

## Identification and Characterization of Polycystin-2, the *PKD2* Gene Product\*

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***PKD2*, the second gene for the autosomal dominant polycystic kidney disease (ADPKD), encodes a protein, polycystin-2, with predicted structural similarity to cation channel subunits. However, the function of polycystin-2 remains unknown. We used polyclonal antisera specific for the intracellular NH<sub>2</sub> and COOH termini to identify polycystin-2 as an ~110-kDa integral membrane glycoprotein. Polycystin-2 from both native tissues and cells in culture is sensitive to Endo H suggesting the continued presence of high-mannose oligosaccharides typical of pre-middle Golgi proteins. Immunofluorescent cell staining of polycystin-2 shows a pattern consistent with localization in the endoplasmic reticulum. This finding is confirmed by co-localization with protein-disulfide isomerase as determined by double indirect immunofluorescence and co-distribution with calnexin in subcellular fractionation studies. Polycystin-2 translation products truncated at or after Gly<sup>821</sup> retain their exclusive endoplasmic reticulum localization while products truncated at or before Glu<sup>787</sup> additionally traffic to the plasma membrane. Truncation mutants that traffic to the plasma membrane acquire Endo H resistance and can be biotinylated on the cell surface in intact cells. The 34-amino acid region Glu<sup>787</sup>-Ser<sup>820</sup>, containing two putative phosphorylation sites, is responsible for the exclusive endoplasmic reticulum localization of polycystin-2 and is the site of specific interaction with an as yet unidentified protein binding partner for polycystin-2. The localization of full-length polycystin-2 to intracellular membranes raises the possibility that the *PKD2* gene product is a subunit of intracellular channel complexes.**

failure in ~50% of patients by age 60 (1). The second gene for ADPKD, *PKD2*, is mutated in ~15% of affected families (2) and is predicted to encode an integral membrane protein, polycystin-2, with six membrane spanning domains and intracellular NH<sub>2</sub> and COOH termini (3). An EF-hand domain with potential calcium-binding and regulatory function is described in its carboxyl-terminal region. Polycystin-2 bears some amino acid similarity to the family of voltage-activated  $\alpha_1$  calcium channel proteins, to polycystin-1 (3) and to the *trp* family of store operated calcium entry channels (4). Polycystin-2 is highly conserved in multicellular organisms including *Caenorhabditis elegans* (3). Recently, another novel member of the *PKD2* gene family, *PKD2L*, has been identified (5, 6).

Transcripts for the *PKD2* gene are widely expressed in fetal and adult tissues (3) while at least one study suggests that *PKD2L* shows a more restricted pattern of expression (6). Polycystin-2 interacts directly with polycystin-1, the protein product of the gene mutated in the majority of ADPKD families (7, 8). In addition, polycystin-2 is capable of homomultimerization (7). It has been hypothesized that these proteins are components of a signaling cascade with polycystin-1 acting as a receptor for either cell-matrix or cell-cell interactions (9–11) that may, in turn, regulate the activity of polycystin-2 through their direct interaction (3). The observation that ADPKD patients with mutations in either *PKD1* and *PKD2* share virtually identical clinical phenotypes, including the full spectrum of extrarenal manifestations (12, 13), is consistent with this formulation.

Polycystic kidney disease resulting from mutations in either *PKD1* or *PKD2* occurs via a cellular recessive mechanism in which the somatic acquisition of “second hits” on the respective normal allele in a single cell gives rise to cyst formation. For *PKD1*, this evidence was obtained from studies using human cystic kidneys (14) and cells derived from cystic kidneys (15) whereas for *PKD2*, evidence for this mechanism was derived from an animal model of the disease produced by targeted gene disruption (16) and confirmed in human studies (17). The loss of functional polycystin-2 in renal tubular and biliary epithelial cells causes these cells to proliferate, lose their normal structure within tissue architecture, and go on to form cysts. The function of polycystin-2, however, remains unknown.

An essential step toward understanding the function of polycystin-2 is a molecular dissection of its important functional domains and the characterization of its cellular expression pattern. In this study, we use a pair of polyclonal antisera against intracellular domains of polycystin-2 to characterize polycystin-2 as an integral membrane glycoprotein whose predominant subcellular location is in the endoplasmic reticulum in cultured cells. We show that a 34-amino acid domain containing a pair of putative phosphorylation sites in cytoplasmic

Autosomal dominant polycystic kidney disease (ADPKD)<sup>1</sup> is one of the most common potentially lethal inherited human diseases. Its principal clinical manifestation is bilateral renal cyst formation in all affected individuals and chronic renal

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<sup>1</sup> The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; ER, endoplasmic reticulum; GST, glutathione S-transferase; PDI, protein-disulfide isomerase; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide:N-glycosidase F; Endo H, endoglycosidase H.

COOH terminus is necessary for the exclusive ER localization of polycystin-2.

#### MATERIALS AND METHODS

**Antibodies**—Fusion protein C2 (amino acids 687–962) contains most of the cytoplasmic COOH-terminal portion of polycystin-2, and B9 (amino acids 103–203) contains part of the cytoplasmic NH<sub>2</sub>-terminal portion of polycystin-2 (3). These recombinant proteins were generated by subcloning polymerase chain reaction-generated fragments of *PKD2* into the pGEX2T expression vector (Amersham Pharmacia Biotech, Piscataway, NJ). Polymerase chain reaction primers were designed with a *Bam*HI and *Eco*RI linkers for the forward and reverse primers, respectively. The primer sequences are as follows: C2, 5'-CGCGGATC-CGTGAAATCTGACTTGGCACA-3' (forward), 5'-CGCGAATTCATT-TCCACCTGCACCTTCC-3' (reverse); B9, 5'-CGCGGATCCGTGGAAG-GGAAGAAGGC-3' (forward), and 5'-CGCGAATTCATGTTCCCGAG-AGACCTCG-3' (reverse). Subclones were sequenced to confirm reading frame and fidelity to *PKD2*. Recombinant proteins were expressed in bacteria and purified with glutathione-agarose according to the manufacturer's protocol (Amersham Pharmacia Biotech). The purified fusion proteins were used to immunize New Zealand rabbits to raise polyclonal antisera (HRP, Denver, PA). Antiserum YCC2 raised against C2 fusion protein and YCB9 raised against B9, were tested by enzyme-linked immunosorbent assay and high titer antisera were used for subsequent studies.

Preabsorbed antisera were prepared by mixing 400  $\mu$ l of PBS containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml pepstatin, 4 mM benzamide), 4  $\mu$ l of antiserum, and either 5  $\mu$ g of GST or 5  $\mu$ g of the respective fusion protein. After incubation for 1 h at 4 °C, 40  $\mu$ l of 50% glutathione-agarose was added, mixed repeatedly for 15 min at room temperature, and centrifuged at 12,000  $\times$  g for 5 min. The supernatants were used at a 1:10 dilution for immunohistochemistry. Affinity purified antibody, YCC2-P, was obtained using Hi-Trap affinity columns (Amersham Pharmacia Biotech). Briefly, GST-C2 fusion protein was cleaved by thrombin and GST protein was removed by binding to glutathione-agarose. The purified C2 polycystin-2 fragment in the eluate was coupled to the HiTrap affinity columns and the rabbit antiserum was affinity purified according to the manufacturer's recommendations.

The following reagents were obtained from commercial sources: mouse monoclonal antibodies for protein-disulfide isomerase (PDI) (StressGen, Victoria, BC, Canada),  $\alpha$  subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase (National Institutes of Health, NICHD Hybridoma Bank), and  $\beta$ -COP (clone M3A5, Sigma); rabbit polyclonal antiserum for calnexin (StressGen).

**Expression Plasmids**—A full-length *PKD2* cDNA, TM4-FL was constructed by taking advantage of the unique *Bam*HI in the open reading frame. The *Xba*I-*Bam*HI fragment from the K1-1 clone and *Bam*HI-*Xho*I fragment from cTM4B3-3 clone (3) were cloned into the pCDNA3.1 expression vectors (Invitrogen, Carlsbad, CA). The construction of TM4-FL was confirmed by restriction enzyme digestion and DNA sequencing.

We introduced naturally occurring human *PKD2* mutations, R742X (3) and R872X,<sup>2</sup> into TM4-FL by site-directed mutagenesis using the QuickChange kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. Additional truncation constructs were produced by introducing stop codons at glutamate 787 (E787X) and glycine 821 (G821X). The mutagenesis primers used were as follows: 1) R742X: 5'-GTTAAACTTTGACGAACCTTGACAAGATCTCAAAGGGAAG-3' (forward) and its reverse complement (reverse); 2) E787X: 5'-GACTTGGAGAAAGAGAGGTAAGACCTGGATTGGATC-3' (forward) and its reverse complement (reverse); 3) G821X: 5'-GACGATGAAGATAGCTAACATAGCTCCAGAAGGAGG-3' (forward) and its reverse complement; 4) R872X: 5'-GAAGCTAGAGATTATGAGTGTAGCCAAACTGAGAAGGAG-3' (forward) and its reverse complement (reverse). The sequence change(s) from wild type is shown in bold and underlined. These four mutagenesis clones were constructed in pCDNA3.1 and were confirmed by direct sequencing.

In addition, we produced a *PKD2* clone truncated after leucine 703 (Leu<sup>703</sup>) by introduction of an influenza virus hemagglutinin protein epitope tag and an in-frame stop codon. For this, TM4-FL cloned into pCDNA3 was linearized by double digestion with *Bgl*II and *Xho*I (the former cutting the insert at Leu<sup>703</sup>) and re-ligated in the presence of an

annealed adaptor: 5'-GATCTTCTTAAGTACCCATACGATGTTCCAG-ATTACGCTTAAC-3' (upper strand) and 5'-TCGAGTTAAGCGTAATC-TGGAACATCGTATGGGTACTTAAGAA-3' (lower strand). The Leu<sup>703</sup> codon is indicated in the upper strand by bold underline and the HA tag sequence is shown in italics followed by a TAA stop codon. The clones were sequence verified.

A COOH terminus clone containing codons 652–968 of *PKD2* was generated by polymerase chain reaction amplification with forward and reverse primers containing *Eco*RI and *Sal*I linkers (underline), respectively. The primer sequences are: 5'-CTTGAATTCGCTAATCGAGTTT-TGGGAC-3' (forward), and 5'-ACGCGTCGACTCATACGTGGACATT-AGAAC-3' (reverse). The amplification product was subcloned in-frame into the *Eco*RI-*Sal*I sites of a modified pFLAG-CMV-2 vector (Kodak, Rochester, NY).<sup>3</sup> The reading frame was confirmed by sequencing. The expression product contains the sixth transmembrane domain and the entire intracellular COOH terminus of *PKD2* downstream of a FLAG tag.

**Cell Culture and Transfections**—HEK293 cells (ATCC number CRT 1573) were used for most transfection experiments in this study. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For transient transfections,  $\sim 1 \times 10^6$  cells were transfected with 2  $\mu$ g of the appropriate expression plasmid using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Cells were continuously cultured for 48–72 h before being used for analysis. For HEK293 stable cell lines expressing TM4-FL in pCDNA3.1, selection with 50  $\mu$ g/ml Zeocin (Invitrogen, Carlsbad, CA) was begun 48 h after transfection. After 2 weeks, individual colonies were selected and continuously cultured under selection and tested by immunoblotting and immunocytochemistry for expression of polycystin-2 using YCC2 and YCB9 antisera. Other cell lines were used in selected experiments to assess the generality of the findings: AtT-20 cells (ATCC number CCL89), COS-7 cells (ATCC number CRL 1651), HeLa cells (ATCC number CCL2), LLC-PK<sub>1</sub> cells (ATCC number CRL 1392), and Madin-Darby canine kidney cells (ATCC number CCL 34).

LLC-PK<sub>1</sub> cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum for stable transfections. Cells at 70% confluence in 25-cm<sup>2</sup> flasks were transfected with 8  $\mu$ g of expression plasmid (TM4-FL or L703HA in pCDNA3) using a calcium phosphate protocol (19). After 16 h, cells were replated into 100-mm dishes and selection with G418 400  $\mu$ g/ml (Life Technologies, Inc., Gaithersburg, MD) was begun after 48 h in culture. Approximately 3 weeks later, resistant colonies were cultured individually and tested for the expression of the full-length and truncated *PKD2* proteins.

**Western Blots and Immunoprecipitation**—For Western blot analysis, 10–80  $\mu$ g of protein solubilized in sample buffer (125 mM Tris (pH 6.8), 200 mM dithiothreitol, 6% SDS, 20% glycerol, and 0.2% bromophenol blue) was subjected to electrophoresis on 8% SDS-PAGE gels without boiling. Fractionated proteins were electrotransferred to Polyscreen polyvinylidene difluoride membranes (NEN Life Science Products Inc., Boston, MA) and detected with the YCC2 (1:5000) or YCB9 (1:4000) polyclonal antisera, using ECL enhanced chemiluminescence (NEN Life Science Products).

Immunoprecipitation was carried out as described previously with modifications (20). Cell lysates were prepared in NET buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA (pH 8.0), 0.02% sodium azide), and protease inhibitors. One ml of total cell lysate was preincubated with preimmune serum and a 10% slurry of protein A-Sepharose beads at 4 °C for 1 h, then centrifuged at 500  $\times$  g. The supernatant was immunoprecipitated with purified YCC2-P antibody, washed extensively by NET buffer, and finally eluted with 1  $\times$  SDS sample buffer. The eluted supernatant was subjected for SDS-PAGE and Western blot analysis.

**Membrane Fractions and Subcellular Fractionation**—Membrane proteins were prepared as described previously with modifications (21). Tissues of human fetal or adult kidneys or cultured cells were washed and homogenized with a motor driven Teflon pestle homogenizer in ice-cold SBE buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES/KOH (pH 7.5)) containing aprotinin (90  $\mu$ g/ml), benzamide (1 mM), leupeptin (4  $\mu$ g/ml), phenylmethylsulfonyl fluoride (0.8 mM), *p*-tosyl-L-arginine methyl ester (20  $\mu$ g/ml), *N*<sup>c</sup>-benzoyl-L-arginine methyl ester (20  $\mu$ g/ml), and soybean inhibitor of trypsin (20  $\mu$ g/ml). The homogenates were centrifuged twice at 500  $\times$  g for 15 min. The resulting supernatant was centrifuged at 100,000  $\times$  g for 1 h or treated with Nonidet P-40, 0.2 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.0) or 1.5 M NaCl prior to centrifugation. The membrane pellet (M) was resuspended in ice-cold SMB buffer (250 mM sucrose, 1

<sup>2</sup> Reynolds, D. M., Hayashi, T., Cai, Y., Veldhuisen, B., Watnick, T. J., Lens, X. M., Mochizuki, T., Qian, F., Fossdal, R., Coto, E., Wu, G. W., Breuning, M. H., Germino, G. G., Peters, D. J. M., and Somlo, S. (1999) *J. Am. Soc. Nephrol.*, in press.

<sup>3</sup> Y. Maeda, unpublished data.

mm MgCl<sub>2</sub>, and 10 mM HEPES/KOH (pH 7.5)), aliquoted and stored at -80 °C. The supernatant (S) fraction was stored similarly. All steps were performed at 4 °C. Protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad).

Subcellular fractionations were performed as described (22) with modifications. Cultured cells were rinsed twice with Buffer A (0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, pH 7.4) and harvested by gentle scraping into 5 ml of homogenization buffer (85% Buffer A and 15% Buffer B (10 mM Tris, pH 7.4, 5 mM KCl, 1 mM EDTA, 128 mM NaCl)). Cells were spun at 300 × *g* and resuspended in 0.5 ml of homogenization buffer, followed by homogenization at 4 °C by a motor driven Teflon pestle homogenizer. Post-nuclear supernatants were prepared by centrifugation at 1000 × *g*. Supernatants were loaded on pre-formed 0–26% Optiprep (Life Technologies, Inc.) linear gradients that had been pre-cooled to 4 °C. Gradients were spun for 120 min at 40,000 rpm at 4 °C in a SW41 rotor in a Beckman ultracentrifuge. Twenty fractions (~600 μl each) were recovered by continuous elution from the bottom using a peristaltic pump. Samples containing equal volumes from each fraction were subjected to SDS-PAGE followed by immunoblotting analysis.

**Glycosylation and Cell Surface Biotinylation Analysis**—Membrane fraction protein or cell lysates were treated with peptide-*N*-glycosidase F (PNGase F) or endoglycosidase H (Endo H) following the manufacturer's protocols (New England Biolabs, Beverly, MA). Reactions were incubated at 37 °C overnight with 1 μl (500 units) of PNGase F or Endo H in the appropriate buffer in 20–40 μl of total reaction volume. After the overnight incubation, an equal volume of 2 × SDS sample buffer was added to each reaction, and incubated at 37 °C for 30 min prior to SDS-PAGE and Western blot analysis.

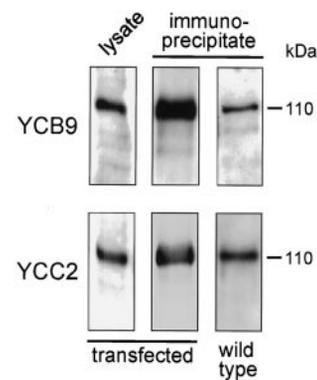
Biotinylation of cell surface proteins was carried out using EZ-Link NHS-SS-Biotin (Pierce, Rockford, IL) as described (23). *N*-Hydroxysuccinimide-SS-biotin was applied to living cultured stable LLC-PK<sub>1</sub> cell lines expressing either the L703HA mutant or full-length PKD2. Biotinylated proteins were recovered using streptavidin-agarose beads. The eluted proteins were analyzed by SDS-PAGE and detected by immunoblotting with the NH<sub>2</sub>-terminal antisera, YCB9.

**Immunofluorescent Cell Staining**—Cultured cells were washed in serum-free medium and PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100/PBS for 10 min before incubation with the primary antibodies. fluorescein isothiocyanate-conjugated anti-rabbit IgG and Cy3-labeled anti-rat or anti-mouse IgG were used as the secondary antibodies. Immunofluorescent staining was detected using Zeiss Axiophot microscope or a Bio-Rad MRC 600 scanning laser confocal microscope.

## RESULTS

**Characterization of Polycystin-2 with Anti-PKD2 Antibodies**—Rabbits were immunized with a pair of recombinant GST fusion proteins, B9 and C2, containing portions of the cytoplasmic NH<sub>2</sub> and COOH termini of polycystin-2, respectively. Each of the resultant antisera gave high enzyme-linked immunosorbent assay titers (>10,000) with their respective immunogens and were shown to specifically detect the corresponding GST fusion proteins on immunoblots (data not shown). Preimmune sera did not detect this band. YCC2 and YCB9, but not preimmune sera, specifically immunostained cultured cells transfected with full-length PKD2 while endogenous PKD2 staining was much weaker (see below). This signal was completely competed by the immunizing fusion peptide but not by GST alone (data not shown).

Initial characterization of the YCB9 and YCC2 antisera was performed using HEK293 cells transfected with full-length PKD2 as well as wild type cells. YCB9 and YCC2 detected a prominent band migrating at ~110 kDa in the crude lysate of transfected cells (Fig. 1). The ~110-kDa signal was detected by YCB9 and YCC2 preabsorbed with GST protein but was abolished when using antisera preabsorbed with the respective immunizing fusion peptides (data not shown). Affinity purified YCC2-P antibody immunoprecipitated a ~110-kDa band from transfected and untransfected cells that was detected by both YCB9 and YCC2 (Fig. 1). We have previously shown that mice homozygous for a null mutation in the *Pkd2* gene lack the ~110-kDa band on immunoblots of kidney tissue (16).

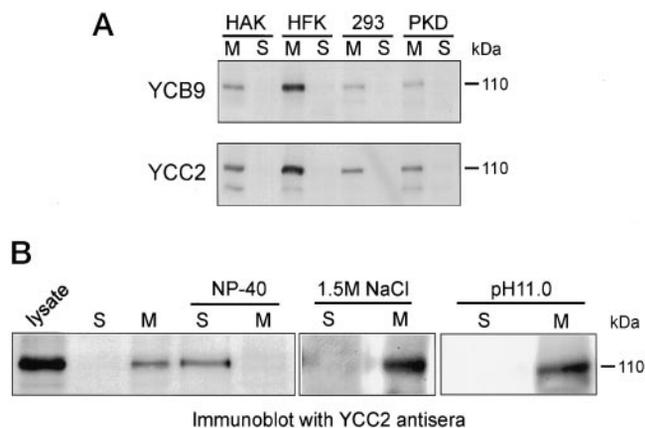


**FIG. 1. Identification of polycystin-2.** The YCC2 and YCB9 antisera both detected a single ~110-kDa band in crude lysates of HEK293 cells transfected with the full-length PKD2 expression clone, TM4-FL (left lane, 5 μl of total cell lysate loaded). Affinity purified YCC2-P antibody was used to immunoprecipitate polycystin-2 from both transfected and wild type HEK293 cells (two right lanes, respectively, 90 μl of total lysate from transfected cells and 200 μl of lysate from untransfected cells in each immunoprecipitation). The two antisera specifically detect only polycystin-2 in the immunoprecipitates.

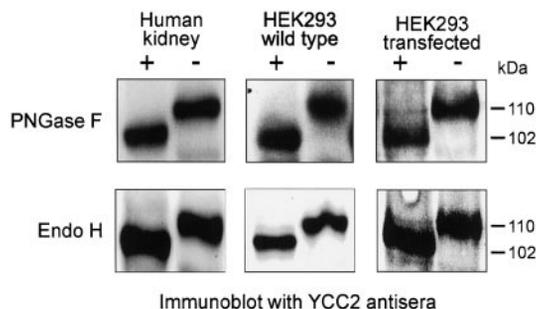
We prepared 100,000 × *g* membrane (M) and supernatant (S) fractions from adult and 22-week fetal normal human kidneys, 6 anonymous PKD kidneys of unknown genotype and from wild type HEK293 cells. Immunoblot analysis of endogenous PKD2 expression from these sources showed an abundant ~110-kDa immunoreactive band in the membrane, but not supernatant, fractions of fetal, adult, and PKD kidney tissues and HEK293 cells using both YCB9 and YCC2 antisera (Fig. 2A). Treatment of crude HEK293 cell lysates with 1% Nonidet P-40 prior to sedimenting at 100,000 × *g* completely shifted the polycystin-2-specific band from the membrane to supernatant fraction (Fig. 2B). Treatment with 1.5 M NaCl or 0.2 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.0) prior to centrifugation of 100,000 × *g* did not alter the membrane association of polycystin-2 (Fig. 2B). These results indicate that polycystin-2 is an integral membrane protein that is not anchored to the cytoskeleton.

**Analysis of *N*-Linked Glycosylation of Polycystin-2**—Our predicted secondary structure model of polycystin-2 allows for the existence of 5 putative external Asn-glycosylation sites at positions 299, 305, 328, 362, and 377 of the primary amino acid sequence (3). In order to examine whether polycystin-2 is indeed a glycoprotein as predicted, membrane fractions from human kidney tissue, and untransfected as well as PKD2-transfected cultured cells were subjected to treatment with PNGase F, an enzyme that cleaves *N*-linked oligosaccharides from proteins. Immunoblot analysis showed a shift in the migration of polycystin-2 in PNGase F-treated membranes compared with untreated membranes from tissue and cell culture sources (Fig. 3). This finding shows that polycystin-2 is *N*-glycosylated in native tissue and in wild type and transfected cells in culture. To further characterize the nature of the *N*-glycosylation in PKD2, the respective membrane preparations were treated with endoglycosidase H (Endo H), an enzyme that cleaves all high-mannose oligosaccharides from proteins. Polycystin-2 from tissue and wild type and transfected cells in culture was completely sensitive to Endo H (Figs. 3 and 7). Typically, proteins that remain sensitive to Endo H are localized to the ER and cis portions of the Golgi. This finding is consistent with a finding that polycystin-2 is a membrane-bound protein whose expression is restricted to the ER and perhaps the pre-middle Golgi.

**Subcellular Fractionation**—To further evaluate the localization of PKD2 in cells, subcellular fractionation using iodixanol-based linear density gradient centrifugation was performed on



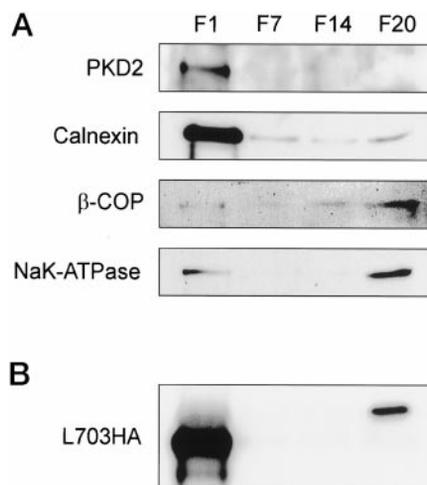
**FIG. 2. Polycystin-2 is an integral membrane protein.** *A*, immunoblots of membrane (*M*) and soluble fractions (*S*) from human fetal kidney (*HFK*), human adult kidney (*HAK*), HEK293 cells (*293*), and a representative human PKD kidney (*PKD*) detected with the YCC2 and YCB9 antisera. The ADPKD genotypes of the six anonymous PKD kidneys used is not known, the majority, if not all, likely represent PKD1 tissues based on the relative preponderance of this genotype in the ADPKD population and the greater clinical severity usually associated with it. All six PKD kidneys (only one is shown) gave similar results. The polycystin-2 immunoreactive band is detected in the membrane fractions but not the supernatants of the tissues tested. *B*, the polycystin-2 immunoreactive band shifts from the membrane to the soluble fraction in lysates of HEK293 cells transfected with full-length PKD2 and treated with 1% Nonidet P-40 prior to 100,000  $\times g$  centrifugation (*left panel*). High salt (1.5 M NaCl) and alkaline extraction (0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.0) did not alter the membrane bound nature of polycystin-2 (*right panels*).



**FIG. 3. Analysis of N-linked glycosylation of polycystin-2.** Immunoblots of 100,000  $\times g$  membrane fractions prepared from normal human kidney, wild type HEK 293 cells, and cells transfected with the full-length PKD2 cDNA. The *top panel* of blots show relative migration of polycystin-2 with (+) and without (-) digestion with PNGase F. Kidney tissue as well as transfected and untransfected cells show evidence of N-linked glycosylation of PKD2. The *lower panel* of blots show the same tissue sources treated with (+) and without (-) Endo H. Polycystin-2 from native kidney tissue and wild type as well as transfected cells in culture show complete sensitivity to Endo H, consistent with a pre-middle Golgi localization of the glycoprotein. All immunoblots are detected with the YCC2 antiserum.

wild type (untransfected) HEK293 cells. Twenty continuously eluted fractions were analyzed by immunoblotting with YCC2 and YCB9 anti-PKD2 antisera and by antibodies directed against calnexin,  $\beta$ -COP, and Na<sup>+</sup>,K<sup>+</sup>-ATPase as markers for ER, Golgi, and plasma membrane fractions, respectively. The distribution of polycystin-2 corresponded with the distribution of calnexin in the denser fractions of the gradient and differed from the distribution of  $\beta$ -COP and Na<sup>+</sup>,K<sup>+</sup>-ATPase (Fig. 4). This finding suggests a predominant ER localization for polycystin-2 and exclusion of prominent cell surface localization in the cultured cells.

**Immunofluorescence Studies on PKD2-transfected Cells**—We examined PKD2-transfected cells by immunofluorescence microscopy. In transiently transfected cells, positive staining by



**FIG. 4. Subcellular distribution of polycystin-2.** *A*, subcellular fractions were prepared from untransfected, wild type HEK293 cells using iodixanol-based linear density gradient centrifugation. Four representative fractions from the 20 collected and analyzed are shown; loading is 60  $\mu$ l (~10%) of each fraction. *F1* corresponds to the highest density, and *F20* to lowest density fraction. Fractions were analyzed by immunoblotting with antisera to marker proteins for ER (*calnexin*), Golgi ( $\beta$ -COP), and plasma membrane (*NaK-ATPase*). PKD2, detected by YCC2 (shown) and YCB9 (not shown), as well as calnexin, were confined to the three highest density fractions, indicating that polycystin-2 co-distributed with ER membranes. *B*, subcellular fractionation of HEK293 cells transiently transfected with the truncated L703HA construct and detected by YCB9 antiserum. The higher density ER fraction retains the bulk of the expressed protein but the lightest, plasma membrane fraction also contains immunoreactive expression product with a slower migration. The variation in migration of the truncated PKD2 proteins in the ER and plasma membrane fractions are due to differences in N-linked glycosylation; the *lower band* (ER fraction) is Endo H sensitive while the *upper band* (plasma membrane) is Endo H resistant (see Fig. 7).

affinity purified YCC2-P was observed in a fine reticular cytoplasmic and perinuclear pattern consistent with expression in the ER (Fig. 5). Cell surface staining was not observed. Similar results were obtained in all transfected cell types tested (HEK293, Madin-Darby canine kidney, LLC-PK<sub>1</sub>, and HeLa) and with the YCB9 antiserum as well (data not shown). The same pattern of expression was observed in LLC-PK<sub>1</sub> and HEK293 cells with stable expression of full-length TM4-FL (data not shown). The lower levels of PKD2 expression per cell in the stable cell lines relative to transiently transfected cells did not influence the staining pattern observed. The level of expression of endogenous PKD2 was so low as to preclude indirect immunofluorescent detection although the protein was detectable on immunoblots. PDI is an ER luminal protein that can be used as a marker for ER localization. Double immunofluorescent staining with anti-PDI monoclonal antibody and YCC2-P in transfected cells showed substantial overlap of the polycystin-2 signal with that of PDI (Fig. 5). Taken together, these immunofluorescence data are consistent with localization of polycystin-2 to the ER compartment.

**The COOH Terminus of Polycystin-2 Is Necessary for ER Localization**—COOH-terminal domains play an important role in the intracellular localization of proteins (24). To test the functional role of this domain in the subcellular localization of polycystin-2, we produced a series of truncation mutant constructs of PKD2 by introducing premature termination codons into the COOH terminus of the cDNA (Fig. 6). Two of these constructs, R742X and R872X, recapitulate naturally occurring human mutations (3).<sup>2</sup> All of the PKD2 truncation products studied are incapable of homomultimerization or association with polycystin-1 (7). Immunofluorescence cell staining of transiently transfected HEK293 showed that the mutants G821X

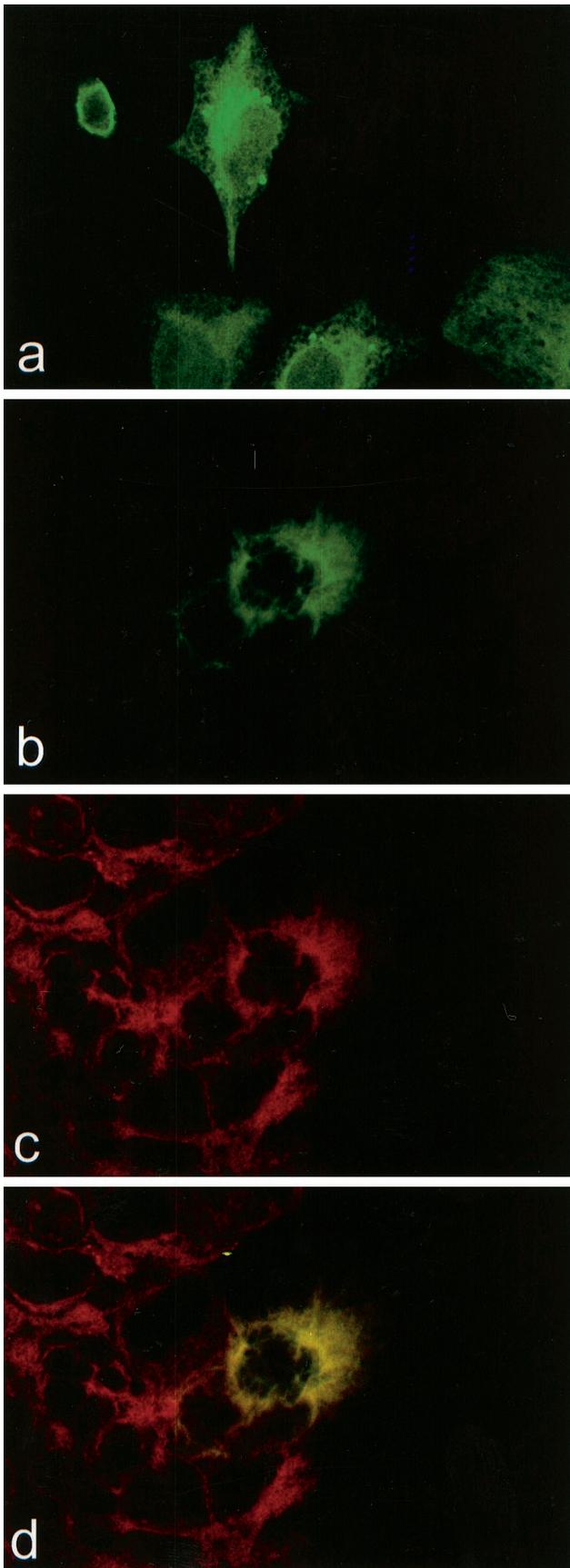


FIG. 5. Indirect immunofluorescent staining of cells transfected with full-length PKD2. *a*, Madin-Darby canine kidney cells transiently transfected with PKD2 were fixed, permeabilized, and stained by indirect immunofluorescence with the YCC2-P antibody. Polycystin-2-specific signal is observed in a fine reticular cytoplasmic and perinuclear pattern consistent with expression in the ER. *b*,

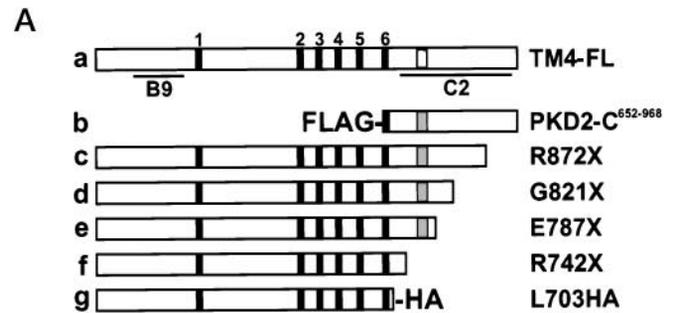
and R872X retained the exclusive ER-associated expression pattern observed for the intact PKD2 protein (Fig. 6). A construct containing only the last transmembrane domain and COOH tail of PKD2 (PKD2-C<sup>652-968</sup>) also demonstrated a reticular cytoplasmic and perinuclear immunofluorescence pattern consistent with an ER localization similar to that of full-length PKD2 (Fig. 6). However, HEK293 cells transiently transfected with the R742X and E787X truncation mutants and LLC-PK<sub>1</sub> cells with stable expression of the L703HA mutant showed truncated PKD2 expression at the cell surface in addition to the cellular staining pattern observed for the wild type clone (Fig. 6). These results suggest that the 34-amino acid domain from Glu<sup>787</sup> to Gly<sup>821</sup> (Fig. 6) plays an important role for the ER localization of polycystin-2. PKD2 translation products containing this region are localized exclusively to the ER (and perhaps early Golgi) compartment while those lacking this region are additionally trafficked to the cell surface.

We sought biochemical evidence for altered trafficking of the truncated proteins to confirm the immunofluorescence findings. The shift in mobility observed after PNGase F treatment of lysates from HEK293 cells transiently transfected with R742X and lysates of LLC-PK<sub>1</sub> stable transfectants expressing L703HA indicates that both proteins are *N*-glycosylated (Fig. 7). Endo H digestion of lysates from these two cell systems showed the existence of Endo H-resistant and -sensitive species (Fig. 7). Similarly, E787X acquired an Endo H-resistant component upon transient transfection (data not shown). This is distinct from the complete Endo H sensitivity of full-length polycystin-2 from both cell and tissue sources (Figs. 3 and 7). The formation of Endo H-resistant translation products is consistent with a post-middle Golgi distribution of a fraction of the polycystin-2 translation product truncated at or before Glu<sup>787</sup>.

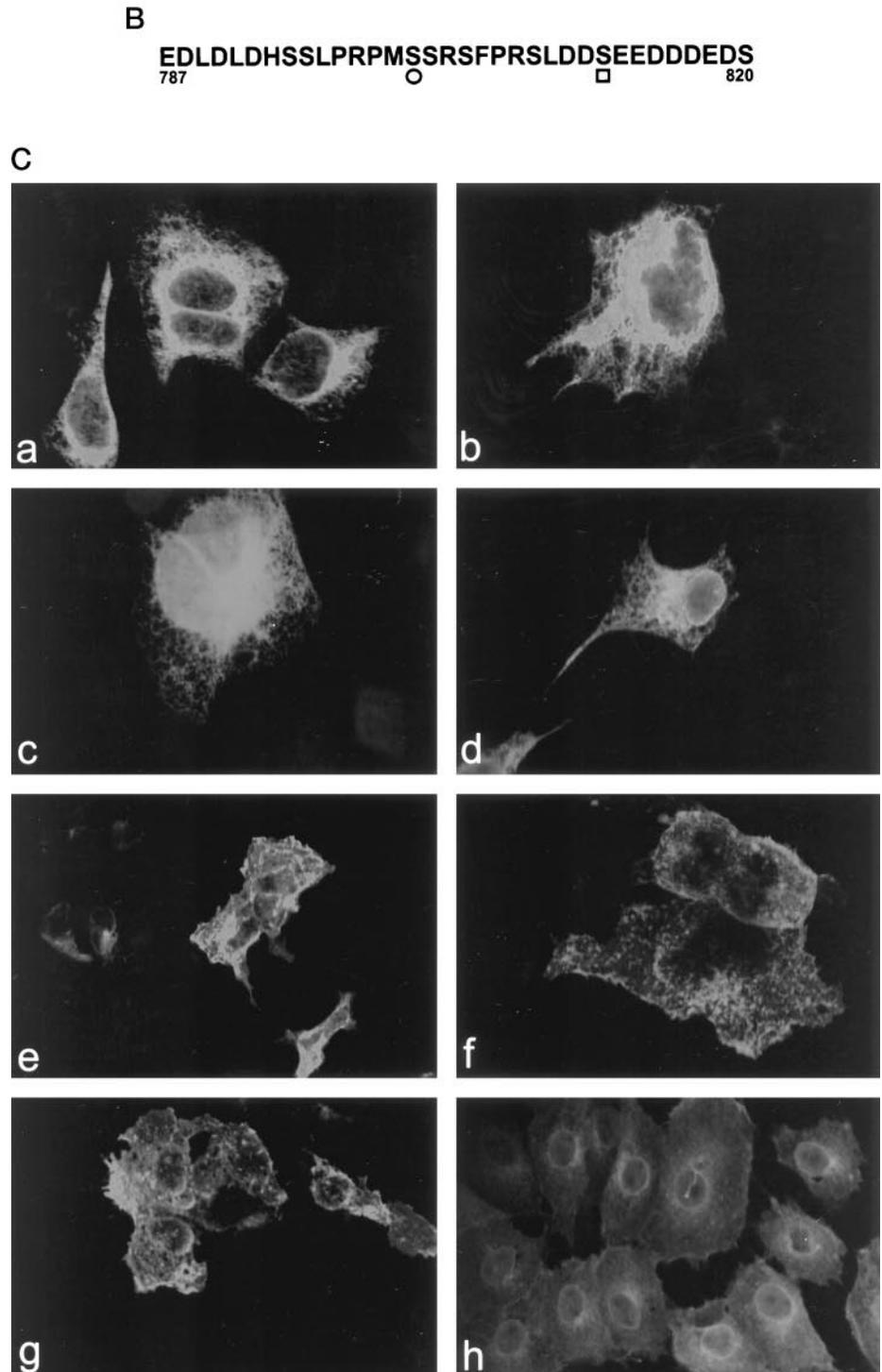
The cell surface localization of the L703HA mutant was directly demonstrated by biotinylation studies. Stable LLC-PK<sub>1</sub> lines stably expressing either the full-length or L703HA constructs were biotinylated at the cell surface in intact cells. The cells were then lysed and proteins from the cell surface that had been accessible to the biotinylation reaction were precipitated with streptavidin. Immunoblots of the biotinylated proteins showed appropriately sized immunoreactive bands detected with the NH<sub>2</sub>-terminal YCB9 antiserum in the L703HA-expressing, but not full-length PKD2-expressing cells. In similar experiments using transient transfection, the R742X truncation product was also biotinylated on the cell surface (data not shown). This finding is consistent with the accessibility of a fraction of the L703HA and R742X truncated proteins, but not full-length protein, at the cell surface (Fig. 7). Consistent with the Endo H resistance of the slower migrating L703HA and R742X translation products (Fig. 7A), only the upper band was biotinylated at the cell surface (Fig. 7B and data not shown). These results are also consistent with the results of the linear density gradient cell fractionation studies for L703HA in which the slower migrating, Endo H-resistant biotin-accessible band co-distributed with the plasma membrane fraction (Fig. 4B). These results prove that a COOH-terminal domain in polycystin-2 plays an important role in its normal localization in the pre-middle Golgi or ER compartments.

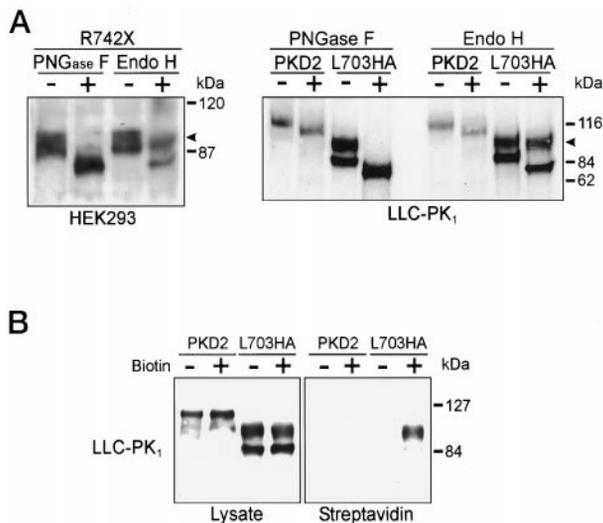
Finally, we took advantage of the plasma membrane localization of R742X to confirm the predicted topology at the NH<sub>2</sub>

HEK293 cells transiently transfected with PKD2 show the same pattern of expression. *c*, the same field as in *B* stained with a monoclonal antibody for endogenous PDI, an ER luminal resident protein. *d*, merged confocal images from *B* and *C* showing colocalization of endogenous PDI with polycystin-2 in one transfected cell in the field. Fluorescein isothiocyanate-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG were used as the secondary antibodies.



**FIG. 6. A COOH-terminal domain signals the exclusive ER localization of polycystin-2.** *A*, schematic representation of the full-length (*TM4-FL*) and COOH-terminal truncated PKD2 constructs (R872X, G821X, E787X, R742X, and the HA-tagged L703HA). PKD2-C<sup>652-968</sup> is a FLAG-tagged construct containing only the 6th transmembrane domain and cytosolic COOH terminus of PKD2. The relative locations of the immunizing peptides B9 and C2 are shown. The predicted transmembrane domains are indicated by the *black bars* and are numbered 1–6. The EF-hand in the COOH terminus is shaded. Both the NH<sub>2</sub> and COOH termini are predicted to be intracellular (3). *B*, the amino acid sequence of the 34-residue region, Glu<sup>787</sup>-Ser<sup>820</sup>, that confers exclusive ER localization to PKD2. Expression products containing this segment show exclusive ER localization indistinguishable from full-length polycystin-2 while truncation products lacking this domain are partially trafficked to the plasma membrane (see below). Predicted phosphorylation sites: *open circle*, protein kinase C; *open square*, casein kinase II. *C*, indirect immunofluorescent localization of mutant polycystin-2 expression products. HEK293 cells transiently transfected with PKD2-C<sup>652-968</sup> (*b*), the naturally occurring human mutation R872X (*c*), and G821X (*d*) show a cellular distribution indistinguishable from that observed for the full-length TM4-FL PKD2 clone (*a* and Fig. 5). The staining is reticular cytoplasmic and perinuclear; no plasma membrane-associated staining is observed. HEK293 cells transiently transfected with E787X (*e*) and the naturally occurring human mutant R742X (*f*), as well as HEK293 cells (*g*) and LLC-PK<sub>1</sub> cells (*h*) with stable expression of L703HA show cell surface expression of the mutant polycystin-2 protein in addition to the aforementioned cytoplasmic staining pattern. PKD2-C<sup>652-968</sup> was detected with the YCC2 antiserum; all others were detected with the YCB9 antiserum. Fluorescein isothiocyanate-conjugated anti-rabbit IgG was used as the secondary antibody. All images except *c* are by confocal microscopy.





**FIG. 7. Glycosylation and cell surface biotinylation analyses of COOH-truncated polycystin-2 expression products.** *A*, total cell lysates from HEK293 cells transiently transfected with R742X cDNA incubated without (–) and with (+) PNGase F and Endo H, respectively (*left panel*). Lysates of LLC-PK<sub>1</sub> cells with stable expression of TM4-FL (PKD2) or L703HA treated similarly with PNGase F and Endo H (*right panels*). Full-length polycystin-2 expressed in stable LLC-PK<sub>1</sub> cell lines is completely sensitive to both PNGase F and Endo H as is the case for endogenous polycystin-2 in kidney and HEK293 cells (Fig. 3). The truncated products R742X and L703HA migrate as 2 species which digest to a single band after PNGase F. However, only the lower band is Endo H sensitive while the upper band (*arrowheads*) shows resistance to digestion with Endo H suggesting that a portion of the truncated proteins are trafficked past the middle Golgi. YCB9 was used for immunoblotting. *B*, cell surface biotinylation of expressed PKD2 constructs. Cultured living cells were treated without (–) or with (+) NHS-SS-biotin. Cell lysates were precipitated by streptavidin, eluted by SDS-loading buffer, and subjected to immunoblot analysis by YCB9. Unprecipitated total cell lysates from the same preparation of cells were used as controls. LLC-PK<sub>1</sub> cells with stable expression of either TM4-FL or L703HA show cell surface availability of only the truncated polycystin-2 protein. Indeed, only the upper, Endo H-resistant band of the L703HA translation product is biotinylated at the cell surface. These findings are consistent with the Endo H results for the same cell lines in part *A*.

terminus of PKD2. Fixed, permeabilized R742X-transfected cells immunostained with YCB9 gave the typical plasma membrane-associated pattern of immunofluorescence. Unpermeabilized cells failed to immunostain with YCB9, consistent with the predicted intracellular localization of the portion of the NH<sub>2</sub> terminus that contains the epitope for YCB9.

#### DISCUSSION

PKD2, the gene responsible for the second form of ADPKD, was discovered by positional cloning. This mode of gene discovery presupposes no knowledge of function, and polycystin-2 is indeed a protein of unknown function although a role as a channel subunit has been suggested based on its sequence properties (3). In the current study, we analyzed several of the predictions regarding polycystin-2 structure. Using antisera directed against cytoplasmic domains of polycystin-2, we find that PKD2 is an integral membrane protein that is widely expressed and highly conserved in the mammalian cells and tissues tested. We were able to detect it by immunoblotting using anti-human-PKD2 antisera in cell lines from human, mouse, dog, monkey, and pig and in all mouse organ tissues.<sup>4</sup> The NH<sub>2</sub> terminus appears to be intracellular as had been predicted. While we found no evidence that polycystin-2 is associated with the cytoskeleton, it remains possible that func-

tional alterations in native tissues may result in anchoring of PKD2 to cytoskeletal components.

Polycystin-2 is an Endo H-sensitive glycoprotein in both native tissue and cells in culture. This finding is strongly suggestive of a pre-middle Golgi localization for the PKD2 gene product. The current study shows that both native and heterologously expressed full-length polycystin-2 is predominantly localized in the endoplasmic reticulum of cells in culture. It is not detected in the plasma membrane. Expression of truncation mutants containing at least the first 820 amino acids of polycystin-2 show a subcellular distribution indistinguishable from the full-length protein. Mutants truncated at or before amino acid Glu<sup>787</sup>, on the other hand, showed altered trafficking with a portion of the expressed protein appearing on the plasma membrane. Our series of mutants define a 34-amino acid region, Glu<sup>787</sup>-Ser<sup>820</sup>, in the cytoplasmic tail of polycystin-2 necessary for the exclusive ER retention/retrieval of the protein. Heterologous expression of a peptide containing the last membrane span and the entire COOH terminus of polycystin-2 shows the same exclusive ER localization as full-length protein. Taken together, these data suggest that the carboxyl terminus of polycystin-2 is necessary and sufficient for ER localization, although it does not exclude a possible role for other domains in functional trafficking of the protein. Interestingly, the region Glu<sup>787</sup>-Ser<sup>820</sup> is not conserved in PKD2L (5, 6), suggesting that the subcellular location of this related protein is controlled by different sequences and may be different from the ER localization observed for polycystin-2.

The persistence of Endo H-sensitive translation products in intracellular membranes despite truncation at or before Glu<sup>787</sup> may indicate the existence of additional determinants of subcellular localization which are incompletely removed in the truncation product. Alternatively, misfolding of the mutant protein or the very high levels of translation achieved in the overexpression system may result in partial retention of truncation products in the ER. Conversely, the consistent trafficking of the shorter truncation products past the middle Golgi makes less likely the possibility that the subcellular localization observed in heterologous expression of full-length PKD2 is solely the result of the capacity of the ER to retain misfolded proteins (25). The latter conclusion is supported by the complete Endo H sensitivity of endogenous polycystin-2 from untransfected cells and native tissues. The Endo H sensitivity observed in native tissue suggests that polycystin-2 is an ER protein in the whole organism as well. Immunohistochemistry studies on formalin immersion-fixed, paraffin-embedded native tissues<sup>4,5</sup> (16) have, to date, not provided adequate resolution to conclusively establish the subcellular localization of polycystin-2 *in vivo*. In some tissues, including the proximal tubule of the nephron, a cytoplasmic granular staining pattern has been observed while in the distal nephron, a more intensely staining basal distribution is described. It remains possible that provocative environments such as specific segments of the nephron or association with particular binding partners can effect re-localization of polycystin-2 under special circumstances and that such re-localization may be functionally significant.

The rules governing ER recovery or retention of proteins are not completely understood. The (H/K)DEL motif is known to be responsible for retrieval of soluble ER resident proteins, while dilysine or diarginine motifs result in retrieval of transmembrane proteins (reviewed in Ref. 24). Transmembrane domains, themselves, have been implicated in ER retention (22, 26) and retrieval (27); other cytoplasmic sequences have also been implicated as signals for retention (27). In addition, cytoplasmic

<sup>4</sup> Y. Cai and V. E. Torres, unpublished observations.

<sup>5</sup> R. Witzgall, unpublished observations.

motifs can signal exit from the ER for proteins that passage through the intermediate compartment to the *cis*-Golgi before being retrieved back to the ER (28). The 34-amino acid region (Glu<sup>787</sup>-Ser<sup>820</sup>) which we identified as critical to the exclusive ER localization of polycystin-2 lacks "classical" ER localization motifs but has several distinguishing features. Most notably, it is rich in acidic residues and contains two predicted phosphorylation sites, a protein kinase C site at Ser<sup>801</sup> and a casein kinase II site at Ser<sup>812</sup> (3).

Diacidic sorting signals have been shown to be important in export of proteins from the ER (reviewed in Ref. 29). In addition, acidic motifs that are also sites for casein kinase II phosphorylation have been implicated in localization of integral membrane proteins to the *trans*-Golgi network (reviewed in Ref. 30). The phosphorylation/dephosphorylation state at the casein kinase II site can act as a trafficking signal (31). Acidic motifs have not been directly linked to ER retention/retrieval. However, the paradigm that acidic domains bearing putative phosphorylation sites in the cytoplasmic tails of transmembrane proteins have a role in localization and trafficking of proteins is consistent with the data presented regarding the involvement of such a region in the ER localization of polycystin-2. Phosphorylation at Ser<sup>812</sup> (and Ser<sup>801</sup>) may effect the affinity of association between polycystin-2 and an unknown linker protein. This linker protein in turn may mediate ER retrieval or retention of polycystin-2 by association with ER "anchor" proteins in a manner analogous to that by which PACS-1 mediates the localization of furin to the *trans*-Golgi network (31). A corollary to such a hypothesis would be that there may be functional states within the cell (*e.g.* one resulting in dephosphorylation of polycystin-2) that may alter the specific localization, trafficking, or functioning of polycystin-2. The series of mutants in the present study argue against mislocalization of polycystin-2 as the sole pathogenic mechanism in polycystic kidney disease. While the naturally occurring human mutation R742X (3) does traffic differently from the wild type protein, the R872X mutant, which is also pathogenic in humans,<sup>2</sup> does not.

The truncation mutant R872X is unable to interact with polycystin-1 (7). Despite this, expression of this truncated product in cells in culture shows the typical ER localization seen with the full-length protein. Therefore, failure to interact with polycystin-1 alone is not sufficient to alter the ER localization of polycystin-2 in cells. Removal of an additional domain, Glu<sup>787</sup>-Ser<sup>820</sup>, results in trafficking of polycystin-2 to the plasma membrane. Therefore, if polycystin-1 is expressed in the cell types studied, the current evidence argues against a role for polycystin-1 as the effector of any possible functional relocation of polycystin-2. If the cell lines do not express polycystin-1 endogenously, then it remains possible that binding of polycystin-1 to polycystin-2 displaces an unknown binding partner responsible for ER retention/retrieval and thus has the same effect as truncating polycystin-2 at Glu<sup>787</sup>. Either formulation provides evidence for at least one additional protein binding partner interacting with the COOH terminus of polycystin-2 and defines the interacting domain as Glu<sup>787</sup>-Ser<sup>820</sup>. It can be concluded that at least three virtually contiguous functional domains exist in the cytoplasmic tail of polycystin-2: (a) the Ca<sup>2+</sup> coordinating EF-hand (Glu<sup>754</sup>-Leu<sup>782</sup>), (b) the presently defined ER localization domain (Glu<sup>787</sup>-Ser<sup>820</sup>), and (c) the overlapping polycystin-1 and -2 interacting domains (Ser<sup>832</sup>-Ser<sup>858</sup>)<sup>6</sup> (7, 8).

The discovery that polycystin-2 is restricted to intracellular membrane compartments has implications for the association

of polycystins-1 and -2. Polycystin-2 may interact with polycystin-1 in the ER and be active in shepherding polycystin-1 through the ER to Golgi transition on its way to cell surface. Polycystin-1, whose subcellular distribution remains somewhat controversial, has been localized to intracellular membranes as well as the cell surface (32). The loss of functioning polycystin-2 (16) may result in improper association with polycystin-1 in the ER and lead to instability or improper trafficking of polycystin-1 (33). Consistent with this scenario, the only report to test immunostaining of anti-polycystin-1 antibodies on known *PKD2* cystic kidney and liver tissues showed weak or no polycystin-1 immunoreactivity in the cyst lining cells, in marked contrast to what is observed in *PKD1*-derived cyst lining cells (20). Alternatively, the two proteins may interact and be resident in different membrane compartments. Polycystin-1 has significant similarity to the sea urchin sperm egg jelly receptor which functions to mediate a specialized membrane fusion event, the acrosome reaction (34, 35). This process, like many membrane fusion events, involves calcium signaling (18). It is therefore possible that the evolutionarily related polycystin-1 protein may also mediate membrane fusion events and do so through calcium signaling. Polycystin-2 may be part of the latter pathway but the two proteins may reside in opposite membrane compartments. It is known that mutations underlying the ADPKD phenotype result in perturbation of fundamental cellular processes in epithelia that lead to abnormal epithelial cell structure and this, in turn, underlies the process of cyst formation. Investigations into the specific processes now need to consider the presence of polycystin-2 in the endoplasmic reticulum membrane.

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