitive, relative to controls, suggesting that the overexpressed Pc1extra post-translational maturation is delayed.

To further investigate Pc1 and Pc1extra localization, we established MEF cells from transgenic Pkd1extra11 and control mice. MEF cells were grown in serum-free media and Pc1/Pc1extra proteins monitored in the media for secretion and in the cells. From cell culture media, the exosomes and exosome-free subfractions were isolated via ultracentrifugation or Exoquick protocol. As shown in Figure 3E, Pc1/Pc1extra proteins were present in exosomes from both non-transgenic control and Pkd1extra11 MEFs. In addition, exosomes from Pkd1extra11 MEFs displayed substantially higher levels (~10- to 12-fold) of Pc1/Pc1extra than the controls, consistent with Pc1extra overexpression. In non-transgenic control and Pkd1extra11 media devoid of exosomes, free Pc1/Pc1extra protein levels seem increased (~3-fold) in Pkd1extra11 relative to controls, though in decreased proportion based on total expression levels of Pkd1extra11 and controls (Fig. 3B). An aliquot of MEF cells from Pkd1extra11 and non-transgenic controls were fractionated in cytosol and triton-soluble membrane (Fig. 3F). Marked increase in Pc1/Pc1extra expression (>10-fold) was detected in total MEF extracts of Pkd1extra11 in comparison with controls. In both the cytosol and the triton-soluble membrane fractions, Pc1/Pc1extra was detected in Pkd1extra11 as well as in controls (Fig. 3F), correlating with the renal Pc1/Pc1extra glycosylation analysis.
Figure 3. Analysis of Pc1extra expression in mice. (A) Expression of Tsc2 protein (tuberin) was monitored in kidneys of the lowest- and highest-expressing Pkd1extra lines 39 and 2 (n = 3 per line). Expression of Gapdh served as internal loading control. No detectable difference in Tsc2 expression was observed in the Pkd1extra compared with the non-transgenic control kidneys. (B) Endogenous and transgenic Pc1/Pc1extra protein expression levels analyzed on a gel gradient by western blot are shown for adult kidneys (n = 2–3) of three Pkd1extra mouse lines. Pc1 was detected using 7e12 monoclonal antibody with both long and short exposures along with internal control Gapdh. Expression of the truncated Pc1 protein, Pc1extra, was within similar range as transcript levels. Highest levels were obtained in line 2 and lowest in line 39. The Pc1extra protein band appears to migrate as the Pc1 protein from endogenous control and transgenic Pkd1TAG26. (C) Analysis of transgenic Pc1/Pc1extra protein expression levels in adult extrarenal tissues of Pkd1extra39 transgenic mice. Pc1 was detected using the 7e12 monoclonal antibody with both long and short exposures along with internal Gapdh control. Transgenic kidneys of Pkd1extra39 showed approximately equivalent Pc1 protein levels as Pkd1TAG26 transgenic kidneys. Lu, lung; Ki, kidney; Pa, pancreas; Br, brain; He, heart; Li, liver. (D) Analysis of Pc1 and transgenic Pc1extra protein glycosylation status. Pc1/Pc1extra was detected with the 7e12 monoclonal antibody and β-tubulin as internal control. Adult transgenic renal extracts of Pkd1extra2 (3.5 μg), 11 (15 μg) and 39 (35 μg), control Pkd1TAG26 (35 μg) and control non-transgenic (90 μg) were treated with PNGase (P) and Endo H (E) and compared with untreated non-deglycosylated (ND). The larger Pc1/Pc1extra band corresponds to the mature glycosylated form in all transgenic and controls. Both controls and transgenic mice have Endo-H-sensitive and -resistant Pc1/Pc1extra, the ratio of Pkd1TAG and non-transgenic control appear similar, whereas it seems increased in Pkd1extra. (E) Pc1 and transgenic Pc1extra protein secretion from MEF cells. Pc1/Pc1extra was detected with the 7e12 monoclonal antibody and as internal controls, β-tubulin and β-actin. Pc1/Pc1extra was detected in total MEF extracts, the cytosolic fraction and the triton-soluble membrane fraction. Levels of Pc1/Pc1extra were substantially elevated in MEFs obtained from the transgenic Pkd1extra11 compared with the non-transgenic control.
Histopathologic anomalies in Pkd1\textsubscript{extra} mice

To evaluate progression of the cystogenic disorder in the Pkd1\textsubscript{extra} transgenic mouse lines, we carried out non-invasive imaging by ultrasound measurements using a VisualSonics Vevo 660 Imaging System with a 40 MHz scanhead. Analysis of Pkd1\textsubscript{extra} transgenic lines 11 and 39 (n = 8) at ≥15 months showed readily detectable multiple macro- and micro-cysts (Fig. 4A and B).

To investigate the phenotype induced by the transgene expression, we subjected the four Pkd1\textsubscript{extra} transgenic mouse lines (n > 16 per line; n = 141) including founders and progenies to complete histologic analysis. Although our analysis was performed on various organs, anomalies were mainly confined to the kidneys. All transgenic mouse lines exhibited characteristic features of PKD, whereas non-transgenic controls did not develop these disorders. Interestingly, very mild renal tubular dilatation can be detected in 2-month-old Pkd1\textsubscript{extra} mice. With advancing age, the renal tubular dilatation phenotype progressed similarly for both the lowest and highest Pkd1\textsubscript{extra}-expressing lines, a key characteristic of ADPKD (Supplementary material, Fig. S2). Both kidneys of Pkd1\textsubscript{extra} transgenic mice over time became pale with an embossed surface and were severely affected by macro- and micro-cysts (Fig. 4C and D). Glomeruli displayed prominent cysts associated with hyperplasia of the parietal epithelial cells (Fig. 4F, G and H). The glomerular tufts were hypocellular and became sclerotic. The renal parenchyma displayed tubular cysts, but also loss of tubules as well as the presence of mild to extensive interstitial fibrosis (Fig. 4I and J), with localized lymphoid infiltrates often perivascular. Thus, the Pkd1\textsubscript{extra} transgenic mice developed typical PKD manifestations that progressed akin with age in all four lines.

Because defects in tubulogenesis can occur from inhibition of Pc1 cleavage (5), \textit{ex vivo} incubation of peptides against the Pkd1 extracellular domain (37), and was recently associated with renal cysts formation (38), we queried whether Pkd1\textsubscript{extra} expression could alter the regulation of ureteric branching \textit{in vivo}. To evaluate ureteric branching or tubulogenesis, we quantified the number of glomeruli/nephron per square millimeter from stained sections in young Pkd1\textsubscript{extra} transgenic mice at 1.5 months of age as described (39). Analysis of mice with low Pkd1\textsubscript{extra} expression (line 9: 13.9 ± 0.91 glomeruli/mm\textsuperscript{2}; n = 3) revealed that Pkd1\textsubscript{extra} transgenic kidneys have similar number of glomeruli relative to wild-type controls (12.9 ± 1.55 glomeruli/mm\textsuperscript{2}; n = 5), suggesting that the renal developmental program is not affected by expression of the Pkd1\textsubscript{extra} transgene. This finding was also consistent with the kidney size, which was comparable in the Pkd1\textsubscript{extra} and the wild-type control at this age.

Figure 4. Renal pathology in Pkd1\textsubscript{extra} transgenic mice. (A and B) High-resolution ultrasound imaging of kidneys. Comparison of control non-transgenic (A) with Pkd1\textsubscript{extra} mice (B) was performed during lifespan. Attached video imaging shows representative renal echographies used to monitor cyst progression in Pkd1\textsubscript{extra} transgenic mice relative to control, at similar age (≈20 months). (C and D) Kidneys from a non-transgenic control mouse (C) were compared with kidneys of Pkd1\textsubscript{extra} transgenic mouse line (D). Transgenic kidneys were pale and displayed pronounced macro-cysts with completely remodeled renal architecture. Red arrowhead indicates protruding cysts. (E and F) Histologic analysis of renal cortex from adult non-transgenic control (E) and transgenic Pkd1\textsubscript{extra} (F) mice (≈20 months). Pkd1\textsubscript{extra} transgenic mice showed numerous tubular and glomerular cysts (black arrow) as well as the presence of hyperplasia (double arrow), lymphoid infiltrates (star) and protein deposits (arrowhead) in tubular cysts. Sections were stained with H&E. Original magnification, ×50. (G) Higher magnification of the boxed area from (F). Original magnification, ×320. (H) Higher magnification of an adjacent region from the same transgenic Pkd1\textsubscript{extra} renal section shown in (F). Original magnification, ×400. (I and J) Analysis of interstitial fibrosis in kidney sections from adult non-transgenic control (I) and transgenic Pkd1\textsubscript{extra} (J) mice (≈20 months). Pkd1\textsubscript{extra} transgenic mice exhibited intense Sirius red staining in comparison with control indicative of marked fibrosis. Original magnification, ×50. Insets show higher magnification of the boxed area. Original magnification, ×320.
Evidence of altered renal physiology in Pkd1extra transgenic mice

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<th>n</th>
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<th>n</th>
<th>Urea nitrogen (mmol/l)</th>
<th>n</th>
<th>Creatinine (mmol/l)</th>
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<td>1316.5 ± 570.3</td>
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<td>8.6 ± 3.8</td>
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<td>407.7 ± 86.9*</td>
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Control, non-transgenic mice used as normal control; SBM, transgenic PKD mouse model used as control for renal insufficiency; four Pkd1extra transgenic lines, Pkd1extra2, 9, 11 and 39. The mice age range was for controls (21–22 months), Pkd1extra2 (23–25.5 months), Pkd1extra9 (17.5–22 months), Pkd1extra11 (21.5–22.5 months), Pkd1extra39 (19.5–23.5 months), SBM (5–5.5 months). Values are mean ± standard deviation.

\*P ≤ 0.05; \**P ≤ 0.005.

Table 2. Chronic renal insufficiency in Pkd1extra transgenic mice

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<tr>
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<th>Creatinine (µmol/l)</th>
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<th>Hematocrit (%)</th>
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Control, non-transgenic mice used as normal control; four Pkd1extra transgenic lines, Pkd1extra2, 9, 11 and 39. The mice age range was for controls (22 months), Pkd1extra2 (20–22 months), Pkd1extra9 (26 months), Pkd1extra11 (21–24 months), Pkd1extra39 (19.5–23.5 months). Values are mean ± standard deviation.

\*P ≤ 0.05; \**P ≤ 0.005.

Since Pkd1TAG mice displayed anomalies prior to overt cystogenesis (32), cilia of renal epithelial cells were monitored by α-acetylated tubulin staining in Pkd1extra and control mice. The cilia size distribution measured at micrometer interval in Pkd1extra39 and 2 lines at precystic age showed that most cilia (n = 389) were of 2–4 µm in length (55–61%) similar to controls (n = 428; 54%). At cystic age, cilia in Pkd1extra11 and 2 lines displayed no difference in cilia size distribution or structure relative to controls. These data suggest that the cilia length is not implicated in Pkd1extra cystogenesis.

Altered renal physiologic function in Pkd1extra transgenic mice

Mice from the Pkd1extra transgenic lines exhibited renal functional abnormalities resembling those in ADPKD compared with age-matched controls. The renal physiology was investigated by urine analysis in comparison with the severe PKD mouse model SBM (40) as well as by blood analysis (Tables 1 and 2). Transgenic mice displayed 3–4-fold increase in urine volume, a concentrating defect that progressed with age (> 15 months). Consequently, renal parameters in these mice also exhibited decrease in urine osmolality, urea nitrogen, creatinine, protein and ion excretion (Table 1). Notably, these alterations are frequent also in human ADPKD. In addition, we qualitatively analyzed the urine protein composition from the four Pkd1extra transgenic mouse lines by SDS–PAGE. As shown in Figure 5A, transgenic mice of all lines displayed non-selective proteinuria. This abnormal protein leakage was already detectable at 12 months and increased further with age. Consistent with these altered urine parameters, the levels of serum blood urea nitrogen (BUN) and serum creatinine were elevated, indicating renal insufficiency (Table 2). Since renal insufficiency frequently causes hematologic anomalies in both mice and humans, hematocrit was measured in all of our Pkd1extra transgenic mouse lines. These mice showed important decrease in hematocrit levels, providing evidence for chronic renal insufficiency (Table 2), similar to human ADPKD pathogenesis.

To determine whether Pc1extra in Pkd1extra transgenic mice is present in urinary exosomes and/or free in the urine, as native Pc1 in non-transgenic and Pkd1TAG26 controls, we isolated urinary exosomes. Pci1/Pc1extra was detected in exosomes of Pkd1extra lines 2, 11 and 39 and of non-transgenic control and transgenic Pkd1TAG26. In transgenic Pkd1extra lines and Pkd1TAG26 mice, exosomal Pci1/Pc1extra levels appeared to increase in parallel with the range of overexpression (Fig. 5B). In exosome-free urine, secreted Pc1/Pc1extra was detected in transgenic Pkd1extra and Pkd1TAG26 mice (Fig. 5C). Since non-transgenic control mice had very low to barely detectable Pc1 levels, this implies that mainly Pc1extra is the secreted form in the urine of Pkd1extra mice. Noticeably, levels of Pci1extra in exosome-free urine, upon comparison of total expression in Pkd1extra lines with Pkd1TAG26 (Fig. 3B), suggest that the Pc1extra protein is not as efficiently secreted as the native Pc1.

Animals from these Pkd1extra transgenic lines manifested late-onset of renal failure. Indeed, the mean lifespan in mice of line 2 (n = 10) was ~17.3 ± 4.5 months of age, line 9 (n = 9) 23.0 ± 5.5, line 11 (n = 20) 17.7 ± 4.4 and line 39 (n = 15) 17.4 ± 6.9 months, whereas for controls of the same genetic background the mean survival age ranged between 23 and 29 months.

Functional analysis of Pkd1extra mice on hemizygous Pkd1 background

In an attempt to genetically mimic the human context, the Pkd1extra39 line was mated to mice with a Pkd1 null allele (41) to produce compound heterozygous Pkd1extra39/Pkd1+/− mice. Histomorphologic analysis of kidneys in the double-heterozygote adult mice (n = 12; 11–23 months) was compared with Pkd1extra39 (n = 22; 15–29 months) and Pkd1+/− mice (n = 9; 13–26 months). Despite some
variability between mice, the Pkd1extra39;Pkd1<sup>+/−</sup> kidneys exhibited tubular and glomerular cysts in late adulthood reminiscent of Pkd1extra mice perhaps with a trend toward more pronounced phenotype as evidenced by general tubular dilations (Fig. 6). Although the Pkd1<sup>+/−</sup> mice displayed limited renal cysts in a minority of old individuals as reported previously (42,43), both the double-heterozygote and the Pkd1extra39 adult mice develop a severe phenotype. To assess whether this phenotype results from the transgene expression or from the imbalanced between Pkd1extra to Pkd1 expression, the strongest expressing line Pkd1extra2 was mated onto the Pkd1<sup>+/−</sup> background. The Pkd1extra2;Pkd1<sup>+/−</sup> kidneys (n = 11; 7–26 months) exhibited slightly more severe phenotype than in the Pkd1extra2 (n = 31; 13–31 months), as observed in the Pkd1extra39;Pkd1<sup>+/−</sup> kidneys. This experiment supports that the Pkd1extra transgene is likely responsible for the phenotype. These double-heterozygous mice seem to display a more severe PKD phenotype than the Pkd1extra transgenic mice by exhibiting more invasive phenotype and/or occasional earlier onset as a possible effect of Pkd1<sup>+/−</sup> phenotype.

**Identification of Pkd1extra renal molecular targets**

Since Pc1 is implicated in a large complex with Pc2 in the kidneys (44–48) and Pkd2 dysregulation can cause cysts
we queried whether Pkd2 renal expression levels might be altered in Pkd1extra mice. Expression analysis was performed by northern on kidneys of all transgenic Pkd1extra mouse lines (\( n = 16 \)). In comparison with control (\( n = 6 \)), there is no increase in the expression of endogenous Pkd2 transcript in Pkd1extra mice (Fig. 7A). In contrast, the protein expression levels of Pc2 determined by immunoblot was increased (mean \( \approx 6-8 \)-fold) in pre-cystic kidneys of all four Pkd1extra lines (\( n \geq 4/\text{line}; n = 18 \)) relative to control (\( n = 4 \)) mice (Fig. 7B). Similar range of Pc2 increase was observed in compound heterozygous Pkd1extra;Pkd1\(^{+/-}\) mice (data not shown). These experiments suggest that the Pc1extra could prevent Pc2 degradation or could modulate Pc2 protein stabilization, and thereby lead to cyst formation. Underlying these hypotheses is whether Pc1extra can interact potentially with Pc2. To monitor this interaction, coimmunoprecipitation experiments were performed with Pc2 in Pkd1extra mice in comparison with Pkd1\(^{TAG}\) and control mice (Supplementary material, Fig. S3). Our results show that Pc1 interacts with Pc2 in kidneys of Pkd1\(^{TAG}\) line in higher proportion than wild-type controls, consistent with increased Pc1 expression. Intriguingly, there also seems increased interaction in kidneys of Pkd1\(^{extra}\)39 mice relative to controls. Although we demonstrate the existence of a protein complex, this result does not distinguish the interaction of Pc1\(^{extra}\) and/or Pc1 with Pc2, but both could be possible based on cellular studies (48). Of significance, the Pc1\(^{extra}\) protein at the very least does not hamper Pc1–Pc2 interaction. Overall, the analogous Pc2-enhanced expression in these Pkd1extra transgenic lines is particularly striking and correlates with the similar slow progression and development of PKD phenotype in these mice.

Because we have previously detected increased c-myc renal expression in human ADPKD kidneys and in a PKD transgenic mouse model due to Pkd1 overexpression, we next evaluated renal c-myc expression levels in the four transgenic Pkd1extra mouse lines. When very mild renal phenotype was observed at 2–4 months of age, these mice had little-to-no difference in endogenous c-myc transcript expression relative to control (data not shown). However, c-myc expression increased up to 6-fold at 19–26 months of age associated with cystogenesis defects (Fig. 7C). This result is also consistent with the increased c-myc expression detected in the Pkd2 transgenic mice (51).

**DISCUSSION**

This study reports the first Pkd1 mutant mouse model, Pkd1\(^{extra}\), of ADPKD. This mutant Pkd1\(^{extra}\) model addresses concomitantly the pathogenic role of the Pc1 extracellular domain since the Pkd1 gene in a BAC was truncated to delete the protein membrane spanning and intracellular domains. Four Pkd1\(^{extra}\) transgenic mouse lines with \( \approx 2- \) to
The design of our truncated Pc1, Pc1extra, homologous to characterized human ADPKD mutations, corresponds to the encoded form documented in four patients with different truncated mutations at the extracellular domain/membrane junction or in the proximity of the natural GPS/GAIN cleavage site (11,14). This Pc1extra mutant form devoid of the membrane-spanning and intracellular regions should be normally glycosylated, maintain its structural conformation/folding, potentially tethered to the membrane and/or secreted, based on previous truncated receptor analysis (5,52–55). Consequently, we have addressed the role of Pc1extra function within the Pkd1 systemic regulation. The four different Pkd1extra transgenic founder mice and lines expressed the transgene in a copy-dependent manner. The expression pattern of the Pkd1extra transgene in various tissues generally paralleled that of the endogenous Pkd1 gene, although for slightly lower levels in the brain as observed in Pkd1 full-length transgenic mice (32). This pattern suggests that the flanking regions of the Pkd1extra transgene contain most of the necessary regulatory elements for proper expression and regulation. Of interest, expression of the Pkd1extra transgene at 2–80-fold that of endogenous did not affect the transcriptional regulation of the endogenous Pkd1 gene, consistent with no autoregulatory feedback as suggested (46).

The Pkd1extra transgenic mouse lines in the presence of the endogenous gene had complete penetrance of the phenotype and shared several physiopathologic features with ADPKD. These include the development of cysts in the cortex, medulla and glomeruli, together with epithelial hyperplasia, interstitial fibrosis and focal interstitial inflammation. In parallel, alterations in renal function progressed with time, and at mid-age, Pkd1extra mice had signs of renal insufficiency. Since the PKD phenotype was consistently observed in all four different transgenic mouse lines and the transgene integration into the mouse genome is a random phenomenon, the phenotype cannot simply be a consequence of chromosomal position effect but results from Pkd1extra expression. Moreover, we showed that Pkd1extra cystogenesis does not occur from an indirect role that targets a stage in the process of ciliogenesis or of ureteric branching during development. Hence, our results provide clear evidence that overexpression of the mutated Pkd1 transgene, Pkd1extra, can produce multiple renal cysts. These Pkd1extra mice are the most valuable orthologous mouse models generated by mimicking natural, physiologically relevant, mutations of the human PKD1 gene that can also recapitulate the slow progression of PKD.

Notably, the protein levels of Pc1extra in the kidneys correlated with the Pkd1extra transcript levels, indicating that a stable truncated Pc1extra protein was produced and reached substantial expression levels in some lines. The cellular levels of Pc1extra were, however, in considerable excess relative to native Pc1 for similar transcript levels. The higher levels of Pc1extra protein than expected in comparison with native Pc1 suggested at least two explanations that are not mutually exclusive. The first is that Pc1extra is protected from degradation, potentially from the loss of the PEST motif localized in the truncated intracellular domain. The second possibility is that Pc1extra is not efficiently secreted and/or excreted from the kidneys.
renal epithelial exosomes in contrast to the native Pc1 (32). This latter mechanism is consistent with the lower levels of secreted Pc1_{extra} relative to the native Pc1 in urine and MEF cells and correlated with the apparent delay in Pc1_{extra} post-translational maturation determined by glycosylation status. These results correlated with cell studies that expressed an analogous truncated human PKD1 due to premature termination codon at p130/E3020X and showed a small amount of PC1 extracellular domain secreted in media, whereas most remained tethered or intracellular (5). Furthermore, we show that the difference in levels of secreted Pc1_{extra} relative to native Pc1 in urine and MEF cells occurred independently of Tsc2 dysregulation previously associated with mislocalization of Pc1 (56). Nevertheless, Pc1_{extra}, like some truncated receptors, appears to proceed via the typical secretory pathway, as it is heavily glycosylated, excreted in exosomes and secreted. In fact, a considerable amount of Pc1_{extra} was excreted in exosomes and freely secreted, which may interfere with the normal Pc1 extracellular function (57). In addition, altered Pc1_{extra} protein distribution may be pathogenic by not localizing to a site of functional task.

Few hypothetical mechanisms for Pkd1_{extra} cystogenesis may prevail. One plausible mechanism is that the Pc1_{extra} protein virtually indistinguishable from the naturally cleaved GPS/GAIN-Pc1 may interfere with the native Pc1 autoproteolytic cleavage and/or alter Pc1 downstream signaling. Alternatively, one mechanism may be attributed to the imbalance between the cleaved Pc1 extracellular domain and the transmembrane and intracellular domains. A dynamic interaction between the extra- and intra-cellular domains may require tight regulation to transmit changes from the microenvironment through channel activity or signaling pathways. Nonetheless, an intriguing finding is that expression of the Pkd1_{extra} transgene in the kidneys at various RNA or protein levels leads to similar progression and severity of the phenotype. This result indicates that Pc1_{extra} could be sufficient to produce the phenotype but may be limited by the availability of ligands, interacting partners and/or downstream targets and therefore the disruptive signal cannot be increased further in a dose-dependent mechanism as reported for the Pkd1 full-length transgenic mice (32). Moreover, the onset and phenotypic progression of PKD in the Pkd1_{extra} mice are milder than in the Pkd1 full-length transgenic mice. Consequently, this raises the question of Pkd1_{extra} pathogenic mechanism(s) triggered in these mice in comparison with the Pkd1-overexpressing mice.

Insights into these Pkd1_{extra} potential pathogenic mechanism(s) were provided by analysis of renal Pc1-interacting partner Pc2. Although no difference in Pc2 expression was noted in the Pkd1_{extra} and Pkd1_{+/-} embryos (29), all four Pkd1_{extra} transgenic lines displayed increased Pc2 expression which argues for a Pc1_{extra}-specific effect. Interestingly, the significantly enhanced Pc2 expression occurred despite similar Pkd2 transcript levels and indicates that Pc1_{extra} may modulate Pc2 protein levels directly or indirectly through a post-transcriptional mechanism. This response may result from a compensatory mechanism, reduced Pc2 protein secretion and/or decreased Pc2 degradation mechanism. Of importance, the remarkably similar range of Pc2 elevated levels correlated with the comparable PKD developmental progression in these Pkd1_{extra} transgenic lines. Compelling support for this interrelation is provided from the Pc2-overexpressing transgenic mice that developed very similar onset and progression of renal PKD phenotype (50). These correlations extend to the absence of liver or pancreatic anomalies in Pkd1_{extra} mice as observed in transgenic Pkd2 mice (51). Such results, combined with the observation that Pkd1 expression in cells was able to modulate the localization and perhaps the function of Pc2 (47), suggest that Pkd1_{extra} pathogenic mechanism may be triggered by the dysregulation of Pc2 that in turn control cystogenic threshold.

Both Pc1 and Pc2 form complexes with many signaling molecules, hence abnormally high Pc1 or Pc2 levels likely disrupt downstream signaling. Consistent with this rationale, Pkd2 transgenic mice revealed stimulation of c-myc expression (51), one of the major mediators of cystogenesis in both humans and mice (21,40). Likewise, c-myc expression was also elevated in Pkd1_{extra} kidneys (herein) as well as in kidneys of Pkd1-overexpressing transgenic mice (31,32). These data indicate that Pc1_{extra} directly and/or via Pc2 can modulate a downstream signaling pathway that causes dysregulation of c-myc.

From the Pkd1_{extra} mice analyses, it is plausible that the interactions of the expressed mutant Pc1_{extra} promote Pc2 expression. Activation of such crosstalk regulation provides evidence for a novel cystogenic mechanism. These findings suggest that other truncated PC1 mutants may also trigger this pathogenic crosstalk. Together with previous human and mouse studies on PKD mutational mechanism, this cystogenic crosstalk mechanism is not only consistent, but also substantiates a dosage-dependent mechanism for ADPKD.

In conclusion, the Pkd1_{extra} mice constitute the first Pc1 extracellular mutant that can reproduce the clinical renal phenotype progression of human ADPKD disease. This model has established the basis for future studies to dissect the functions of the multimodular Pkd1 extracellular domain. Most importantly, our results provide major insights into a novel PKD pathogenic mechanism implicating a regulatory crosstalk between a truncated Pc1 mutant and Pc2. This model will be invaluable for the characterization of multiple facets of Pkd1 pathophysiology and devise innovative therapeutic strategies.

**MATERIALS AND METHODS**

**DNA constructs and homologous recombination in Pkd1_{extra}-BAC**

List of all primers used is provided in Supplementary Material, Table S1.

We have previously isolated a murine Pkd1-BAC derived from a murine 129/Sv library that contained ~129 kb insert with the entire Pkd1 gene (31) as well as ~37 kb of upstream and ~39.1 kb of downstream flanking sequences including the Tsc2 gene body. To create a BAC with Pc1 extracellular domain only, we removed Pc1 transmembrane and cytoplasmic domains by homologous recombination in the Pkd1-BAC, adjoining amino acid 3042 (exon 25, nucleotides 9442–9444, accession number: U70209), a translation termination codon (UAA) and the native 3’ UTR of the Pkd1 gene (exon 46, nucleotide 13819). To produce the truncation, Exon23–25 fragment
including the stop codon was obtained by amplification (1.25 kb), digested with Clal (NEB) and BamHI (Invitrogen) (nucleotide 9445) and introduced into the same corresponding sites of p-Bluescript. A second fragment containing the 3’ UTR Pkd1’/3’UTR Tsc2-intron39 was amplified (1.163 kb) and then was subsequently digested with BamHI/BclII (nucleotide 13 820/4980) and inserted in BamHI of p-Bluescript. The novel junctions of the resulting exon23–25/3′ UTR Pkd1’/3’ UTR Tsc2-intron39 plasmid were sequenced to confirm appropriate cloning. This plasmid was then digested with PvuII (2.45 kb) and introduced into the SmaI site of the BAC recombination vector (PLD53.SC-AB) (58). This BAC recombination vector containing Pkd1 exon23–25/3’ UTR Pkd1’/3’UTR Tsc2-intron39 served to modify the wild-type Pkd1-BAC by homologous recombination protocol as previously described (31,32). DNAs from resolved BACs were analyzed by Southern for appropriate modifications by four probes: exon 1 (nucleotides 1–509); exon 7–15 (nucleotides 1752–6622); exon 23–25 (nucleotides 8579–9443) and exon 46 (nucleotides 12 760–13 820). The integrity of the modified Pkd1extra-BAC was also confirmed by PFGE and by sequencing the junction between exons 25 and 46.

### Production and genotyping of transgenic mice

The new Pkd1extra-BAC was digested to isolate the Pkd1extra gene away from the murine Tsc2 gene and the BAC vector sequences with MluI, a unique site ~24.8 kb upstream of Pkd1 initiation codon and Clal site ~21.6 kb downstream of Pkd1 stop codon. The 77 kb Pkd1extra fragment was isolated and purified for micro-injection into fertilized (C57BL/6JXCBA/J)F2 mouse eggs as described previously (40).

Transgenic mice were analyzed for transgene integrity. The 5′ upstream sequence was monitored by a polymorphic sequence located in the murine Pkd1 gene at 4.47 kb upstream of the ATG translation initiation codon. A PCR protocol was designed to distinguish the 129/Sv of the Pkd1-BAC from the C57BL/6J and CBA/J inbred strains that served to produce transgenic mice (32). The integrity of Pkd1extra transgene sequences was monitored by signal intensity from Southern blot of genomic DNA digested with EcoRI and hybridized with six genomic probes: exon 1, exons 2–3, exons 7–15, exons 15–20, exons 25–34, exon 46. To verify transgene integrity in the 3′ downstream sequences, genomic DNA from founder mice and progenies of the four transgenic mouse lines were analyzed by Southern blot. Three restriction enzymes [EcoRI, BamHI and HindIII (Invitrogen)] that distinguished the endogenous Pkd1 gene from the Pkd1extra transgene were used with the exons 23–25 Pkd1 genomic probe.

### Renal function analysis

BUN, creatinine, urinary proteins and osmolality levels were determined from the urine of transgenic (n > 3/line) and non-transgenic age-matched control mice (n > 4). For urine analysis, 350–500 μl was collected per mouse. BUN and creatinine were measured with a CX9 Beckmann apparatus, whereas osmolality was evaluated with a radiometer. Qualitative assessment of urinary proteins was also performed with ~50 μg of proteins on 8% SDS–PAGE as described (59) and stained with Coomassie Blue. Blood serum from transgenic and control mice was also analyzed for BUN and creatinine levels.

### Histopathologic analysis

Transgenic and non-transgenic age-matched control mice were sacrificed and tissues of the kidney, lung, liver, brain, spleen, heart and pancreas readily removed. Tissues were immediately placed in formalin or paraformaldehyde and following fixation were embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H&E) for morphologic evaluation. Fibrosis analysis was carried out on both kidneys of transgenic and age-matched control mice (n = 4 – 5 mice/line). Five micrometer sections were stained with Sirius red to evaluate fibrosis. Levels of renal fibrosis were quantified in function of square micrometer with a 10 × objective from four cortical fields of each kidney using the Northern Eclipse software.

### RNA expression analysis

Total RNA from transgenic and age-matched control tissues including kidneys, brain, lung, spleen, heart, liver and pancreas was extracted using the guanidium thiocyanate or TRIzol/ chloroform method (60). RNA integrity was verified by electrophoresis on formaldehyde-agarose gel (61). Expression of the Pkd1extra transgene was analyzed by both semi-quantitative RT–PCR and real-time PCR approaches. Each pair of primers was designed such that only spliced mRNA would produce the predicted amplification products of 290, 94 and 103 bp for Pkd1extra, Pkd1 total (endogenous and transgene) and S16, respectively. For semi-quantitative PCR, control reactions were first performed using a wide range of RT aliquots to test for linearity amplification. PCR reactions were carried out for both Pkd1extra and S16 internal control, were run on 10% polyacrylamide gel (PAGE)/TBE1X gel and visualized by ethidium bromide staining. The image Quant 5.0 software was used for the semi-quantitative evaluation. For real-time PCR, all reactions were carried out in triplicate in a Quantech SYBR Green PCR master mix (Qiagen and Quanta) using Mx4000/3005 multiplex PCR apparatus (Stratagene).

Expression analyses of Pkd1extra transgene and Pkd1 endogenous gene were monitored by Northern blotting. Total kidney RNA from adult transgenic and non-transgenic mice of approximately same age (30 μg) was DNase-digested and separated by electrophoresis on a two-step 0.8%/1.5% formaldehyde-agarose gel and transferred to nylon membranes (Osmomics, Inc.) and was hybridized with a Pkd1 exon 15–20 cDNA probe (1.55 kb) for transgene, Pkd1 exon36–45 cDNA probe (1.6kb) for endogenous Pkd1 and a Gapdh cDNA probe for internal control (1.2 kb) as previously described (62). Pkd2 expression analysis was performed by Northern blotting and evaluated by hybridization with a Pkd2 cDNA probe (2.2 kb) (generous gift from Dr S. Somlo) and Gapdh as control. Expression analysis of c-myc in Pkd1extra mice was evaluated by Northern blot analysis as described for Pkd2. RNA (20 μg) was hybridized with a c-myc probe consisting of exons 2 and 3 and the ribosomal protein L32 (rpl32), which served as loading control. Both scanned films and phosphoimager were quantified by with the Image Quant 5.0 software.
Protein analysis

Total protein extract was prepared from transgenic and non-transgenic renal and extrarenal tissues, homogenized in RIPA buffer and cocktail of proteases inhibitors (Sigma). Protein extracts (36 µg extrarenal, 48 µg renal) were loaded on 4–12% Tris–glycine SDS–PAGE (NuPAGE, Invitrogen) and transferred at 4°C overnight, 30 V, using Biotrace™ PVDF membrane (Pall). Membranes were hybridized with the monoclonal 7e12 antibody (16) for the detection of Pc1 (a generous gift of Drs C. Ward and P. Harris). Secondary HRP-conjugated antibodies allowed detection by ECL Plus western blotting detection system (Amersham Biosciences). Deglycosylation analysis was performed with PNGase and Endo H (New England Biolabs) as described in Kurbegov et al. (32) and monitored on 4–12% Bis-Tris gels. For Tsc2 protein/tuberin analysis, proteins (8 µg) were loaded on a two-step 6.25%/12.25% PAGE and detected using a rabbit polyclonal SC893 that recognizes the C-terminal fragment (20 amino acids). For Pc2 analysis, immunoblotting was similar to Pc1, protein extracts (200 µg) were used on 8% Tris–glycine SDS–PAGE (Bio-Rad) and transferred on nitrocellulose membrane. Hybridization was carried out with the YCC2 polyclonal antibody (a generous gift of Drs Y. Cai and S. Somlo) (41). Gapdh (Abcam), β-tubulin and β-actin (Sigma) served as loading controls.

MEF cells were isolated from Pkd1extra transgenic and non-transgenic embryos (e14.5) and incubated for 2–3 days with FBS until confluency. MEF cells were replated in serum-free media for 3 days to analyze free-secreted Pc1 in the media and Pc1 in exosomes. Both Exoquick protocol™ (System Biosciences, Medcorp) for exosomes as described above and the ultracentrifugation for exosomal and exosomal-free fractions were carried out as described in Kurbegov et al. (32). PBS-washed MEF cells were centrifuged at 100 000 g, supernatant fraction (cytosolic) was collected and the insoluble pellet resuspended and incubated in lysis buffer containing 1% Triton (Triton-soluble fraction, membrane). Proteins were analyzed on 3–8% Tris acetate gel containing 1% Triton (Triton-soluble fraction, membrane). Proteins were analyzed on a two-step 6.25%/12.25% PAGE and detected using a rabbit polyclonal SC893 that recognizes the C-terminal fragment (20 amino acids). For Pc2 analysis, immunoblotting was similar to Pc1, protein extracts (200 µg) were used on 8% Tris–glycine SDS–PAGE (Bio-Rad) and transferred on nitrocellulose membrane. Hybridization was carried out with the YCC2 polyclonal antibody (a generous gift of Drs Y. Cai and S. Somlo) (41). Gapdh (Abcam), β-tubulin and β-actin (Sigma) served as loading controls.

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Coimmunoprecipitation analysis was performed on adult kidney samples (600 µg) incubated with 2 µg of the monoclonal anti-rat Pc2 antibody, generously provided by the University of Texas PKD core facility and with Protein A/G Plus-Agarose beads (SantaCruz Biotechnology). The material was analyzed on 4–12% Bis-Tris (Invitrogen); controls consist of non-specific binding to the beads alone (−) without the Pc2 antibody.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


