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Identification, Characterization, and Localization of a Novel Kidney Polycystin-1-Polycystin-2 Complex*

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The functions of the two proteins defective in autosomal dominant polycystic kidney disease, polycystin-1 and polycystin-2, have not been fully clarified, but it has been hypothesized that they may heterodimerize to form a “polycystin complex” involved in cell adhesion. In this paper, we demonstrate for the first time the existence of a native polycystin complex in mouse kidney tubular cells transgenic for *PKD1*, non-transgenic kidney cells, and normal adult human kidney. Polycystin-1 is heavily *N*-glycosylated, and several glycosylated forms of polycystin-1 differing in their sensitivity to endoglycosidase H (Endo H) were found; in contrast, native polycystin-2 was fully Endo H-sensitive. Using highly specific antibodies to both proteins, we show that polycystin-2 associates selectively with two species of full-length polycystin-1, one Endo H-sensitive and the other Endo H-resistant; importantly, the latter could be further enriched in plasma membrane fractions and co-immunoprecipitated with polycystin-2. Finally, a subpopulation of this complex co-localized to the lateral cell borders of *PKD1* transgenic kidney cells. These results demonstrate that polycystin-1 and polycystin-2 interact *in vivo* to form a stable heterodimeric complex and suggest that disruption of this complex is likely to be of primary relevance to the pathogenesis of cyst formation in autosomal dominant polycystic kidney disease.

disorder, have been identified (1, 2). Nonetheless, the functions of the two gene products, polycystin-1 and polycystin-2, remain unclear. Sequence analysis and hydropathy plots suggest that the *PKD1* protein, polycystin-1, is a novel integral membrane glycoprotein containing a number of extracellular domains previously described in other proteins involved in cell-cell and cell-matrix interactions (3). Polycystin-1 is also highly homologous to the sea urchin sperm receptor for egg jelly (suREJ) protein, which triggers extracellular Ca^{2+} influx in the acrosome reaction (4). A recent study demonstrated that polycystin-1 overexpression stimulated tubulogenesis and inhibited proliferation in Madin-Darby canine kidney cells (5). Therefore, it seems likely that polycystin-1 plays a multifunctional role in the regulation of cell differentiation, proliferation, and cation transport. Polycystin-2, the *PKD2* protein, has significant homology to voltage-activated and transient receptor potential K^+ , Na^+ , and Ca^{2+} channels (2). A polycystin-2 homologue, PKD-L, has been shown to reconstitute non-selective Ca^{2+} channel activity in *Xenopus* oocytes (6), and recent studies suggest that polycystin-2 can also reconstitute a non-selective Ca^{2+} channel under certain conditions (7, 8).

The overlapping phenotype of PKD1 and PKD2 patients has led to the hypothesis that polycystin-1 and polycystin-2 could function as members of the same signaling pathway or as interacting partners in a heterodimeric protein complex. Consistent with this hypothesis, co-ordinate expression of polycystin-1 and polycystin-2 in the kidney and other tissues has been described (9) and recombinant fusion proteins containing the C-terminal domains of both proteins shown to heterodimerize in yeast two-hybrid systems (10, 11). Nevertheless, the existence of a native polycystin-1-polycystin-2 complex has yet to be demonstrated and is an area of current controversy. There are two main reasons for this uncertainty. First of all, the subcellular location of both proteins appears to be very different. There are convincing data demonstrating an exclusive endoplasmic reticulum (ER) location for polycystin-2 (12), but yeast two-hybrid studies have shown that polycystin-2 can also interact with actin-binding proteins such as Hax-1 and CD2-AP via its C-terminal tail (13, 14). Polycystin-1 immunoreactivity has been observed in different plasma membrane domains as well as within cytoplasmic vesicles but not predominantly in the ER (reviewed in Ref. 15). This non-overlapping distribution has made it seem less likely that both proteins form a complex at least within the same membrane domain (see “Discussion”). Second, there is conflicting evidence as to whether polycystin-2 can reconstitute a functional channel independently of polycystin-1. A recent paper showed that transient overexpression of polycystin-1 and polycystin-2 but neither one alone increased a non-selective whole-cell conductance in transfected CHO cells (16). In this study, surface expression of a putative polycystin-

Autosomal dominant polycystic kidney disease (ADPKD)¹ is the most common inherited human renal disease, affecting up to 15% of all patients on renal replacement therapy. In the past 7 years, *PKD1* and *PKD2*, the two genes defective in this

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¹ The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; CHO, Chinese hamster ovary; Endo H, endoglycosidase H; mAb, monoclonal antibody; ER, endoplasmic reticulum; LRR, leucine-rich repeat; NHK, normal adult human kidney; PNGase F, *N*-glycosidase F; MOPS, 4-morpholinepropanesulfonic acid; TRITC, tetramethylrhodamine isothiocyanate; afp30, affinity-purified p30.

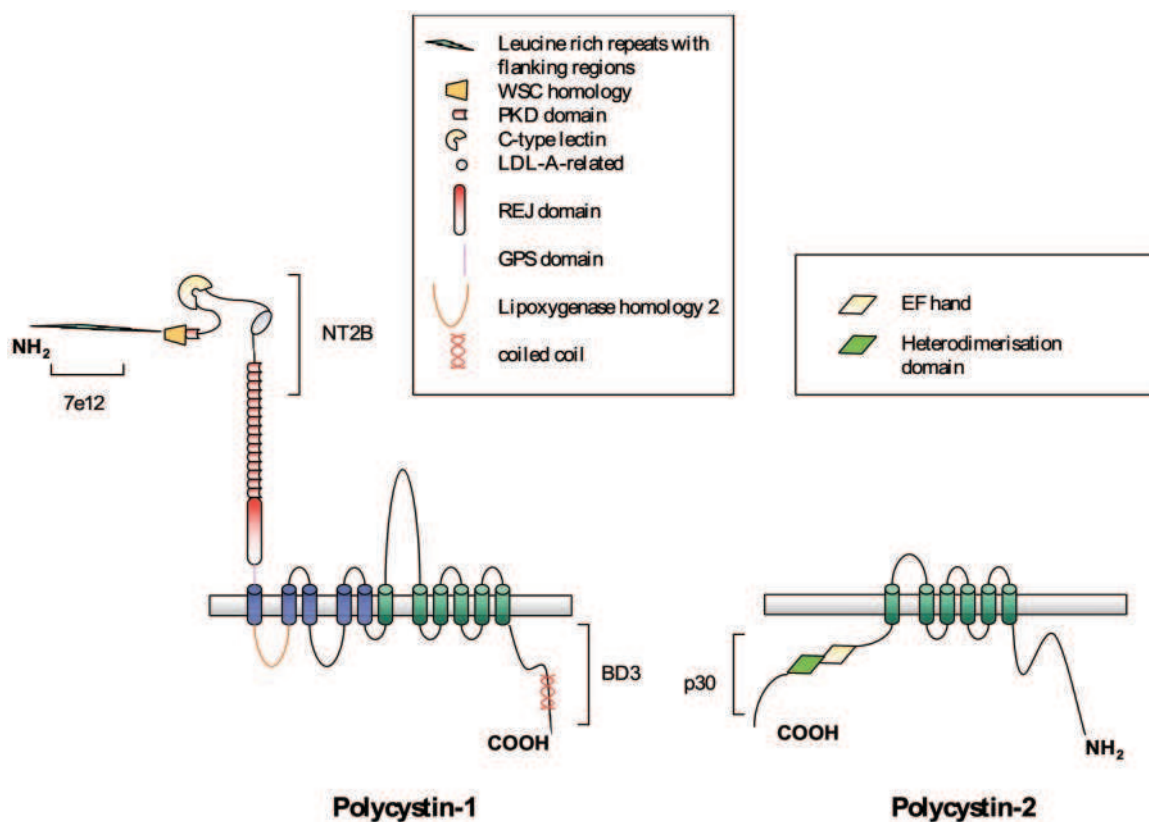


FIG. 1. Modular structure of polycystin-1 and polycystin-2 showing the different domains of each protein to which polycystin-1 (7e12, NT2B, BD3) and polycystin-2 (p30) antibodies were generated. For further details, refer to "Experimental Procedures."

1-polycystin-2 channel was inferred by overlapping signals with a co-transfected surface marker protein (CD4) in individual cells. However, using different experimental systems, two other groups later described surface Ca^{2+} channel activity reconstituted by polycystin-2 alone (7, 8). These differences might well be cell- or tissue-specific, but the demonstration of polycystin-1-independent polycystin-2 activity could also imply that the two proteins do not interact directly. This conclusion would be consistent with one observation of differential polycystin-1 and polycystin-2 expression (17).

The low levels of native polycystin-1 in tissues and cells have made functional studies of endogenous polycystin-1 very difficult (15). To overcome this problem, we have generated renal cell lines from newborn mice transgenic for human *PKD1* (18). These mice express a transgene comprising the entire human *PKD1* gene with its 5'- and 3'-flanking regions (20 kb upstream, 43 kb downstream) but excluding the HG loci (18). Expression of transgenic polycystin-1 was developmentally regulated in different tissues including the kidney, and expression of transgenic polycystin-1 could rescue the embryonically lethal *Pkd1* knockout phenotype observed in *pkd1del34* mice (18). We reasoned, therefore, that cells derived from these animals might be good models to study polycystin-1 function because the transgene appeared to be appropriately regulated and the gene product processed, transported, and expressed as a functional protein *in vivo*. Here we report for the first time the isolation, biochemical characterization, and subcellular localization of a polycystin-1-polycystin-2 protein complex in *PKD1* transgenic kidney cells and also describe the existence of this complex in non-transgenic kidney cells and normal adult human kidney.

EXPERIMENTAL PROCEDURES

Generation of *PKD1* Transgenic Lines—The generation of three founder *PKD1* transgenic mice has been described in a previous paper (18).

Like the endogenous murine protein, expression of transgenic polycystin-1 is markedly down-regulated between 2 and 4 weeks postnatally (18). We decided therefore to generate cell lines from newborn kidneys (embryonic day 18). Renal cells were cultured using standard techniques from eight newborn (embryonic day 18) mice (M1–M8) resulting from an F1 cross between a heterozygote *PKD1* transgenic mouse (TPK3) and a transgenic mouse homozygous for the temperature-sensitive T antigen (Immortomouse) (19). In brief, renal tissue was minced and digested with type 1 collagenase, and tubular fragments plated onto collagen-coated flasks. Cells were grown in low serum selective medium to encourage epithelial outgrowth (Dulbecco's modified Eagle's medium/F-12 supplemented with 5% Nuserum) and cultured at 33 °C in the presence of recombinant mouse γ -interferon (Roche Molecular Biochemicals, Mannheim, Germany) to activate T antigen expression (20).

Two lines (M7 and M8) were selected for further study. Because collecting ducts consistently show the highest polycystin-1 expression in developing and mature kidney (15), cells of collecting duct origin were isolated from primary culture by lectin affinity cell separation using the lectin DBA, which is exclusively expressed at the apical cell surface of collecting duct cells in mouse kidney (21). Cells positive for biotinylated DBA (Vector) were then purified using streptavidin-conjugated magnetic beads (M280 beads, Dynal) as previously described (21). These cells were found to express DBA at the cell surface but not the lectin, *Lotus tetragonolobus*, which is expressed by proximal tubular segments (data not shown). Cells were studied mainly at early passage (up to passage 15) and retained epithelial morphology throughout.

Northern Blotting—The isolation of the *PKD1* probes 3A3 (single copy) and JH8 (duplicated region) and *Pkd1* probe MS9 (exons 3–10) has been reported in previous papers (1, 18). A new N-terminal probe (PKD1NT) was generated as a 1.4-kb *Bgl*II-*Not*I fragment (nucleotides 1–1232). A 0.9-kb mouse utrophin *Eco*R1 fragment (nucleotides 425–1330) was used to detect a full-length utrophin transcript (13 kb) (gift of Dr. N. Y. Loh and Prof. K. E. Davies). Total RNA was extracted using TRIzol[®] reagent (Invitrogen, Paisley, UK) from cultured cells as described previously (22). The extracted RNA was quantified both by optical density at 260 nm and UV densitometry of the 18 S rRNA subunit. 15 μg of total RNA was run on a 1.2% (w/v) agarose/MOPS/formaldehyde gel, capillary-blotted onto Hybond N (Amersham Biosciences, Bucks, UK), and cross-linked with 70 mJ/cm² UV radiation (UV cross-linker, Amersham Biosciences). This membrane was probed

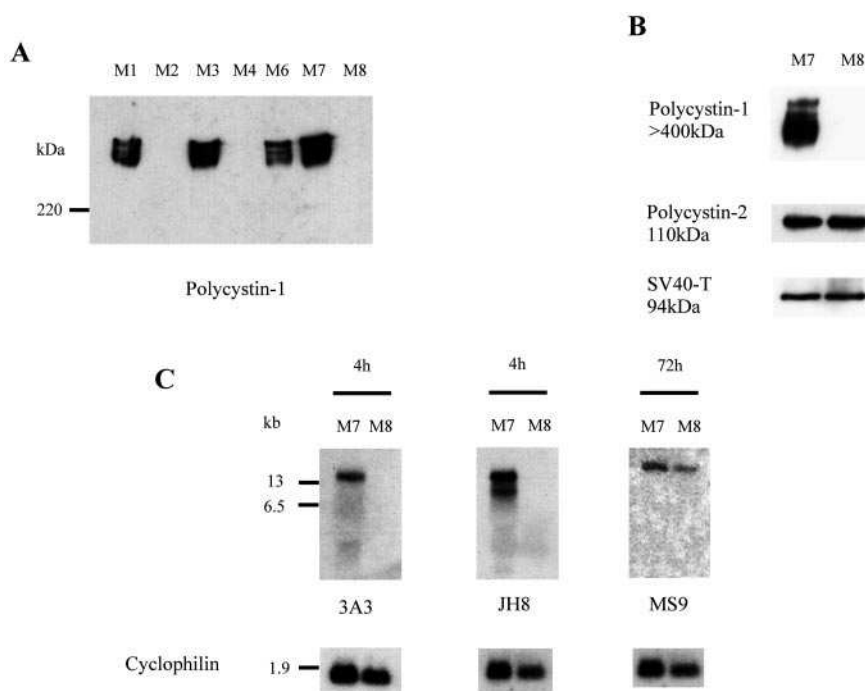


FIG. 2. Polycystin-1, PKD1, and Pkd1 expression by PKD1 transgenic and non-transgenic kidney cells. Four animals (M1, M3, M6, and M7) from a litter of eight newborn mice were found to carry the *PKD1* transgene by Southern blot. **A**, Western blotting of cell lysates with a polycystin-1 N-terminal mAb (7e12) showing the presence of several high molecular mass bands (>400 kDa) in these four lines (6% gel, SDS-PAGE). Data for M5 is not displayed on this gel. **B**, Western blotting of a transgenic (M7) and non-transgenic line (M8) for polycystin-1 (7e12), polycystin-2 (p30), and the SV40-T antigen (pAb416). Note that, although polycystin-1 expression remains undetectable in the non-transgenic line M8, equal amounts of polycystin-2 and SV40-T antigen were present in both lines. 20 μ g of total cell protein was loaded per lane. **C**, Northern analysis of RNA extracted from M7 and M8 lines. A single 14-kb band was detected in M7 alone with the C-terminal *PKD1* probe 3A3, but an additional band of 9.5 kb was also detected using probes to the middle (JH8) portion of *PKD1*. On prolonged exposure (72 h), single 14-kb bands can be seen in both lines with a *Pkd1* probe, MS9. Cyclophilin (1.8 kb) was used as an internal control for loading and RNA integrity.

with a [³²P]dCTP random-primed (Prime a gene, Promega, UK) specific DNA probe. Specific signals were obtained by exposing the blot to Biomax MS film for up to 72 h. The resulting autoradiograph was quantitated by scanning densitometry using a Bio-Rad GS-690 densitometer and Molecular Analyst version 4 software. Values were corrected for loading using repeat probing with the housekeeping gene cyclophilin (23).

Generation of Antibodies to Polycystin-1 and Polycystin-2—The generation of a mAb to the leucine-rich repeat (LRR) motif of human polycystin-1 (7e12), specific rabbit antisera to the C-terminal 258 amino acids of human polycystin-2 (p30), and C-terminal 205 amino acids of human polycystin-1 (BD3) have been described in previous papers (9, 24, 25). In this paper, we report the generation of new rabbit polyclonal antisera (PKD1NT2A, PKD1NT2B) to a large N-terminal region (*HincII* fragment, bp 358–3790) of human polycystin-1 (amino acids 50–1144) containing the LRR motif up to the fifth PKD repeat domain (3). A bacterial protein containing this region fused to a thioredoxin histidine tag was prepared in *Escherichia coli* (BL21 DE3) using the expression vector pET32a+ (Invitrogen, Groningen, The Netherlands). The protein was initially prepared as inclusion bodies, solubilized in 6 M guanidine, and purified by nickel affinity chromatography (9). It was then dialyzed against phosphate-buffered saline and the soluble protein used to immunize rabbits. The domains to which different polycystin-1 (7e12, NT2B, BD3) and polycystin-2 (p30) antibodies were raised are illustrated in Fig. 1.

Affinity Purification—The generation and characterization of p30, a specific antiserum to a fusion protein containing the C-terminal 258 amino acids of human polycystin-2, has been described in a previous publication (9). More recently, this antiserum has also been found to block cation channel activity reconstituted by recombinant polycystin-2 (7). p30 was further purified (afp30) by affinity purification to the same fusion protein coupled to cyanogen bromide (CNBr)-activated Sepharose beads (Sigma) according to the manufacturer's instructions. The generation of rabbit antisera to a large N-terminal region of human polycystin-1 (PKD1NT2A, PKD1NT2B) is described above. These antisera were affinity-purified (afNT2A and afNT2B) to the same fusion protein immobilized on a polyvinylidene difluoride membrane using a previously published method (26).

Antibody Biotinylation—An IgG fraction of p30 was isolated using

Protein A beads and biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce, Chester, UK) according to the manufacturer's instructions.

Immunoblotting—Plasma membrane enriched fractions from human kidney were prepared and processed for immunoblotting as previously described (9, 24). The binding of biotinylated antibody was detected with horseradish peroxidase-conjugated streptavidin (Amersham Biosciences) after an additional blocking step with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) to remove endogenous biotin signals.

Immunoprecipitation—Initial experiments demonstrated that the polycystin-1-polycystin-2 complex was equally present in cells cultured at permissive (33 °C) and non-permissive (37 °C for 48 h omitting γ -interferon) temperatures (data not shown). Subsequent immunoprecipitation experiments were therefore performed on cells grown at 33 °C. In brief, cell lysates or membrane fractions were obtained by extraction at 4 °C using detergent lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM sodium chloride, 25 mM sodium phosphate, pH 7) supplemented with a commercial protease inhibitor mixture (Complete™, Roche Molecular Biochemicals) and precleared for 1 h using recombinant Protein G-Sepharose beads (Gamma-bind, Amersham Biosciences). Protein G-antibody conjugates were then added to the cleared lysates and allowed to incubate on a rotator overnight at 4 °C. After extensive washes with the binding buffer, the beads were incubated with either reducing (containing 5% β -mercaptoethanol) or non-reducing sample buffer at 50 °C for 30–60 min and processed for immunoblotting.

Glycosylation Analysis—Membrane fractions from kidney or cultured cells were solubilized in 1% SDS, 10 mM Tris-HCl (pH 7.5) supplemented with a Complete™ mini protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride. Lysates corresponding to 10–50 μ g of total protein were treated with 500 units of endoglycosidase H (Endo H) or *N*-glycosidase F (PNGase F) according to the manufacturer's protocols (Roche Molecular Biochemicals) prior to SDS-PAGE and immunoblot analysis.

Subcellular Fractionation—Different membrane fractions were prepared from transgenic cells or human kidney tissue by density gradient ultracentrifugation. In brief, a postnuclear supernatant was first prepared after Dounce homogenization of a cell pellet or tissue homogenate by centrifugation at 1000 rpm for 10 min. A total cell membrane pellet was then isolated by centrifugation at 100,000 \times g for 1 h at 4 °C. Cell

membranes or post-nuclear supernatant (human kidney) were then layered onto a continuous 0–15% Optiprep (Sigma, Poole, UK) gradient and centrifuged at $200,000 \times g$ for 3h at 4 °C using a swinging bucket rotor. Seventeen fractions were recovered from the top, concentrated, and analyzed for the presence of polycystin-1 (7e12), polycystin-2 (p30), and marker organelle proteins such as calnexin (ER), GM130 (Golgi), and Na^+/K^+ -ATPase $\alpha 1$ subunit (plasma membrane) by immunoblotting. The relative abundance of each protein within individual fractions was quantitated by scanning densitometry and expressed as relative specific activity ($F_i P_i / P_i F_i$) where F_i = quantity of the marker in fraction i (arbitrary units), P_i = protein content of fraction i (mg), P_i = total cellular protein (mg), and F_i = total cellular content of the marker (arbitrary units).

Immunofluorescence—Cells were grown on collagen-coated glass coverslips and were fixed with freshly prepared 4% paraformaldehyde or ice-cold methanol-acetone. Alternatively, they were extracted for 10 min with 0.5% Triton X-100 prior to fixation using a previously published protocol (27). Dual-color immunofluorescence was performed using 7e12 (polycystin-1) and afp30 (polycystin-2), respectively. Controls included cells stained with the primary antibody omitted, an irrelevant mouse IgG1 mAb (Serotec, Kidlington, UK) as a control for polycystin-1, or a non-immune rabbit IgG fraction (Dako, Ely, UK) as a control for polycystin-2. The specificity of polycystin-2 staining was also demonstrated by pre-incubating afp30 with a polycystin-2 C-terminal fusion protein as previously described (9). TRITC-labeled wheat germ agglutinin (Vector Laboratories) was used to label the Golgi and an antibody to Sec 61 β (gift of Dr. T. Rapoport, Harvard Medical School, Cambridge, MA) was used to label the ER. Antibody binding was visualized using fluorescein isothiocyanate-conjugated goat anti-mouse IgG and TRITC-labeled goat-anti-rabbit secondary antibodies.

Materials—All chemicals were purchased from Sigma (Poole, UK) unless otherwise stated. Antibodies to calnexin, E-cadherin, and GM130 were purchased from BD Transduction Laboratories (Oxford, UK). mAbs to utrophin (MANCHO3, MANCHO7) and SV40-T antigen (pAb416) were gifts of Prof. G. E. Morris and Dr. P. Jat, respectively. COS-1 cells were a gift of Dr. D. L. Simmons (University of Oxford, Oxford, UK).

RESULTS

Transgenic Renal Cells Express Full-length Human Polycystin-1—Four *PKD1* transgenic cell lines were generated from a litter of eight newborn mice resulting from an F1 cross between a heterozygote *PKD1* transgenic mouse (TPK3) and a mouse homozygous for the temperature-sensitive T antigen (Immortomouse). These lines were shown to express high levels of full-length human polycystin-1 by immunoblotting with the N-terminal mAb, 7e12 (Fig. 2A). Polycystin-1 was detected as several (3–4) bands of high molecular mass (>400 kDa) in these cells and also as several smaller minor bands (<220 kDa) visible on prolonged exposure (compare Figs. 2A and 4A). The polycystin-1 antibody used (7e12) was raised to the flank-LRR-flank region within the N terminus of human polycystin-1 (Fig. 1) (24) but does cross-react with murine polycystin-1 (Fig. 5A). However, the native murine protein was only detectable by immunoblotting at much higher protein concentrations (300 μg of protein; data not shown) in non-transgenic cells (M8) but not at the lower protein concentrations (20 μg) shown here (Fig. 2B). In contrast, polycystin-2 and the SV40-T antigen were present in equivalent amounts in both transgenic (M7) and non-transgenic (M8) cells (Fig. 2B). Thus, overexpression of polycystin-1 had no effect on steady-state levels of polycystin-2.

Using probes to coding sequences within the C-terminal tail (3A3), the middle extracellular portion (JH8), and the extreme N terminus (PKD1NT) of *PKD1* (data not shown), the presence of a full-length 14-kb human transcript was demonstrated on Northern analysis in M7 cells but not in M8 (Fig. 2C). The 13-kb murine dystrophin transcript was used as a size marker. M7 cells also express a major RNA species of approximately 9.5 kb in size (detectable with JH8 and PKD1NT), which contains the predicted extracellular portion of *PKD1* but which lacks the C-terminal tail. This is likely to represent a splice form of the gene and may account for a form of the protein (180 kDa)

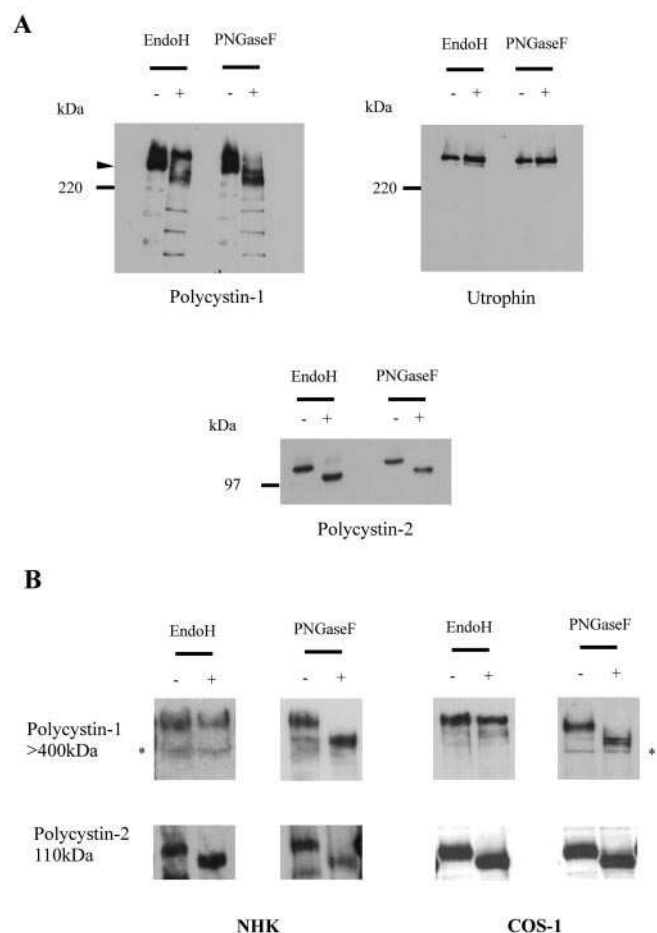


FIG. 3. Glycosylation analysis of polycystin-1 and polycystin-2 in transgenic cell lysates (A), NHK (B), and COS-1 membranes. Polycystin-1 and polycystin-2 expression was detected using 7e12 and p30, respectively. Both polycystin-1 and polycystin-2 are significantly *N*-glycosylated, as shown by their apparent shift in molecular mass following PNGase F treatment. Significantly, an Endo H-resistant polycystin-1 fraction (>400 kDa) was observed in M7 lysates, NHK, and COS-1 cells. With prolonged exposure, smaller (<220 kDa) Endo H- and PNGase F-sensitive fragments can be visualized in M7 lysates by immunoblotting with 7e12; these are likely to represent N-terminal polycystin-1 species. Note also that a minor unglycosylated cross-reactive band (*) can sometimes be seen in NHK and COS-1 cells above the 220-kDa size marker with longer exposure times; this is unlikely to represent a polycystin-1 species. In contrast, polycystin-2 was sensitive to Endo H and PNGase F in all three preparations. The arrowhead in A shows the position of the unglycosylated endogenous protein, utrophin, used as a size marker (400 kDa) and as an internal control for protein integrity and loading. 30–100 μg of total protein were loaded per lane (5% gel, SDS-PAGE). The results shown are a representative experiment of three performed.

especially prominent in transgenic brain (18). Several potential splice sites of the *PKD1* transcript have been identified: intron 12 (mouse *Pkd1*), intron 16 (human), and exon 24 (human) (3, 28), but the latter would seem to fit best with the estimated size of the alternative transcript. The absence of other smaller RNA species also suggests that not all the small N-terminal fragments detected by immunoblotting can be related to alternative splicing. Using a mouse-specific probe (MS9), we found that both lines expressed equally low levels of full-length *Pkd1* mRNA of the same size after prolonged exposure (72 h) but no other splice forms were detected under these conditions (Fig. 2C). Thus, expression of transgenic *PKD1* did not affect steady-state expression of the endogenous *Pkd1* gene, even though expression of *PKD1* was up to 22 times greater than that of *Pkd1* by densitometry. This is consistent with a previous esti-

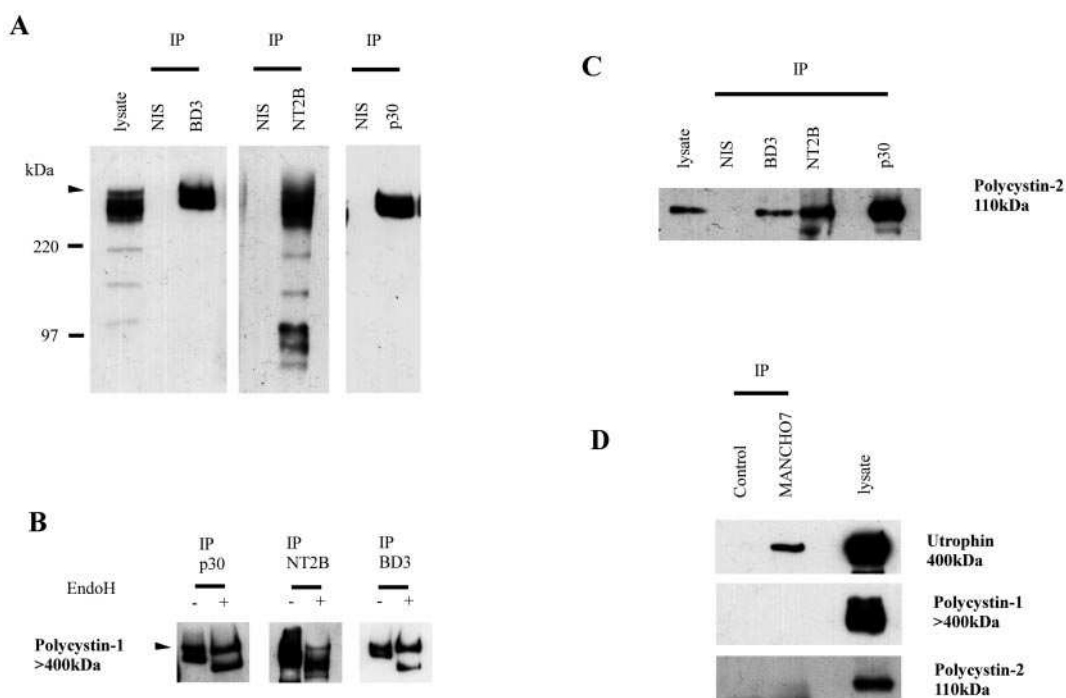


FIG. 4. Isolation of a polycystin-1-polycystin-2 protein complex from M7 transgenic cell lysates. Immunoprecipitated polycystin-1 and polycystin-2 were detected by immunoblotting with 7e12 and biotinylated p30, respectively. *A*, the N-terminal polycystin-1 antibody PKD1NT2B precipitated several high (>400 kDa) and low (<220 kDa) molecular mass polycystin-1 bands, whereas polycystin-2 co-immunoprecipitated with the two highest molecular mass polycystin-1 bands normally present as minor bands in total cell lysate. These two bands correspond to full-length polycystin-1 because they are also immunoprecipitated by a C-terminal polycystin-1 antibody, BD3. Non-immune rabbit serum (*NIS*) and pre-immune sera (data not shown) served as negative controls. The *lysate lanes* contained 30 μ g of protein, and each *IP lane* represents protein immunoprecipitated from 125 μ g (PKD1NT2B) or 500 μ g (p30) of total cellular protein (5% gel, SDS-PAGE). A representative gel of three experiments is displayed. The *arrowhead* indicates the highest molecular weight polycystin-1 band detected that is Endo H-resistant (see below). *B*, immunoprecipitated polycystin-1 was treated with the deglycosylating enzyme Endo H (+) or enzyme buffer (-). The identical results with p30 and BD3 confirm that polycystin-2 co-immunoprecipitated with two full-length polycystin-1 species, one Endo H-resistant (*arrowhead*) and the other Endo H-sensitive. A single Endo H-resistant band (*arrowhead*) and up to three Endo H-sensitive high molecular weight polycystin-1 bands were immunoprecipitated by PKD1NT2B. *C*, polycystin-2 was immunoprecipitated by p30 and co-immunoprecipitated by the polycystin-1 antibodies, PKD1NT2B and BD3. Each *IP lane* represents protein immunoprecipitated from 250 μ g (p30) or 500 μ g (PKD1NT2B, BD3) of total cell lysate. *D*, a mAb to utrophin (MANCHO7) does not immunoprecipitate polycystin-1 or polycystin-2 from M7 lysates. An irrelevant mouse IgG2a mAb (*Control*) was used to show the specificity of MANCHO7. Utrophin was detected by immunoblotting with MANCHO3. The *lysate lanes* represent 30 μ g of total protein loading, and the *IP lanes* represent protein immunoprecipitated from 500 μ g of total lysate.

mate of up to 28 copies of the transgene in this founder animal (TPK3) by Southern analysis (18).

Glycosylation Analysis of Polycystin-1 and Polycystin-2—Analysis of the secondary structure of human polycystin-1 predicts 60 putative external *N*-linked glycosylation sites, but the glycosylation profile of polycystin-1 has not been previously studied (3). Using the enzyme PNGase F, which efficiently cleaves *N*-linked carbohydrate groups, we demonstrate that human polycystin-1 is heavily *N*-glycosylated as indicated by a significant size shift in its molecular mass following enzymatic deglycosylation (Fig. 3A). Of interest, both larger (>400 kDa) and smaller (<220 kDa) forms of the protein detected by this antibody showed a major size shift after PNGase F treatment, indicating that they are likely to represent specific *N*-glycosylated polycystin-1 species. The dystrophin-related protein, utrophin, is unglycosylated and has a molecular mass of approximately 400 kDa (29). Reprobing these membranes with a utrophin antibody (MANCHO3) revealed that the highest forms of polycystin-1 had a molecular mass of ~400 kDa after PNGase F treatment (Fig. 3A). The finding that utrophin was unaffected by enzyme treatment with PNGase F (and Endo H) also confirms that the existence of these smaller polycystin-1 bands is unlikely to have resulted from proteolytic degradation either during extraction or by contaminating proteases in the commercial enzyme preparations. The predicted unglycosylated molecular mass of polycystin-1 is 460 kDa (3).

Because several high molecular weight polycystin-1 bands

(up to four) were consistently detected, we wondered if other differences in glycosylation might account for some or all of these species. Endoglycosidase H is an enzyme that specifically cleaves high mannose type oligosaccharides from the protein backbone (30). Resistance to Endo H is typically acquired as the protein undergoes further post-translational modification of carbohydrate residues in the Golgi (30). Using this enzyme, we found that the highest molecular weight band was Endo H-resistant, whereas the other three bands were Endo H-sensitive (Fig. 3A). Biochemically, this Endo H-resistant fraction should have reached the cis-Golgi and could potentially be targeted to the plasma membrane.

To confirm that these findings were not restricted to transgenic human polycystin-1, normal adult human kidney (NHK) membrane fractions were subjected to enzymatic deglycosylation with either PNGase F or Endo H. Parallel studies were also performed with COS-1, a cell line that we had previously shown to express both polycystin-1 and polycystin-2 (9, 24). As shown in Fig. 3B, a high molecular weight Endo H-resistant polycystin-1 fraction was also detected in NHK and COS-1 cells. However, an Endo H-sensitive polycystin-1 fraction was not clearly visualized in NHK and faintly seen in COS-1 cells, possibly because of the much lower levels of native polycystin-1 compared with that expressed in transgenic cells.

Analysis of the secondary structure of polycystin-2 predicts five external *N*-linked glycosylation sites in this protein. PNGase F treatment confirmed that both human and murine

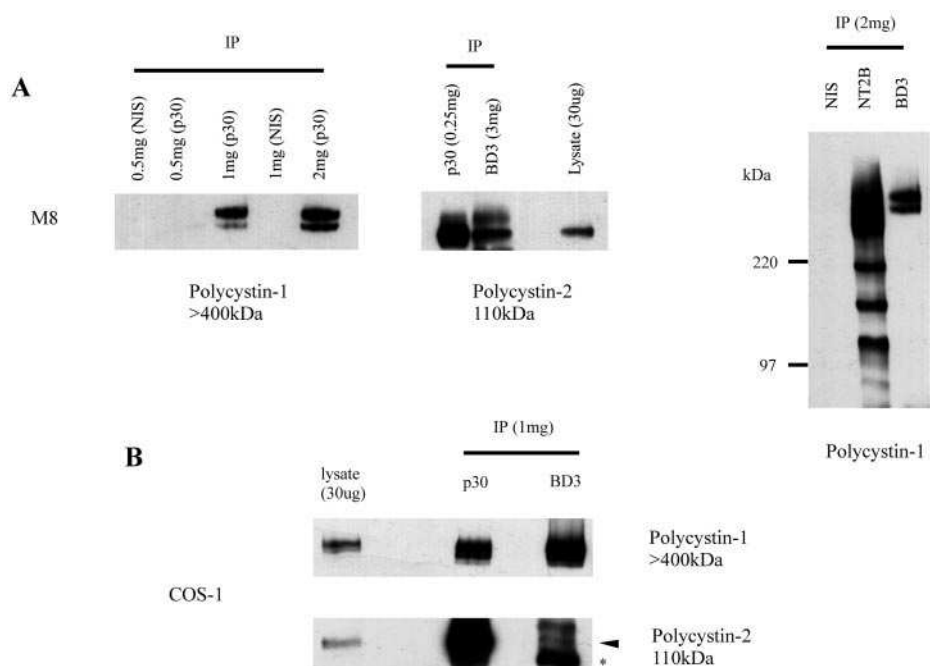


FIG. 5. Isolation of a native polycystin-1-polycystin-2 protein complex from M8 non-transgenic cells and COS-1 cells. *A*, polycystin-2 co-immunoprecipitated with native polycystin-1 in M8 cells but the abundance of this complex was only detectable with higher protein concentrations, higher antibody concentrations, and prolonged exposure. *Left panel*, starting material of 0.5, 1, and 2 mg of total cell lysate for immunoprecipitation (IP) with p30. *Middle panel*, 12 times the starting material was used for immunoprecipitation of polycystin-2 with BD3 (3 mg) compared with p30 (0.25 mg). *Right panel*, smaller molecular mass bands (<220 kDa) are seen to be enriched by NT2B from M8 cell lysates. In contrast, like p30, BD3 enriched only two high molecular weight polycystin-1 bands in M8 cells. These findings are identical to those from M7 cells (see Fig. 4). *B*, co-immunoprecipitation of polycystin-1 and polycystin-2 from COS-1 cells. Equal amounts of starting material (1 mg) were used for immunoprecipitation with p30 and BD3. The arrowhead indicates the position of monomeric polycystin-2. Two other cross-reactive bands are visible with longer exposure; the lower band (~100 kDa, *) in the BD3 lane represents partially reduced rabbit immunoglobulin heavy chains; the higher band may represent an endogenous biotinylated protein.

polycystin-2 are significantly *N*-glycosylated, but, unlike polycystin-1, native polycystin-2 was sensitive to Endo H in *PKD1* transgenic cells, COS-1 cells, and normal adult human kidney (Fig. 3). These results are in agreement with a previous study of polycystin-2 (12). The calculated unglycosylated molecular mass of polycystin-2 is 110 kDa, so this suggests that the glycosylated molecular mass is >110 kDa (2).

Polycystin-2 Interacts with Full-length Polycystin-1—In preliminary studies, 7e12 did not efficiently immunoprecipitate polycystin-1. To study the potential interaction between the two polycystin proteins, new polycystin-1 antiserum was therefore generated to a large N-terminal region of human polycystin-1 (PKD1NT2A, PKD1NT2B) (Fig. 1). Both antisera were found to be equally efficient in precipitating both large (>400 kDa) and smaller (<220 kDa) polycystin-1 bands from transgenic cells and tissues (Fig. 4A); PKD1NT2B was used in subsequent experiments. Although up to four high molecular weight bands were enriched by PKD1NT2B, we found that p30 immunoprecipitated only the two highest molecular weight polycystin-1 bands (Fig. 4A). Identical results were found with affinity-purified antisera afp30 and afNT2B (data not shown). Using a previously described polycystin-1 C-terminal antibody (BD3), we subsequently confirmed that the two bands co-immunoprecipitated by p30 represent full-length polycystin-1 (Fig. 4A). Pre-immune sera from PKD1NT2A, PKD1NT2B, and p30 rabbits did not immunoprecipitate polycystin-1 or polycystin-2 (data not shown).

Further analysis using the enzyme Endo H enabled us to distinguish two subpopulations of full-length polycystin-1, *i.e.* Endo H-resistant and Endo H-sensitive species capable of interaction with polycystin-2 (Fig. 4, *B* and *C*). This suggests that polycystin-2 first associates with polycystin-1 in the ER/cis-Golgi, and this complex undergoes further maturation by un-

dergoing complex glycosylation through the Golgi prior to insertion into the plasma membrane. In addition to these two bands, two other Endo H-sensitive high molecular weight polycystin-1 bands were immunoprecipitated by PKD1NT2B but not recognized by BD3 (Fig. 4B). Because BD3 recognizes an epitope within a 205-amino acid C-terminal sequence of polycystin-1 (Fig. 1), it is highly likely that these two bands represent truncated forms of polycystin-1 containing most of the polycystin-1 sequence but lacking the C-terminal tail. The specificity of these findings is indicated by the negative results obtained using non-immune serum (Fig. 4, *A* and *C*), pre-immune serum (data not shown), a mAb to an abundant cellular protein, utrophin (Fig. 4D), and an irrelevant mouse mAb (Fig. 4D) for combined immunoprecipitation and immunoblotting studies.

To exclude the possibility that the polycystin-1-polycystin-2 complex identified from transgenic cells might be the result of overexpression of polycystin-1, the same co-immunoprecipitation experiments were carried out on non-transgenic cells (M8), COS-1 cells, and NHK membranes. As illustrated in Fig. 5, polycystin-1 and polycystin-2 also co-immunoprecipitated in non-transgenic M8 cells. Native polycystin-1 expression in M8 was very low and could only be demonstrated by using 4–6 times as much protein in a combined immunoprecipitation/immunoblotting protocol and by increasing both the concentration and incubation time of primary and secondary antibodies used for detection (Fig. 5A). Nevertheless, immunoprecipitation with BD3 and p30 enriched two high molecular mass (>400 kDa) bands as observed in the M7 transgenic cells, whereas NT2B enriched both high (>400 kDa) and low (<220 kDa) molecular mass species in these cells (Fig. 5A). In both M7 and M8 cells, only a minor fraction of polycystin-2 appeared to associate with either transgenic or native polycystin-1 (com-

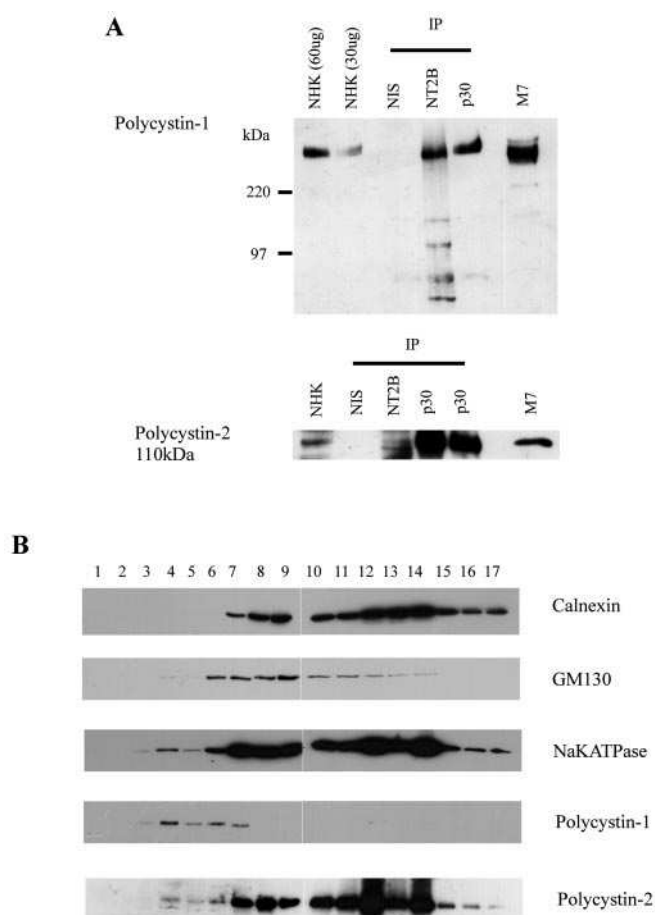


FIG. 6. Isolation of a polycystin-1-polycystin-2 protein complex from normal human kidney. A, polycystin-1 and polycystin-2 were present in very low abundance in NHK but could be immunoprecipitated by PKD1NT2B and p30, respectively. As with M7 cells, both antibodies also co-immunoprecipitated the other polycystin partner, although the polycystin-2 signal with PKD1NT2B was much weaker. No specific band was detected after immunoprecipitation with non-immune serum (NIS). Each IP lane represents protein immunoprecipitated from 0.5 mg (p30) to 2 mg (NT2B) of total cell lysate. B, using similar conditions for density gradient centrifugation as described in Fig. 7, polycystin-1 and polycystin-2 could be detected in plasma membrane fractions (F3–F5) from NHK and both proteins were also present in Golgi fractions (F6–F9). Polycystin-2 was, however, most abundant in the denser ER fractions (F12–F14), as seen in the M7 cells. A representative experiment of two is displayed.

pare Figs. 4C and 5A). We estimate by densitometry that, in both cells, this is less than 10% (7–8%) of total steady-state polycystin-2 concentrations. These results imply that polycystin-2 binding to polycystin-1 remains tightly regulated in transgenic cells despite the large increase in polycystin-1 expression.

Similarly, a polycystin-1-polycystin-2 complex was identified in COS-1 cells (Fig. 5B) and NHK membranes (Fig. 6A). As with M8 cells, NT2B also enriched low molecular weight polycystin-1 species in COS-1 (data not shown) and NHK (Fig. 6A), confirming that their existence was not the result of polycystin-1 overexpression. As they are minor species in all these native preparations, they are more abundant in transgenic cells and therefore more easily detectable.

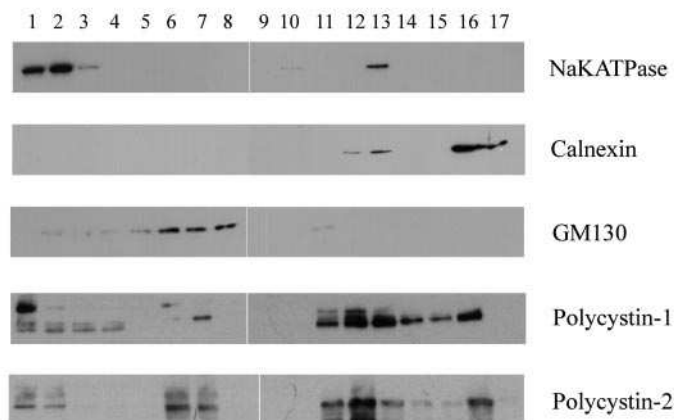
Co-sedimentation and Co-immunoprecipitation of Endo H-resistant Polycystin-1 and Polycystin-2 from Plasma Membrane Fractions of Transgenic Cells—To further define the subcellular location of the Endo H-resistant and Endo H-sensitive polycystin-1 species capable of interaction with polycystin-2, different subcellular membrane compartments were prepared from

transgenic cell membranes by density gradient centrifugation. We obtained a consistent and clear separation between the lightest fractions (F1–F3) enriched for plasma membrane proteins ($\alpha 1$ subunit, Na^+/K^+ -ATPase), the middle fractions (F6–F8) for a Golgi protein GM130, and the heaviest fractions (F12–F17, especially F16 and F17) for an ER-resident protein calnexin (Fig. 7A). Differential distribution of polycystin-1 subpopulations was found between these fractions. In particular, the highest molecular weight Endo H-resistant polycystin-1 band was enriched in plasma membrane fractions, whereas the high molecular weight Endo H-sensitive bands were found mainly in the heavier ER fractions (Fig. 7A). Polycystin-2 was detected mainly in ER fractions but was visible in Golgi fractions and a small subpopulation also clearly detectable in plasma membrane fractions (Fig. 7, A and B). Significantly, these plasma membrane fractions completely excluded calnexin but, as expected, were highly enriched for the $\alpha 1$ subunit of Na^+/K^+ -ATPase (82% of total). Importantly, Endo H-resistant polycystin-1 could be selectively co-immunoprecipitated with polycystin-2 from the lightest fractions (F1 and F2), confirming that they form a heterodimeric complex located in plasma membranes (Fig. 7C).

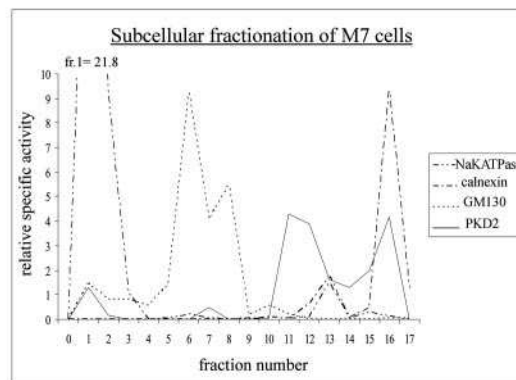
Co-sedimentation of Polycystin-1 and Polycystin-2 in Plasma Membrane Fractions of Normal Human Kidney—Although we demonstrated the existence of a complex of polycystin-1 and polycystin-2 in plasma membrane-enriched fractions from normal human kidney (Fig. 6A), the method of isolation used does not reliably exclude a minor contamination with ER membranes (24). Density gradient centrifugation was therefore used to obtain clearer separation between plasma membranes and ER membranes to demonstrate the presence of populations of polycystin-1 and polycystin-2 in plasma membranes. As shown in Fig. 6B, human kidney membranes separated in slightly different peaks to that seen with M7 cells (Fig. 7A) under the same gradient conditions. The $\alpha 1$ subunit of Na^+/K^+ -ATPase was more evenly distributed throughout the gradient than with M7 cells, although it was clearly detected in the lightest fractions (F3–F5), which excluded Golgi (GM130)- and ER (calnexin)-resident proteins. Calnexin was not detected in these fractions even after prolonged exposure (30 min, Fig. 7A). Thus clear separation between these lighter plasma membranes (F3–F5) and heavier ER membranes (F8–F17, peak in F12–F14) was achieved. As expected, Golgi membranes sedimented in the middle fractions (F6–F9), but calnexin was detected in F7–F9 after prolonged exposure. Under these conditions, polycystin-1 was found mainly in plasma membrane and Golgi fractions, whereas polycystin-2 was found predominantly in the ER. Nevertheless, as observed with M7 cells, significant polycystin-2 expression was also detectable in Golgi fractions and a minor fraction clearly present in plasma membranes (Fig. 6B). The highest relative specific activities for polycystin-2 and calnexin were found in F14 (3.59, 4.27), confirming that most of kidney polycystin-2 is present in the ER. However, a minor fraction of polycystin-2 was also found in plasma membrane fractions that excluded calnexin, e.g. F4 (relative specific activity 0.21 and 0), respectively. This fraction also had the highest relative specific activity for polycystin-1 (12.4).

Higher Molecular Mass Forms of Polycystin-2—In addition to a band of ~ 110 kDa corresponding to monomeric polycystin-2 from transgenic cell lysates, p30 immunoprecipitated at least two other high molecular mass polycystin-2 bands, which were more prominent under non-reducing conditions (Fig. 8A); similar results were found in non-transgenic cells (Fig. 8A). To ascertain if these higher molecular mass bands might represent physiological homodimers of polycystin-2, NHK membranes were run under non-reducing conditions and detected

A



B



C

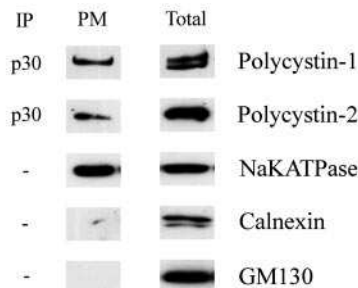


FIG. 7. Isolation of a plasma membrane polycystin-1-polycystin-2 complex from M7 transgenic cells by subcellular fractionation and co-immunoprecipitation. *A*, using density gradient centrifugation, fractions enriched for the plasma membrane protein subunit α -Na⁺-K⁺-ATPase (F1–F3), the Golgi protein GM130 (F6–F8), and the ER protein calnexin (F12–F17) were isolated and analyzed for the presence of polycystin-1 (7e12) and polycystin-2 (p30). The highest molecular weight Endo H-resistant polycystin-1 band was found to be highly enriched in a plasma membrane fraction F1, whereas the lower Endo H-sensitive polycystin-1 bands were enriched in the denser ER fractions F12–F17. Polycystin-2 was present predominantly in the same ER fractions but was also detectable in Golgi (F6–F8) and plasma membrane fractions (F1 and F2). A representative experiment of three is displayed. *B*, the abundance of polycystin-2 (PKD2) in relation to specific organelle marker proteins in each fraction was quantified by scanning densitometry and expressed as relative specific activity (see “Experimental Procedures”). Note that the distribution pattern of polycystin-2 is very different from that of the ER resident protein calnexin. *C*, unlike total cell membranes (*Total*), a single Endo H-resistant polycystin-1 band was co-immunoprecipitated with polycystin-2 from plasma membrane fractions (*PM*) by p30.

with p30. As shown in Fig. 8*B*, the two higher molecular mass bands became equally prominent as the full-length monomeric polycystin-2 band detected by p30 under reducing conditions (110 kDa), suggesting that they could represent native polycystin-2 oligomers. Alternatively, these higher molecular mass species could represent polycystin-2 bound to other (as yet unidentified) proteins.

Immunolocalization of Polycystin-1 and Polycystin-2 in Transgenic Cells—Both polycystin-1 (7e12) and polycystin-2 (p30) antibodies cross-react with the murine proteins. Polycystin-1 expression in M7 cells was clearly more intense than in M8 cells by immunofluorescence (Fig. 9). Most of the signal detected was intracellular and co-localized with both ER and Golgi marker proteins (data not shown), consistent with the results of subcellular fractionation (Fig. 7). Previous studies had shown that three pools of cadherin-catenin complexes (intracellular, at the lateral membrane, and at the apical junctional complex) in Madin-Darby canine kidney cells can be distinguished in part by their differential solubility in the detergent Triton X-100 (27). Taking the same approach, we found detectable labeling for polycystin-1 at the lateral cell surfaces after removal of the intracellular polycystin-1 pool with Triton X-100 (Fig. 9). As previously shown, E-cadherin expression was retained within the basolateral membrane domain under these conditions (Fig. 9) (27).

Like polycystin-1, the polycystin-2 signal was predominantly

intracellular, but, unlike polycystin-1, polycystin-2 expression was of equal intensity in both lines. As with polycystin-1, a subpopulation of membrane-associated polycystin-2 was identified after extraction with Triton X-100 (Fig. 9). Dual immunofluorescence studies further showed co-localization of both proteins with largely but not completely overlapping signals in extracted and non-extracted cells (Fig. 9, *C* and *F*). We also confirmed the retention of a significant proportion of polycystin-1, polycystin-2, and E-cadherin in the insoluble cell pellet by immunoblotting following prolonged (1 h) extraction with 0.5% Triton X-100 (Fig. 8*C*).

Because the polycystin-2 antibody, p30, does not distinguish between different forms of polycystin-2, it is possible that the polycystin-2 signal detected in lateral cell membranes could represent both polycystin-2 bound to polycystin-1 as well as polycystin-2 monomers/multimers. There is disagreement as to whether polycystin-2 can be independently targeted to the plasma membrane (7, 8, 16). However, our data would suggest that the polycystin-2 signal detected is most likely to represent that bound to polycystin-1 as part of a heterodimeric complex.

DISCUSSION

The identification of *PKD1*, the major gene mutated in ADPKD, was a major step in elucidating the pathogenesis of this common hereditary disease. However, progress in understanding the normal function of the *PKD1* protein, polycys-

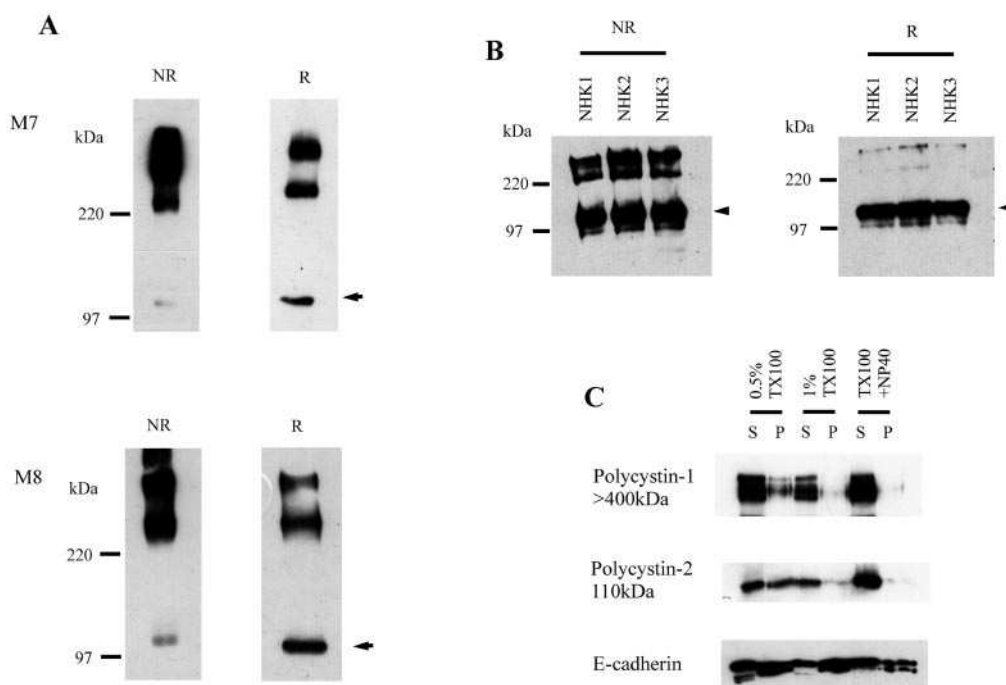


FIG. 8. Polycystin-2 exists as several subpopulations in transgenic cells (M7), non-transgenic cells (M8), and NHK. A, apart from monomeric polycystin-2 (~110 kDa, *arrowhead*), two other high molecular mass bands were consistently detected in M7 and M8 cells by immunoblotting following SDS-PAGE. These *panels* represent native polycystin-2 immunoprecipitated by affinity-purified p30 (afp30) from 250 μ g of total cell lysate. These bands were more prominent under non-reducing (NR) conditions, suggesting a tendency for polycystin-2 to dimerize or to bind other proteins. The results shown are a representative experiment of three performed. B, panels representing three adult NHK samples (NHK1–NHK3) resolved on SDS-PAGE (7.5%) under non-reducing (NR) or reducing (R) conditions. 100 μ g of NHK was loaded per lane. C, differential detergent solubility of polycystin-1 and polycystin-2 present in M7 cells. A residual fraction of both proteins remained in the cell pellet (P) following extraction (S) with 0.5% Triton X-100 but not after extraction with 1% Triton X-100 either singly or in combination with 0.5% Nonidet P-40. In contrast, E-cadherin (120 kDa) was not fully extracted by 1% Triton X-100 and 0.5% Nonidet P-40. 20 μ g of each fraction was loaded per lane.

tin-1 has been slow. This has been in part because of the size and complexity of the protein, the low levels of the native protein, the paucity of specific reagents, and the difficulty in generating cell lines expressing full-length recombinant polycystin-1 (reviewed in Ref. 15).

Progress in understanding the function of the *PKD2* protein, polycystin-2, has been more rapid. Recent studies suggest that it is an *N*-glycosylated integral membrane protein with significant homology to voltage-activated and transient receptor potential Ca^{2+} channels (2). Unexpectedly, glycosylation analysis of native and recombinant polycystin-2 has shown that it is completely Endo H-sensitive, suggesting that most, if not all, of polycystin-2 is normally located within the ER and/or cis-Golgi (12). Consistent with this finding, polycystin-2 has been localized to the ER within cells and tissues by immunohistochemistry, although some signal has also been detected in the basolateral cell membrane of mature mouse and rat kidney tubules (12, 31, 32). This has led to the suggestion that polycystin-2 might function primarily as an intracellular ER calcium channel. These results also imply that polycystin-1 and polycystin-2 could be essential members of the same signaling pathway without directly interacting with each other.

Our results support recent findings showing that transient co-expression of polycystin-1 and polycystin-2 in CHO cells reconstitutes a non-selective whole-cell conductance (16). We extend these findings by demonstrating that polycystin-1 and polycystin-2 interact to form a stable complex in *PKD1* transgenic kidney cells, non-transgenic kidney cells, and normal human adult kidney membranes. In addition, native polycystin-2 was unable to bind truncated forms of human polycystin-1 lacking the C-terminal 205 amino acids in transgenic cells, thus confirming previous data that the two proteins interact via their C-terminal tails (10, 11).

Our results also reveal, for the first time, an Endo H-resistant polycystin-1 population interacting with polycystin-2 and localizing preferentially to the lateral cell membranes of renal epithelial cells. These results are thus in keeping with recent immunolocalization findings for native polycystin-1 in Madin-Darby canine kidney cells (33, 34). Previous studies had revealed intracellular, apical, lateral, and basal cellular locations for native polycystin-1 in a variety of systems (reviewed in Ref. 15). The demonstration of different polycystin-1 subpopulations may in part explain these apparent discrepancies. Although there is likely to be a dynamic exchange between various pools of polycystin-1 similar to E-cadherin-catenin complexes (27), the proteins or signals regulating polycystin-1 trafficking to the plasma membrane are not known. Two studies have suggested that E-cadherin may itself be such a candidate molecule; one described a defect in E-cadherin targeting to the basolateral domain in cystic ADPKD cells, whereas the other found that polycystin-1 could co-immunoprecipitate with E-cadherin from the human pancreatic adenocarcinoma cell line HPAC (35, 36). It will be interesting to investigate whether these interactions are also dependent on polycystin-2 binding in renal tubular cells.

The first descriptions of potential homotypic and heterotypic interactions between the C termini of polycystin-1 and polycystin-2 came from yeast two-hybrid experiments. These studies suggested a tendency for homotypic interactions to occur preferentially to heterotypic interactions (10, 11). In one study, recombinant polycystin-2 was found to homodimerize via a coiled-coil domain in its C-terminal region, distinct from a more distal region responsible for heterodimerization with recombinant polycystin-1 (11). We found that native polycystin-2 could consistently be detected as slower migrating forms even under denaturing SDS-PAGE. The precise molecular weight of these

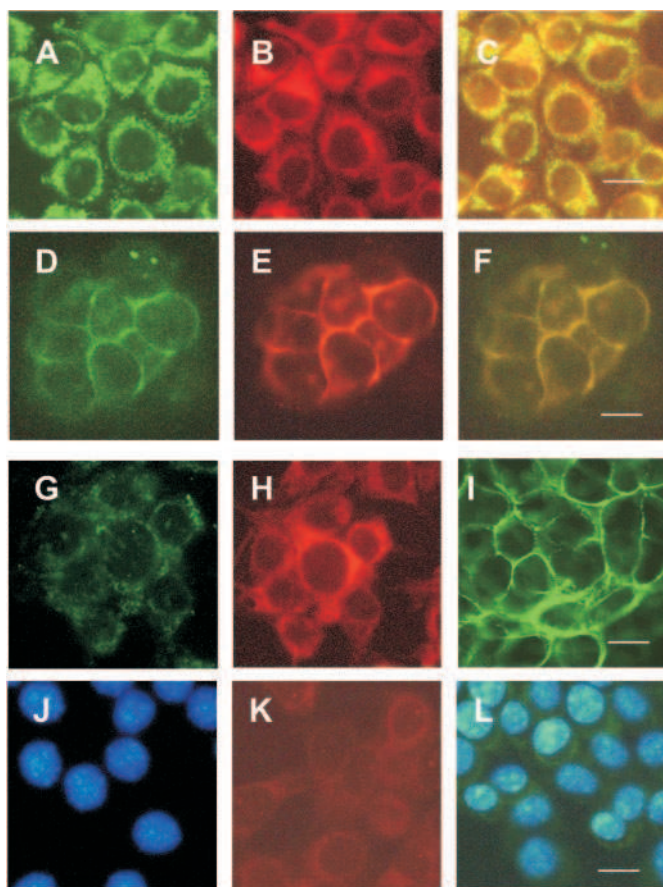


FIG. 9. Polycystin-1 and polycystin-2 expression in PKD1 transgenic and non-transgenic kidney cells. Polycystin-1 and polycystin-2 expression were detected with 7e12 and afp30, respectively, by dual-color immunofluorescence. In fixed M7 cells (A–C), polycystin-1 (A) and polycystin-2 (B) expression was mainly intracellular and largely, although not completely, overlapping (merged image in C). Polycystin-1 expression was more intense in M7 cells (A) than M8 cells (G), but polycystin-2 staining was of similar intensity for M7 (B) and M8 (H). Following differential Triton extraction, distinct staining of the cell-cell borders was detected for polycystin-1 (D), polycystin-2 (E), and E-cadherin (I) in M7 cells. A merged image for polycystin-1 and polycystin-2 is shown in F. Similar results for polycystin-2 and E-cadherin were found in M8 cells following Triton extraction (data not shown). For polycystin-1, a mouse IgG1 control mAb was negative for fixed (L) and extracted (data not shown) M7 cells. For polycystin-2, pre-adsorption of afp30 with a C-terminal polycystin-2 fusion protein led to loss of the polycystin-2 signal in fixed (K) and extracted (J) M7 cells. Nuclear counterstaining with 4,6-diamidino-2-phenylindole is illustrated in J and L. Non-immune rabbit IgG was negative on M7 and M8 cells (data not shown). Original magnification, $\times 400$ for all panels. Scale bars (10 μm) are as shown.

forms is uncertain, but they could represent either polycystin-2 oligomers or stable binding to other unidentified proteins. It is not clear at present whether polycystin-2 (monomers or oligomers) possesses functions independent of polycystin-1. In CHO cells, heterologous polycystin-2 alone was inactive, but another group showed that polycystin-2 could gate a non-selective Ca^{2+} channel independently of polycystin-1 (7, 16). In the latter study, it is of interest that multiple conductance states were observed, implying that the different polycystin-2 oligomers could simultaneously be involved in gating (7). It is also possible that, analogous to the transient receptor potential family of channel proteins, different combinations of polycystin-2 homo- and heteromultimers could be functionally distinct (37).

In contrast to the results for polycystin-1, we were unable to identify an Endo H-resistant fraction of native polycystin-2 in either cells or tissues. These results raise the question as to

where and how the interaction between polycystin-1 and polycystin-2 takes place. Two models can be proposed. In the first, polycystin-2 is co-translated and co-assembled with polycystin-1 and the complex then undergoes normal Golgi maturation (38). Polycystin-2 might undergo a conformational change enabling it to bind to polycystin-1 initially within the ER before being transported to the Golgi. As polycystin-2 remains Endo H-sensitive, this change is likely to be independent of complex glycosylation and could, for example, be mediated by changes in the binding of specific sugars, lipids, cations, or anions. In the second model, polycystin-2 remains anchored within the ER membrane and thus remains Endo H-sensitive. ER membrane microdomains enriched in polycystin-2 could then interact with membrane-anchored polycystin-1 within the plasma membrane or in the membranes of vesicles destined to fuse with the plasma membrane. The latter model is analogous to what has been proposed for the interaction of ER-located inositol trisphosphate receptors with surface-bound transient receptor potential channels (39). Further kinetic studies will be necessary to confirm this, but the presence of Endo H-sensitive and Endo H-resistant forms of polycystin-1 bound to polycystin-2 and the co-sedimentation of polycystin-1 and polycystin-2 in plasma membranes lends support to the first model. Finally, it should also be noted that Endo H-sensitive secretory proteins as well as membrane proteins with a mixture of Endo H-resistant and Endo H-sensitive N-linked glycosylation sites have been described (40, 41). Thus, Endo H sensitivity alone would not exclude normal maturation of polycystin-2 through the Golgi.

Although we have not provided direct evidence of function, the demonstration of this complex in transgenic and non-transgenic cells suggests that the formation of a polycystin complex is physiological. Its presence in normal adult human kidney, moreover, implies that a functional complex is still required for the maintenance of renal tubular structure apart from its role in tubular maturation during nephrogenesis (42, 43). If this is the case, then it seems likely that ADPKD cysts may arise not only in the fetus but also in the adult. The lateral location of this complex further suggests that the complex may play a major role in mediating or stabilizing cell-cell adhesion (33). However, it is probable that it also exerts other functions (5). Finally, the demonstration of a "Triton-insoluble" fraction of both proteins suggests that they may be anchored to the actin cytoskeleton and/or intermediate filaments (27). This finding also reconciles previous yeast two-hybrid data suggesting that the C terminus of polycystin-2 can bind to the actin cytoskeleton via the adapter proteins, Hax-1 and CD2-AP (13, 14).

In conclusion, we have demonstrated that polycystin-1 and polycystin-2 can interact *in vivo* to form a protein complex localized in part to the lateral plasma membrane domain of renal tubular cells. We conclude that disruption of this complex is likely to be of primary relevance to the pathogenesis of cyst formation in ADPKD and that haploinsufficiency at either locus could lead to cystogenesis (44, 45).

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REFERENCES

- European Polycystic Kidney Disease Consortium (1994) *Cell* **77**, 881–894
- Mochizuki, T., Wu, G., Hayashi, T., Xenophontos, S. L., Veldhuisen, B., Saris, J. J., Reynolds, D. M., Cai, Y., Gabow, P. A., Pierides, A., Kimberling, W. J., Breuning, M. H., Deltas, C. C., Peters, D. J., and Somlo, S. (1996) *Science* **272**, 1339–1342
- Hughes, J., Ward, C. J., Peral, B., Aspinwall, R., Clark, K., San Millan, J. L., Gamble, V., and Harris, P. C. (1995) *Nat. Genet.* **10**, 151–160
- Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133**, 809–817

5. Boletta, A., Qian, F., Onuchic, L. F., Bhunia, A. K., Phakdeekitcharoen, B., Hanaoka, K., Guggino, W., Monaco, L., and Germino, G. G. (2000) *Mol. Cell* **6**, 1267–1273
6. Chen, X. Z., Vassilev, P. M., Basora, N., Peng, J. B., Nomura, H., Segal, Y., Brown, E. M., Reeders, S. T., Hediger, M. A., and Zhou, J. (1999) *Nature* **401**, 383–386
7. Gonzalez-Perrett, S., Kim, K., Ibarra, C., Damiano, A. E., Zotta, E., Batelli, M., Harris, P. C., Reisin, I. L., Arnaout, M. A., and Cantiello, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1182–1187
8. Vassilev, P. M., Guo, L., Chen, X. Z., Segal, Y., Peng, J. B., Basora, N., Babakhanlou, H., Cruger, G., Kanazirska, M., Ye, C., Brown, E. M., Hediger, M. A., and Zhou, J. (2001) *Biochem. Biophys. Res. Commun.* **282**, 341–350
9. Ong, A. C., Ward, C. J., Butler, R. J., Biddolph, S., Bowker, C., Torra, R., Pei, Y., and Harris, P. C. (1999) *Am. J. Pathol.* **154**, 1721–1729
10. Qian, F., Germino, F. J., Cai, Y., Zhang, X., Somlo, S., and Germino, G. G. (1997) *Nat. Genet.* **16**, 179–183
11. Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V. P., and Walz, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6965–6970
12. Cai, Y., Maeda, Y., Cedzich, A., Torres, V. E., Wu, G., Hayashi, T., Mochizuki, T., Park, J. H., Witzgall, R., and Somlo, S. (1999) *J. Biol. Chem.* **274**, 28557–28565
13. Gallagher, A. R., Cedzich, A., Gretz, N., Somlo, S., and Witzgall, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4017–4022
14. Lehtonen, S., Ora, A., Olkkonen, V. M., Geng, L., Zerial, M., Somlo, S., and Lehtonen, E. (2000) *J. Biol. Chem.* **275**, 32888–32893
15. Ong, A. C. M. (2000) *Exp. Nephrol.* **8**, 208–214
16. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) *Nature* **408**, 990–994
17. Foggensteiner, L., Bevan, A. P., Thomas, R., Coleman, N., Boulter, C., Bradley, J., Ibraghimov Beskrovnaya, O., Klinger, K., and Sandford, R. (2000) *J. Am. Soc. Nephrol.* **11**, 814–827
18. Pritchard, L., Sloane-Stanley, J. A., Sharpe, J. A., Aspinwall, R. A., Lu, W., Buckle, V., Strmecki, L., Walker, D., Ward, C. J., Alpers, C. E., Zhou, J., Wood, W. G., and Harris, P. C. (2000) *Hum. Mol. Genet.* **9**, 2617–2627
19. Jat, P. S., Noble, M. D., Atalio, P., Tanaka, Y., Yannoutsos, N., Larsen, L., and Kioussis, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5096–5100
20. Jat, P. S., and Sharp, P. A. (1989) *Mol. Cell. Biol.* **9**, 1672–1681
21. Grupp, C., Troche, I., Steffgen, J., Langhans, S., Cohen, D. I., Brandl, L., and Muller, G. A. (1998) *Exp. Nephrol.* **6**, 542–550
22. Ward, C. J., Turley, H., Ong, A. C., Comley, M., Biddolph, S., Chetty, R., Ratcliffe, P. J., Gattner, K., and Harris, P. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 1524–1528
23. Bergsma, D. J., Eder, C., Gross, M., Kersten, H., Sylvester, D., Appelbaum, E., Cusimano, D., Livi, G. P., McLaughlin, M. M., and Kasyan, K. (1991) *J. Biol. Chem.* **266**, 23204–23214
24. Ong, A. C., Harris, P. C., Davies, D. R., Pritchard, L., Rossetti, S., Biddolph, S., Vaux, D. J., Migone, N., and Ward, C. J. (1999) *Kidney Int.* **56**, 1324–1333
25. Ibraghimov Beskrovnaya, O., Dackowski, W. R., Foggensteiner, L., Coleman, N., Thiru, S., Petry, L. R., Burn, T. C., Connors, T. D., Van Raay, T., Bradley, J., Qian, F., Onuchic, L. F., Watnick, T. J., Piontek, K., Hakim, R. M., Landes, G. M., Germino, G. G., Sandford, R., and Klinger, K. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6397–6402
26. Olmsted, J. B. (1981) *J. Biol. Chem.* **256**, 11955–11957
27. Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J., and Nelson, W. J. (1994) *J. Cell Biol.* **125**, 1341–1352
28. Lohning, C., Nowicka, U., and Frischauf, A. M. (1997) *Mamm. Genome* **8**, 307–311
29. Man, N. T., Ellis, J. M., Love, D. R., Davies, K. E., Gatter, K. C., Dickson, G., and Morris, G. E. (1991) *J. Cell Biol.* **115**, 1695–1700
30. Rothman, J. E., and Fine, R. E. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 780–784
31. Obermuller, N., Gallagher, A. R., Cai, Y., Gassler, N., Gretz, N., Somlo, S., and Witzgall, R. (1999) *Am. J. Physiol.* **277**, F914–F925
32. Markowitz, G. S., Cai, Y., Li, L., Wu, G., Ward, L. C., Somlo, S., and D'Agati, V. D. (1999) *Am. J. Physiol.* **277**, F17–F25
33. Ibraghimov Beskrovnaya, O., Bukanov, N. O., Donohue, L. C., Dackowski, W. R., Klinger, K. W., and Landes, G. M. (2000) *Hum. Mol. Genet.* **9**, 1641–1649
34. Scheffers, M. S., van der Bent, P., Prins, F., Spruit, L., Breuning, M. H., Litvinov, S. V., de Heer, E., and Peters, D. J. M. (2000) *Hum. Mol. Genet.* **9**, 2743–2750
35. Huan, Y., and van Adelsberg, J. (1999) *J. Clin. Invest.* **104**, 1459–1468
36. Charron, A. J., Nakamura, S., Bacallao, R., and Wandinger Ness, A. (2000) *J. Cell Biol.* **149**, 111–124
37. Xu, X. S., Li, H., Guggino, W. B., and Montell, C. (1997) *Cell* **89**, 1155–1164
38. Ackermann, U., and Geering, K. (1990) *FEBS Lett.* **269**, 105–108
39. Putney, J. W., Jr. (1999) *Cell* **99**, 5–8
40. Pollack, L., and Atkinson, P. H. (1983) *J. Cell Biol.* **97**, 293–300
41. Green, M. (1982) *J. Biol. Chem.* **257**, 9039–9042
42. Lu, W., Peissel, B., Babakhanlou, H., Pavlova, A., Geng, L., Fan, X., Larson, C., Brent, G., and Zhou, J. (1997) *Nat. Genet.* **17**, 179–181
43. Wu, G., D'Agati, V., Cai, Y., Markowitz, G., Park, J. H., Reynolds, D. M., Maeda, Y., Le, T. C., Hou, H., Jr., Kucheralapati, R., Edelmann, W., and Somlo, S. (1998) *Cell* **93**, 177–188
44. Watnick, T., He, N., Wang, K., Liang, Y., Parfrey, P., Hefferton, D., St. George-Hyslop, P., Germino, G., and Pei, Y. (2000) *Nat. Genet.* **25**, 143–144
45. Ong, A. C., and Harris, P. C. (1997) *Lancet* **349**, 1039–1040