Polycystin: In vitro synthesis, in vivo tissue expression, and subcellular localization identifies a large membrane-associated protein

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Communicated by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, April 11, 1997 (received for review February 18, 1997)

ABSTRACT The primary structure of polycystin predicts a large integral membrane protein with multiple cell recognition motifs, but its function remains unknown. Insight into polycystin’s normal function and its role in the development of autosomal dominant polycystic kidney disease (PKD1) requires the assembly of an extensive collection of molecular reagents to examine its expression and create model systems for functional studies. Development of these crucial reagents has been complicated due to the presence of transcriptionally active homologous loci. We have assembled the authentic full-length PKD1 cDNA and demonstrated expression of polycystin in vitro. Polyclonal antibodies directed against distinct extra- and intracellular domains specifically immunoprecipitated in vitro translated polycystin. The panel of antibodies was used to determine localization of polycystin in renal epithelial and endothelial cell lines and tissues of fetal, adult, and cystic origins. In normal adult kidney and maturing fetal nephrons, polycystin expression was confined to epithelial cells of the distal nephron and vascular endothelial cells. Expression in the proximal nephron was only observed after injury-induced cell proliferation. Polycystin expression was confined to ductal epithelium in liver, pancreas, and breast, and restricted to astrocytes in normal brain. We report clear evidence for the membrane localization of polycystin by both tissue sections and by confocal microscopy in cultured renal and endothelial cells. Interestingly, when cultured cells made cell–cell contact, polycystin was localized to the lateral membranes of cells in contact. These data suggest that polycystin is likely to have a widespread role in epithelial cell differentiation and maturation and in cell–cell interactions.

Mutations within the polycystic kidney disease (PKD1) gene on human chromosome 16p13.3 are responsible for 85% of cases of autosomal dominant polycystic kidney disease (ADPKD) (1, 2). ADPKD is characterized by a progressive increase in size and number of cysts in the kidney, liver, pancreas, and spleen as well as a variety of cardiovascular, cerebrovascular, and connective tissue abnormalities (3–6). However, the precise molecular mechanisms involved in cyst development are unknown. Cloning of PKD1 (7–10) and PKD2 (11–13), a second gene responsible for ADPKD, now provides an important opportunity to determine the primary events in cystogenesis and the pathways involved in maintaining normal epithelial cell structure and differentiation.

The PKD1 cDNA (14 kb) encodes a novel large protein, polycystin, with a predicted molecular weight of at least 462 kDa, which contains a number of recognizable protein motifs, such as leucine-rich repeats (LRR), a C-type lectin domain, immunoglobulin (Ig-like) repeats, and transmembrane regions. This has lead to the prediction that polycystin is a membrane-spanning protein which may be involved in cell–cell/matrix interactions (8–10).

Assembly of the authentic full-length PKD1 cDNA (∼14 kb) has been complicated due to the existence of multiple transcribed copies of homologous sequences (97% sequence identity) present at chromosome 16p13.1 (8–10). Although sequencing of the entire gene, partial cDNAs, and reverse transcription–PCR (RT-PCR) products by several groups have resulted in a predicted full-length PKD1 cDNA and its encoded protein, no bona fide full-length cDNA was recovered (8–10). This deficiency has delayed the development of model systems to study structure/function relationships of polycystin, mechanisms of cystogenesis, as well as the creation of a complex set of immunogens and their corresponding set of antibodies. Limited data using several antipolycystin antibodies has produced conflicting reports in the literature. Although polycystin appears to be expressed in renal tubular epithelial cells in normal and cystic kidneys many other discrepancies are reported (14–16).

To overcome these limitations, we have generated an authentic full-length PKD1 cDNA. We report (i) the characterization of the cDNA by sequencing and in vitro translation, (ii) the creation and use of a set of antibodies directed against both the extra- and intracellular domains, and (iii) the cellular and subcellular localization of polycystin in primary cultures of kidney cells and multiple tissues of fetal, adult, and cystic origin. These results suggest that polycystin may function in epithelial cell differentiation and maturation and in cell–cell interactions. In addition, differential level of expression or absence of polycystin in different cysts suggests that loss of function is one mechanism of cystogenesis.

Abbreviations: PKD1, polycystic kidney disease 1; ADPKD, autosomal dominant polycystic kidney disease; LRR, leucine-rich repeats; RT-PCR, reverse transcription–PCR; HUVEC, human umbilical vein endothelial cells; DCT, distal convoluted tubule; PCT, proximal convoluted tubule; CD, collecting ducts; PCAM, platelet endothelial cell adhesion molecule.

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METHODS

Cloning PKD1 cDNA. Specifically primed RT-PCR. Clone pTEQ6A was amplified from adult human brain cDNA using the primers D47 Lower (CTCCGGCCGTCGACGTT) and Lig40–1R2 (GGACTGCTTGTCGTTGATG): 94°C for 4 min, 35 cycles of 95°C for 30 sec, 64°C for 30 sec, 72°C for 3 min, and a final extension at 72°C for 10 min. Clone 94-3 was amplified from 145.19 cell line cDNA with primers Lig40–1F2 (GCTTGAGCCACGGAGAC) and FQR4 (CCGACTGCACAAACTGCCCTCTCTG): 94°C for 4 min, 35 cycles of 95°C for 20 sec, 65°C for 20 sec, 72°C for 5 min, and a final extension at 72°C for 10 min.

cDNA library construction. Double-stranded cDNA from 145.19 radiation hybrid cell line was cloned into λ ZAP EXPRESS to yield a library of several million independent clones. Clones ZE4 and ZE9 from this library were used for full-length cDNA assembly. Two libraries were constructed using fetal brain cDNA cloned into λ ZAP EXPRESS and the replacement vector, λ DASH (Stratagene). Clones ZEFB7 and DASH 14 resulted from these libraries. Clone brain 15 (see Fig. 1) was recovered from adult brain cDNA library (pSPORT vector, LTI).

Antibody Preparation and Western Blot Analysis. The pGEX fusion proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia), and pMAL-c2 fusion protein was purified on amylose column (New England Biolabs) as recommended by the manufacturers. Polyclonal antibodies against four different fusion proteins were produced in rabbits and affinity purified as described (17). Anti-MAL-BD3 antibodies were affinity purified from rabbits injected with FP-46-1c and FP-46-2 fusions. Both, anti-FP-46-1c and anti-FP-46-2 antibodies were affinity purified from rabbits injected with MAL-BD3 protein. SDS/PAGE was carried out on 3–12% gradient gels in the presence of 1% 2-mercaptoethanol and transferred to nitrocellulose for immunoblot analysis as described (18, 19).

In Vitro Translation and Immunoprecipitation. The in vitro translation of the PKD1 constructs was performed using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [35S]methionine. Polyclonal antibodies anti-FP-LRR or anti-FP-46-1c or anti-FP-46-2 were coupled with protein A Sepharose (Pharmacia). Antibody-coupled beads were equilibrated in immunoprecipitation buffer containing 10 mM Hepes (pH 7.4), 100 mM NaCl, 0.75 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride and incubated with in vitro translated product for 4 h at 4°C. After extensive washing with immunoprecipitation buffer the proteins attached to beads were resolved by SDS/PAGE with starting material and void in parallel.

Immunohistochemistry. A standard two-stage indirect immunoperoxidase staining protocol was used for all tissue sections (Vectastain ABC System; Vector Laboratories). A dilution of 1 in 20 was used for antibodies anti-FP-46-2, anti-FP-46-1c, and anti-MAL-BD3 and a dilution of 1 in 50 for antibody anti-FP-LRR from stocks of 0.1 mg/ml.

Cell Culture, Immunofluorescence, and Confocal Microscopy. Human renal proximal tubular epithelial cells were obtained from Clonetics and cultured according to the supplied protocols. Human umbilical vein endothelial cells (HUVEC) were obtained from fresh human umbilical cords and serially passaged as described (20). Cells were fixed by 1% paraformaldehyde and permeabilized in 0.1% Triton X-100 or by acid ethanol (90% ethanol/5% acetic acid/5% water). Primary antibodies were used at a dilution of 1:100 from stocks of 0.1 mg/ml. Secondary antibodies were fluorescein isothiocyanate labeled goat anti-rabbit antibody and tetramethylrhodamine B isothiocyanate labeled goat anti-rabbit antibody (Sigma) at 1:50. Cells were examined using a Bio-Rad MRC 1000 confocal microscope.

RESULTS

Cloning of the Full-Length PKD1 cDNA. We used RT-PCR and cDNA library screening to assemble the full-length PKD1 cDNA (Fig. 1). RNA for RT-PCR and cDNA library construction was derived from either brain or the 145.19 cell line, a radiation hybrid cell line that contains the PKD1 genomic interval but lacks the homologous loci. Sequence analysis of most RT-PCR clones and cDNA library clones showed significant differences from the published cDNA, including nonsense and nonsense changes along with splicing variations. However, by using appropriate portions of seven different cDNAs, a complete, full-length cDNA was constructed (Fig. 1). The resulting full-length cDNA matched the genomically predicted cDNA with the exception of coding changes for amino acids 463, 739, and 999. Our genomic sequence predicted a cysteine, arginine and serine for these three positions, respectively, while the corresponding positions in the cDNA encoded serine, glutamine, and proline. We compared each sequence difference with all of our isolated cDNAs as well as other published cDNAs (9, 10). In the case of amino acid position 999, only cDNA clone 94-3 showed this coding change while other cDNAs of ours and other groups supported a serine at position 999. Consequently, the serine at position 999 was changed to a serine by site-directed mutagenesis. The glutamine at position 739 is present in all of our cDNAs (brain, 145.19 cell line) as well as that reported by the IPKD1 Consortium (10). Because our genomically predicted arginine is also described by Hughes et al. (9), we believe this variation is a polymorphism. Lastly, the serine we described at position 463 in our assembled cDNA is present in both of our cDNAs which span this interval, as well as those described by other groups (9, 10). This serine codon only differs with our genomic sequence, suggesting this to be an additional polymorphism. In addition to the sequence differences described above, a comparison of our assembled cDNA with that predicted by Hughes et al. (9) and the IPKD1 Consortium (10) shows other differences. Our predicted polycystin sequence more closely matches that of Hughes et al. (9), five differences, than that of the IPKD1 Consortium (10), nine differences. The Hughes et al. (9) polycystin sequence differs from ours as follows: A691P to a 145.19 cell line, which contains the PKD1 locus, but does not include the homologs and ZEFB7. DASH 14 (fetal brain libraries constructed in λ ZAP EXPRESS and λ DASH). Clones pTEQ 6A and 94-3 were derived by RT-PCR from brain mRNA. Restriction endonuclease sites used to assemble seven individual cDNAs are indicated.
a coupled transcription/translation system. The predicted molecular weight of polycystin, 462 kDa, is extremely large for efficient translation in vitro. In addition, SDS/PAGE does not provide an accurate estimate of the size of proteins in this molecular mass range. To overcome these limitations, we have produced a set of PKD1 cDNA constructs with gradually increasing deletions at the 3’ end, which encoded truncated proteins of predicted size (Fig. 2). The in vitro translation products resulting from these constructs are shown in Fig. 2. As expected, the full-length product is not synthesized as efficiently as the truncated products, even though all constructs include the same 5’ untranslated region and initiator AUG. Interestingly, all constructs are expressed as doublets (Fig. 2). This observation is consistent with polycystin synthesis initiating from both the first, predicted initiator AUG and from the second in-frame methionine codon positioned 3’ from the LRR (also, see below).

**Antibody Production and Characterization.** Antibodies were raised in rabbits against fusion proteins containing different domains of polycystin (Fig. 3). All four antibody preparations specifically recognized their corresponding immunogens by Western blot analysis (data not shown). The antisera were tested for their ability to immunoprecipitate the in vitro-translated polycystin (Fig. 3). The BRASH 7 clone contains the C-terminal epitopes as well as transmembrane domains, while Srl delta contains the amino terminus, the LRR and Ig-like domains. The BRASH 7 protein was efficiently immunoprecipitated by antibodies to FP-46-1c and FP-46-2 bound to protein A Sepharose (Fig. 3, lanes 2 and 4), but not by protein A Sepharose alone (Fig. 3, lane 6). As expected, antibodies raised against FP-LRR did not recognize the BRASH 7 protein (data not shown). Furthermore, neither anti-FP-46-1c antibody nor anti-FP-46-2 antibody recognized an irrelevant protein, luciferase (Fig. 3).

The Srl delta protein (N-terminal region of the polycystin) was specifically immunoprecipitated by anti-FP-LRR antibodies (Fig. 3), but not by an irrelevant antibody (data not shown). Anti-FP-LRR did not react with luciferase (Fig. 3). The Srl delta protein, translated in vitro, appears as a doublet. As discussed above, all PKD1 truncations are expressed as doublets as long as they include the *bona fide* N terminus. Antibody to the FP-LRR recognized the top band of the doublet, but not the bottom band. Most likely the top band is initiated from the first, predicted initiator methionine, while the product corresponding to the bottom band starts from the second in-frame methionine codon, located downstream of LRR. Thus, the bottom band would not include the LRR epitopes and hence would not be recognized by anti-FP-LRR antibodies. Interestingly, the sequence preceding the second methionine is a better match to the Kozak consensus sequence than that of the first methionine (21).

**Polycystin Expression in Normal Human Tissues.** Antibodies anti-FP-46-1c and anti-FP-LRR consistently produced the strongest staining in fixed tissues. In fresh tissues staining was observed with anti-FP-46-1c, anti-FP-46-2, and anti-FP-LRR antibodies. Specific tissue staining was identified as that abolished by preabsorption of antibody with the relevant fusion protein.

In formalin-fixed adult kidney, staining was limited to the tubular epithelium with weaker staining in vascular endothelium (Fig. 4). In eight separate normal renal tissues, staining was restricted to the distal convoluted tubule (DCT) and collecting ducts (CD) but was not seen in the proximal convoluted tubule (PCT), interstitium, glomeruli, or renal...
capsule (Fig. 4a and b). Urothelial cell staining was also observed in the bladder (data not shown). Staining in liver was confined to biliary epithelium with no specific staining of hepatocytes (Fig. 4f). In pancreas (Fig. 4g) and breast (data not shown), polycystin expression was confined to ductal epithelium with no staining of glandular tissue. Polycystin expression was not seen in sections from esophagus, small intestine, colon, or rectum. Staining of cervix and skin demonstrated polycystin expression in squamous epithelia and endothelial cells (data not shown). In normal brain tissue sections taken from sites adjacent to resected tumor, polycystin expression was identified in astrocytes of the temporal neocortex, although these cells were negative in frontal cortex and cerebellum (Fig. 4h). Weak staining was also observed in vascular endothelium of surface vessels.

Staining of frozen sections of kidney confirmed polycystin expression in the DCT and CD while the PCT remained negative. In addition, anti-FP-46-2 and anti-FP-46-1c antibodies specifically stained glomerular endothelial cells and endothelium of peritubular capillaries (Fig. 4c). The endothelium of large blood vessels was negative.

Expression of polycystin on proliferating PCT epithelial cells was assessed in two cases of acute tubular necrosis following hypoperfusion of renal allografts. In this condition, ischaemic injury and necrosis of PCT epithelial cells is followed by regeneration. In each case the regenerating cells showed circumferential membrane staining with intensity correlating with the degree of regenerative activity as assessed by the frequency of epithelial cell mitosis (Fig. 4e).

Fig. 4. Polycystin expression in normal human and ADPKD tissues. All sections were formalin fixed and pretreated with trypsin as described, except c, which was fresh tissue. All sections shown are stained with antibody anti-FP-LRR except c, which is stained with anti-FP-46-1c. (a) Normal adult kidney showing staining of DCT, PCT and glomeruli (G) are negative. (b) Higher power view of normal CD demonstrating clear membrane staining. All cell surfaces are positive. (c) A glomerulus (G) from a fresh tissue specimen demonstrates staining of peritubular and glomerular endothelial cells (arrowed). (d) Section of renal cyst from end-stage ADPKD showing staining of cyst-lining epithelial cells (E). C, -cyst lumen. Cytoplasmic and membrane staining is still apparent but there is absence of staining in some cysts (data not shown). (e) Polycystin expression in acute tubular necrosis showing staining of DCT and regenerating and necrotic cells from the PCT. Necrotic cells can be seen in the PCT lumen. (f) Staining in normal liver is confined to the biliary epithelium (B) and not seen in hepatocytes (H). (g) Membrane staining is also seen in pancreatic duct (PD) epithelial cells and not glandular tissue. (h) Astrocytes (A) stain positively for polycystin with more intense staining seen in the perivascular cells. No neuronal or glial cell staining is seen (Gl, glial; BV, blood vessel).
In all tissue sections examined except brain, where a precise subcellular localization for polycystin expression could not be determined, cytoplasmic staining was also accompanied by clear and consistent membrane accentuation (Fig. 4).

**Polycystic Kidney and Liver.** Polycystin expression was examined in ADPKD tissues from individuals whose disease has not yet been assigned to either PKD1 or PKD2. In both renal and hepatic cysts, staining was seen in the epithelial lining of the cyst. Although staining appeared more intense than in normal kidney, it was variable even within sections and within cysts. Some cysts were also negative. Normal nephrons in the same kidney sections displayed the same pattern of staining seen in normal tissue. In ADPKD cysts staining appeared to be predominantly cytoplasmic but areas of membrane staining were also observed (Fig. 4d).

Sections from multicystic dysplastic kidneys were examined and demonstrated staining of some cystic epithelia and normal tubular epithelium in DCT and CD (data not shown). Staining was both cytoplasmic and membrane-associated.

**Polycystin Expression in Fetal Tissues.** Good quality tissue sections were available from a 20-week-old human fetus. Polycystin expression was detected in CD epithelium and DCT. No staining of glomeruli or other structures was observed except for endothelium. In the liver staining was restricted to ductal plates, structures that are the precursors of the intrahepatic biliary tree (data not shown).

**Polycystin Expression in Cultured Cells.** Immunofluorescence microscopy of HUVEC and human renal proximal tubular epithelial cells was carried out with anti-MAL-BD3 and anti-FP-LRR antibodies. Staining was most intense with anti-MAL-BD3 antibody using either paraformaldehyde or acid ethanol fixation. Staining with anti-FP-LRR antibody was only seen with acid-ethanol fixation.

A punctate linear pattern of staining at the cell borders was seen at the lateral cell junctions in both cell types using laser scanning confocal microscopy (Fig. 5c and d). No apical or basal staining was seen. In confluent cultures staining was confined to points of cell–cell contact (Fig. 5c). Free cell borders did not express polycystin. In HUVEC polycystin staining colocalized with platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD-31) which is known to stain cell junctions in this system (22) (Fig. 5 c, f, g, and h). Identical results were obtained with cells derived from renal CD where the level of expression appeared to be greatest. Primary cultures of vascular smooth muscle cells were consistently negative for polycystin expression (data not shown). The linear pattern of staining was completely abolished with preabsorption of the antibodies (Fig. 5b). Nonspecific diffuse cytoplasmic and nuclear staining was observed and shown to be due to the secondary antibodies (Fig. 5a).

**DISCUSSION**

The assembly of a full-length PKD1 cDNA has proven difficult due to the presence of homologous transcripts from several closely related loci (8–10). Generation of a number of partial cDNAs which, in aggregate span the PKD1 cDNA, were described; however, no contiguous full-length cDNA, derived solely from the *bona fide* locus was obtained (9, 10). The protein sequence predicted by our full-length cDNA matches that of the genomic prediction except for two putative poly-morphisms. In 145.19 cells we have found several alternatively spliced PKD1 mRNAs with different exon combinations. The physiological significance of these isoforms is not clear, but is noteworthy, since distinct domains of polycystin (such as both LRR and the C-type lectin domain) are encoded by separate exons. Such correlation of structure and possible function suggests that differential functions of polycystin in different cell types/tissues could be regulated by alternative splicing.

![Fig. 5. Polycystin expression in cultured HUVEC. All figures demonstrate polycystin expression in HUVEC using anti-MAL-BD3 antibody. (a) HUVEC stained with secondary antibody alone demonstrating the nonspecific pattern of nuclear and cytoplasmic staining. (b) HUVEC stained with anti-MAL-BD3 antibody with or without preabsorption with fusion protein MAL-BD3 demonstrating the specific punctate linear expression pattern of polycystin. (c and g) Staining with PECAM (red) a lateral membrane marker colocalizes polycystin to the lateral membrane using two-color fluorescence (yellow). (d, f, and h) Vertical sections of c, e, and g also show the colocalization of polycystin and PECAM to the lateral membranes with no apical or basal staining.](image-url)
tissues. This distinct pattern of staining was observed with antibodies to both extracellular and intracellular domains, thus confirming the authenticity of staining.

One consistent feature in previously published studies is the overexpression of polycystin in ADPKD kidney with all cysts showing strong epithelial staining (14, 16). In contrast with these observations, we detected variable polycystin expression in cystic epithelia, ranging from levels more intense than in normal kidney, up to the absence of staining in some cysts. As cysts in ADPKD arise from all regions of the nephron (23) it might be expected that all cysts from the same patient should stain either positive or negative, as they all carry the same germ-line mutation in the PKD1 gene. Our observation of variable staining suggests that in some cysts no polycystin expression occurs, whilst in others the normal allele is over-expressed (14). The former may be due to a "second hit" as has been proposed (24) and recently reported (25). The significance of the variable cyst epithelial staining of multicystic dysplastic kidneys in the pathogenesis of this heterogeneous group of conditions remains to be determined.

In all tissues examined, except brain, polycystin expression was confined mainly to epithelial cells, which supports a role for polycystin in the developmental regulation of epithelial cell differentiation and morphology. Interestingly, polycystin expression in PCT epithelial cells correlates with their proliferative activity such that expression is seen in PCT injury and in culture. In this tissue polycystin may have a dominant role in cellular differentiation rather than maintenance of the mature phenotype. In fetal liver, expression was confined to the ductal plates from which mature biliary epithelium is derived but not seen in developing or mature hepatocytes. This concurs with the localization to biliary epithelium in adult liver. Whether polycystin is required for initiation and/or maintenance of cellular differentiation will require more detailed studies. The role of polycystin in endothelial cells and astrocytes remains unknown, although it is possible that abnormal endothelial cell function may contribute to the development of vascular abnormalities such as hypertension and saccular aneurysms by interfering with normal endothelial cell–matrix regulation in a manner analogous to the abnormal matrix seen in ADPKD kidneys (26, 27).

In summary, we have characterized a set of unique polycystin-specific reagents and used these molecular tools to define the tissue and subcellular distribution of polycystin. The consistency of our data for tissue distribution and membrane localization strongly supports the structural predictions of polycystin being an integral membrane protein. Moreover, we have demonstrated polycystin localization to the lateral cell junctions in culture. These data suggest a widespread role of polycystin in epithelial cell differentiation and maturation and in cell–cell interactions. In addition, we report variable polycystin expression in cystic epithelia ranging from the absence of staining in some cysts to overexpression in others even in the same tissue preparation. These findings support the hypothesis of a second somatic event as a molecular mechanism of cystogenesis, but do not exclude other possible mechanisms.

We wish to acknowledge the contribution of Margaret McLeish who carried out the immunohistochemical studies. This study was supported in part by grant from the National Institutes of Health (DK4853 to K.W.K.) and by The Medical Research Council. R.S. is a Medical Research Council Clinician Scientific Fellow. J.B. is an National Kidney Research Fund Senior Fellow.