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.

Autosomal dominant polycystic kidney disease (ADPKD) 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 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 (sulfated) 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, PKDL, 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-
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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 (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 JHS (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 BglII-NotI fragment (nucleotides 1–1232). A 0.9-kb mouse utrophin EcoR1 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
with a [32P]dCTP random-primed (Prime a gene, Promega, UK) specific antisera (PKD1NT2A, PKD1NT2B) to a large N-terminal region of human polycystin-1 (BD3) have been described in previous papers (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 preincubated 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 μg 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 × 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 × g for 3 h 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 α1 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 (Fᵢ/Pᵢ/Pᵢ) where Fᵢ = quantity of the marker in fraction i (arbitrary units), Fᵢ = protein content of fraction i (mg), Pᵢ = total cellular protein (mg), and Pᵢ = 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 See618 (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 mouse IgG1 mAb (Serotec, Kidlington, UK) were used as a control for polycystin-1, and an irrelevant 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 See618 (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.

NEW RESULTS

Transgenic Renal Cells Express Full-length Human Polycystin-1—Four 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 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-terminal portion (PKD1NT) of PKD1 (data not shown), the presence of a full-length 14-kb human transcript was demonstrated on Northern blots and 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) 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 (M09), we found that both bands expressed essentially 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 transcisgenic PKD1 did not affect steady-state expression of the endogenous Pkd1 gene, even though expression of PKD1 was up to 20 times greater than that of Pkd1 by densitometry. This is consistent with a previous esti-
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 and that the highest molecular weight polycystin-1 bands normally present as minor bands in total cell lysate. 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 μg of protein, and each IP lane represents protein immunoprecipitated from 125 μg (PKD1NT2B) or 500 μg (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 μg (p30) or 500 μg (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. Urophin was detected by immunoblotting with MANCHO3. The lysate lanes represent 30 μg of total protein loading, and the IP lanes represent protein immunoprecipitated from 500 μg of total lysate.

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
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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 antisera 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 undergrowing 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. 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.
Compared with normal human kidney (NHK), polycystin-1 and polycystin-2 were present at 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. 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–F17) as seen in the M7 cells. A representative experiment of two is displayed.

Polycystin-1 and Polycystin-2 Complexes

We obtained a consistent and clear separation between the lightest fractions (F1–F3) enriched for plasma membrane proteins (α1 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 sub-populations 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 α1 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).

**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.

Polycystin-1 and Polycystin-2 Complexes—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 (α1 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 sub-populations 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 α1 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 those seen with M7 cells (Fig. 7A) under the same gradient conditions. The α1 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...
with p30. As shown in Fig. 8B, 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. 8C).

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-
Polycystin-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 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.

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
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 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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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 (J) 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, ×400 for all panels. Scale bars (10 μm) are as shown.
