

Tissue and Cellular Localization of a Novel Polycystic Kidney Disease–Like Gene Product, Polycystin-L

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Abstract. Polycystin-L (PCL), the third member of the polycystin family of proteins, functions as a Ca^{2+} -modulated non-selective cation channel when expressed in *Xenopus* oocytes. Polycystin-1 and -2 are mutated in autosomal-dominant polycystic kidney disease (ADPKD), but the role of PCL in disease has not been determined. In this study, an anti-peptide polyclonal antiserum was generated against the carboxyl terminal domain of human PCL and used to determine the patterns of expression and distribution of PCL by indirect immunofluorescence in both developing and adult mice. The results show that PCL is predominantly expressed in adult mouse tissues and has a more restricted pattern of expression than either polycystin-1 or -2. In the kidney, PCL expression was first

detected at E16, and levels increased into adulthood. Localization of PCL was predominantly found in the apical region of the principal cells of inner medullary collecting ducts. PCL was also found in discrete cell types of the retina, testis, liver, pancreas, heart, and spleen, but it was not detected in the lung. These data in combination with evidence of PCL channel activity are crucial for elucidating the physiologic role of this novel cation channel and may shed light on the function of inner medullary collecting ducts and polycystins. The expression pattern of PCL suggests that it is unlikely to be a candidate gene for ADPKD, but it remains a potential candidate for other as yet unmapped human cystic disorders.

Polycystins comprise an expanding family of membrane proteins with five members identified to date (1–6). Two members of the polycystin family, polycystin-1 (PC1) and PC2, are mutated in autosomal-dominant polycystic kidney disease (ADPKD). ADPKD is a common hereditary disorder that affects 1 in 1000 individuals and is associated with a 50% incidence of end-stage renal disease (7). A hallmark of this disease is the progressive replacement of renal parenchyma by fluid-filled cysts, although ADPKD also affects other organs such as the liver, spleen, and pancreas.

Polycystin-L (PCL), the third cloned member of the family, is predicted to be an integral membrane protein with 6 transmembrane domains. Its protein sequence shares 71% homology with PC2, and both show a striking structural similarity to Na^+ , K^+ , and Ca^{2+} channels and to the *Drosophila trp* and *trp*-like capacitance calcium entry channels (4,8,9). Both proteins contain recognizable motifs, such as an EF-hand domain, with potential calcium-binding and regulatory functions, as well as a coiled-coil domain that may mediate interaction with

other proteins. The coiled-coil domain in PC2 has been shown to mediate homodimerization (10,11).

A fourth member of the polycystin family, polycystin-REJ, was recently identified. This member shows highest homology to PC1 and to the sea urchin receptor for egg jelly (*suREJ*). Additionally, the distribution of PCREJ appears to be restricted to the testis, and it is hypothesized that it may be involved in human fertilization (5). Most recently, we and others (6,9) have identified a fifth member of the polycystin family, polycystin-L2 (PCL2), encoded by *PKD2L2*. PCL2 shares significant homology with PCL and PC2, with amino acid identities of 58% and 59%, respectively. *PKD2L2* transcript is expressed in human brain, kidney, testis, and HepG2 cells, and there are at least three alternatively spliced variants that were differentially expressed. *PKD2L2* was mapped to human chromosome 5q31 and *Pkd2L2* to mouse chromosome 18 in band C.

Expression studies have shown that the highest levels of PC1 are found during development with low levels in adult life (12–14). PC2 is also expressed during development, but high levels of expression are maintained in the kidney in adult life (15–17), suggesting a more specific role for this protein in the maintenance of differentiated renal tubular architecture. An essential developmental role for these proteins has been demonstrated by targeted disruption of their genes in mice (15,18), because homozygous mice for either mutation develop embryonic renal and pancreatic cysts and die perinatally.

Whether or not PCL plays a role in kidney development remains to be established. The mouse orthologue, *PkdL*, is deleted together with other genes, such as *Pax2*, in a 7-cM

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region in a mouse line presenting with kidney and retinal defects (*Krd*) (19). Homozygous *Krd* mice die early *in utero*, and heterozygotes develop aplastic, hypoplastic, and cystic kidneys. Retinal defects in heterozygotes include a reduction in the cell density of the inner nuclear and ganglion cell layers as well as abnormal electroretinograms (19–20).

An emerging view is that PC1-like proteins are receptors that regulate channel activity of PC2-like proteins through formation of a polycystin channel that can activate a diverse selection of signaling pathways. PCL was the first member of this novel family to be identified to function as a channel and has been shown to be permeable to Na^+ , K^+ , and Ca^{2+} , which can be modulated by Ca^{2+} (21). Subsequently, we and others (22–25) also demonstrated channel activities for PC2 that were shown to be highly similar to PCL. However, the physiologic role of polycystin channels remains unknown. To extend the current knowledge of polycystins and as a first step in determining the physiologic role of PCL, we have raised a polyclonal antiserum against a peptide in the intracellular carboxyl-terminal tail of human PCL. We have determined the patterns of expression and distribution of this newly identified protein in fetal and adult mouse tissues using this antiserum. Our data demonstrate that PCL is expressed in a number of mouse tissues but is restricted to discrete cell types within these organs and that these expression patterns are distinct from those of PC1 and PC2.

Materials and Methods

Generation of PCL Antibodies

Polyclonal antibodies were raised by immunizing New Zealand rabbits against the KLH-conjugated peptide TLRELGHAEHEITEL-TATFTKFD (Research Genetics Inc., Huntsville, AL), which corresponds to amino acids 624 to 646 of the predicted translation sequence for human PCL (4). This region of PCL is located in the intracellular carboxyl-terminal tail, which exhibits low homology to the other known polycystins. Antibody specificity was verified by Western blot analysis with recombinant fusion proteins, including (3.1c, 3.2c, and 3.3c) or excluding (3.4c) the region that contained the peptide sequence used for immunization. The relevant fragments of PCL were amplified by PCR by using primers containing *EcoRI* and *XhoI* linkers. Recombinant fusion proteins were obtained by subcloning the PCR-generated fragments into the *EcoRI* and *XhoI* sites of the pGEX4T-1 expression vector (Amersham-Pharmacia, Piscataway, NJ). Subclones were sequenced to confirm nucleotide and reading frame fidelity with respect to PCL. Recombinant proteins were expressed in DH5 α and were purified by using glutathione-sepharose4B (Amersham-Pharmacia) according to the manufacturer's protocol.

Western Blot Analysis

Purified recombinant fusion proteins were loaded onto a 4 to 20% gradient gel (Biorad, Hercules, CA) and electrotransferred onto Hybond ECL nitrocellulose membranes (Amersham-Pharmacia). Membranes were incubated with anti-PCL (1/1000) or anti-glutathione sulfatransferase (1/2000) before incubation with a peroxidase-conjugated secondary antibody (1/1000; Amersham) and were detected by enhanced chemiluminescence (ECL, Amersham-Pharmacia).

Indirect Immunofluorescence

Kidneys from 129SvJ mice were collected at stages E15, E16, E17.5, newborn (NB), 6 wk, and adult (12 to 18 mo). Other organs were collected at NB, 6 wk, and adult. All tissues were embedded in optimal cutting temperature compound (VWR, Boston, MA). A minimum of two individual mice were tested at each stage indicated. Mouse tissues were cross-sectioned at 5 μm in a cryostat at -20°C and were air-dried for 30 min. Optimization of the anti-PCL antiserum for indirect immunofluorescence with a variety of fixation conditions revealed that this antiserum only worked on frozen sections fixed with methanol. No specific staining was observed on the following types of fixed tissues: frozen sections fixed with 2% paraformaldehyde, acetone, ethanol, or on paraffin sections either with or without antigen-demasking techniques. Positive labeling was eliminated by preincubation of the antiserum with the immunizing peptide (50 μM ; Figure 2C), and no specific signal was observed with preimmune serum (data not shown).

Sections were fixed in methanol (prechilled) at -20°C for 10 min. The sections were then incubated for 1 h at room temperature with primary polyclonal antibodies and diluted as follows: anti-PCL, 1/100; aquaporin-1 (AQP1), 1/100; and AQP3, 1/100 (generous gifts of Dr. Dennis Brown, Charlestown, MA). For colabeling experiments, antibodies were incubated sequentially in the following order: rabbit anti-PCL, goat anti-rabbit-FITC (Vector, Burlingame, CA), AQP1 or AQP3, goat anti-rabbit-CY3 (Sigma Chemical, St. Louis, MO). Control sections were individually incubated with each antibody to establish the patterns of expression. Pictures were taken under fluorescence illumination and a spot digital camera. Sections incubated with anti-PCL and preimmune sera were photographed with the same exposure settings, and contrasts were then identically enhanced with Adobe Photoshop software (Adobe, Mountain View, CA).

Reverse Transcriptase-PCR of *PkdL*

The papilla region was dissected from adult mouse kidneys ($n = 2$). Total RNA was isolated by using Trizol (Life Technologies, Rockville, MD), and 5 μg was subjected to DNase I digestion for 15 min. First-strand cDNA synthesis was performed by using the First-Strand Synthesis System (Life Technologies) for reverse transcriptase-PCR (RT-PCR) with oligo-dT primers. Primers in exon 4 (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] forward: 5'-TCACCATCTTC-CAGGAGCG-3') and exon 8 (GAPDH reverse: 5'-CTGCTTCAC-CACCTTCTTGA-3') of the rat GAPDH gene were used in a PCR reaction to confirm the quality of the cDNA and to standardize the amount of cDNA obtained from different reverse transcription reactions. The GAPDH PCR product was observed after 18 to 24 cycles with an annealing temperature of 52°C for 30 s and an extension at 72°C of 30 s. The *PkdL*-specific fragment (confirmed by gel-isolation of the fragment and direct sequencing) was obtained by using a forward primer in exon 2 of the mouse cDNA (F605: 5'-CACAGC-CGAGAACAGGGAGCTT-3') and a reverse primer in exon 5 (F605: 5'-CAGTCCCTCCTGGAGTCCTTGG-3') with the following parameters: 94°C for 10 s, 67°C for 30 s, 72°C for 40 s. Products were observed between cycles 38 to 40. PCR reactions were repeated at least three times each.

Results

Characterization of PCL Antibodies

Polyclonal antibodies against human PCL were generated to a peptide sequence located in the intracellular carboxyl-terminal tail (Figure 1A), which exhibits low homology to other known polycystins. Recombinant GST-PCL fusion proteins,

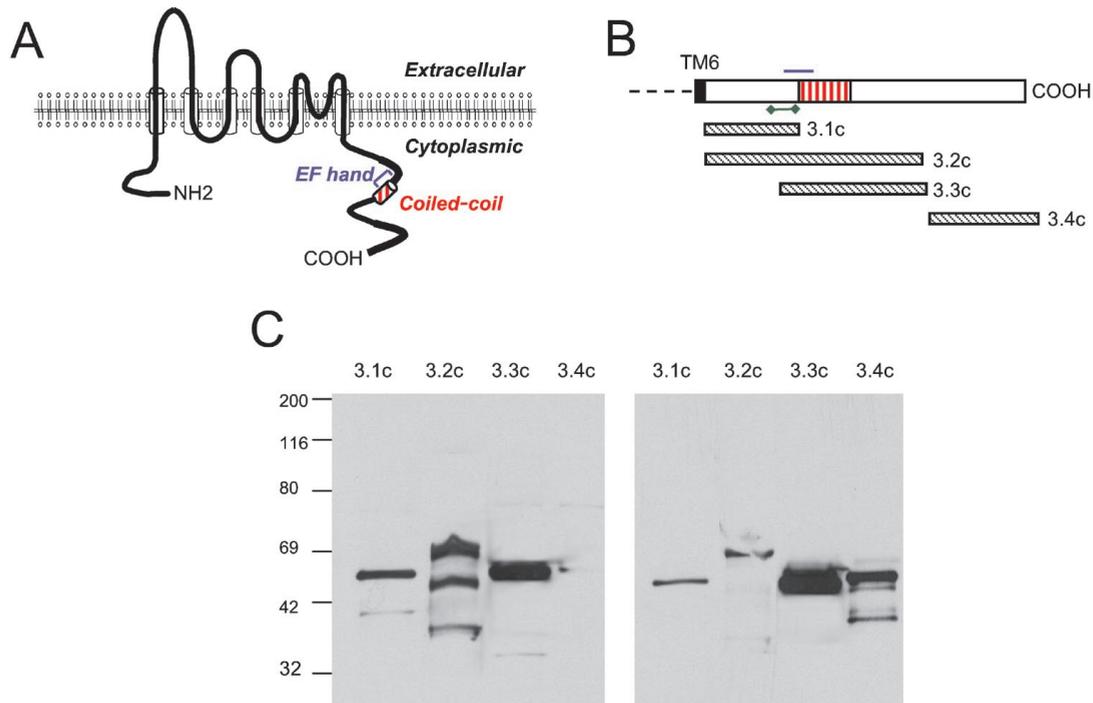


Figure 1. Characterization of anti-human polycystin-L (PCL) antiserum. (A) Proposed membrane topology for PCL. (B) Schematic representation of the regions of the intracellular C tail of human PCL fused with GST. TM6 indicates transmembrane domain 6; ▨ predicted EF-hand; ◆—◆ spans the peptide sequence used for immunization. (C) Western blot analysis of the purified glutathione sulfatransferase fusion proteins shown in panel B; left panel, blotted with an anti-PCL antiserum; right panel, anti-GST.

with or without the immunizing peptide sequence, were generated to test the specificity of the antibody (Figure 1B). Western blot analysis of purified GST-PCL fusion proteins with anti-GST antibodies detected all four fusion proteins (Figure 1C, right panel). However, anti-PCL antibodies recognized only those GST-PCL fusion proteins that contained the epitope (Figure 1C, left panel; 3.1c, 3.2c, and 3.3c) and not the fusion protein lacking the epitope (Figure 1C, left panel; 3.4c), thus confirming the specificity of the antibody. The smaller, less intense bands are likely degradation products, which are commonly seen in fusion protein preparations. We could not detect PCL by Western blot in whole kidney tissue protein homogenates, probably due to the low abundance of this protein, because human PCL transiently overexpressed in HEK293 cells was detected only after immunoprecipitation (data not shown).

Expression of PCL in Mouse Kidney

We first examined PCL expression in the kidney. Optimization of the anti-PCL antiserum for indirect immunofluorescence with a variety of fixation conditions revealed that this antiserum only worked on frozen sections fixed with methanol (see Materials and Methods). PCL expression appeared at E16 in the inner medulla region (Figure 2B) in a small number of tubules. No expression was detected in the nephrogenic zone, including the ureteric bud, comma and S-shaped bodies, and undifferentiated mesenchyme, at all embryonic stages studied (E15-NB). The fluorescence seen in the papillary epithelium is

probably nonspecific, as determined by comparison with the anti-PCL serum neutralized with the immunizing peptide (Figure 2, B and C). In the adult, localization remained much the same, although the number of tubules and staining intensity were increased (Figure 2D). We next isolated the papilla regions from adult kidney samples to amplify the mRNA coding for PCL by RT-PCR. The primers were designed to span several exons to ensure amplification of mRNA as opposed to genomic DNA. A band of the correct expected size, 611 bp, was amplified in the papilla samples (Figure 2E), and sequencing confirmed that this band corresponded to the expected sequence of *PkdL*.

To determine the precise tubular segment that expressed PCL, we carried out double labeling of PCL and AQP1 and AQP3. Aquaporins comprise a family of water channels, the members of which are expressed in a nephron segment-specific pattern in the kidney (26,27). AQP1 was located in the apical membrane of epithelial cells of the proximal tubules and descending thin limbs (Figure 3A, blue box). AQP3 was found exclusively in the basolateral membranes of the principal cells of collecting ducts from the cortex to the inner medulla (Figure 3A, red box). Double labeling of AQP1 and PCL illustrated that PCL expression was found adjacent to the descending thin limbs labeled by AQP1 in the inner medulla, *i.e.*, these two proteins were restricted to distinct segments of renal tubules (Figure 3, B and C). Colabeling of PCL and AQP3, however, revealed that these two proteins were localized in the same tubules in the inner medulla (Figure 3, D and E), where all

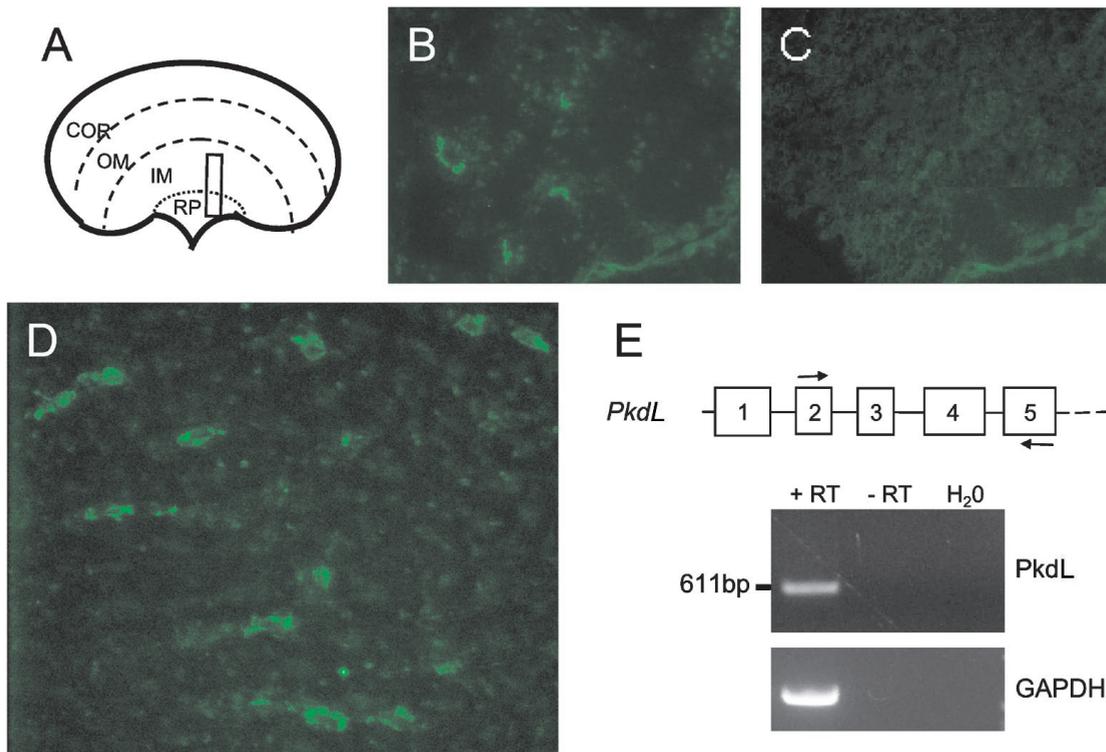


Figure 2. PCL is expressed in the developing and adult mouse kidney. (A) Schematic representation of the different regions of the kidney, PCL expression is restricted to the boxed region. (B) PCL expression at E16. Staining of the papillary urothelium is nonspecific. (C) anti-PCL antiserum preincubated with the immunizing peptide. (D) PCL expression in adult kidney. (E) Reverse transcriptase–PCR (RT-PCR) amplification of *PKD* in adult mouse papilla. *PKD*-specific primers in exon 2 and exon 5 of the mouse cDNA sequence amplify a product of 611-bp (+RT). Each reverse transcription reaction was performed in duplicate with one of the two samples lacking the reverse transcriptase enzyme (RT–). This sample was included in each PCR reaction. The H₂O lane was a PCR control that contained water instead of cDNA. Glyceraldehyde-3-phosphate dehydrogenase–PCR (GAPDH-PCR) reactions were performed in parallel with identical amounts of cDNA. Magnification: $\times 20$ in B through D.

tubules positive for PCL labeling were also positive for AQP3 labeling. PCL expression was restricted to inner medullary collecting ducts (Figure 3A, green box), and no signal was detected in the cortical or outer medulla collecting ducts. Careful inspection of the colabeled tubules further revealed that PCL was predominantly located in the apical region of those cells expressing AQP3 (Figure 3E, arrows). Intercalated cells, *i.e.*, those cells not expressing AQP3, were also negative for PCL expression (Figure 3E, *i*). PCL was not detected in proximal tubules, descending limbs, ascending thick limbs, or in the distal convoluted tubules. Glomeruli, interstitial cells, and small vessels were also negative for PCL expression.

As mentioned above, PCL localization in embryonic mouse kidney at E16 (Figure 4A) was similar to that observed in the adult mouse kidney (Figure 4D), where it appeared to be predominantly expressed in the apical region of tubular epithelial cells. Overlay images, which combine specific PCL indirect immunofluorescence detection with Nomarsky imaging (Figure 4, B and E), clearly showed that PCL expression was indeed concentrated in the apical region (Figure 4, C and F). Some punctuate intracellular staining was also observed (Figure 4, A and D; arrowheads), although the resolution of these images was not sufficient to identify whether PCL was in

the apical membrane or in structures very near the plasma membrane or both.

PCL Expression in Extrarenal Tissue

Extrarenal expression was not observed at the embryonic or newborn stages but was detected in adult tissues only and included a variety of cell types. cDNA for *PKDL* was originally isolated from the retina, and mice with one *PkdL* allele also develop retinal defects (4,19,20). We, therefore, examined PCL expression in this tissue. We found that PCL was indeed expressed in the adult mouse retina and was located in the neurons of the ganglion cell and inner nuclear cell layers (Figure 5A).

We also examined other tissues that are affected in ADPKD. In the liver, PCL was detected in the smaller ducts of the biliary tree (canaliculi) (Figure 5B), although staining in sinusoidal cells cannot be excluded. The larger bile ducts, as well as hepatocytes, were not labeled. In the pancreas, PCL was expressed in the epithelial cells of both the small and large pancreatic ducts (Figure 5C). The islets of Langerhans and pancreatic acini were not stained. Positive staining was also observed in the heart (Figure 5D), particularly in the epicardium and in the endothelial cells of the larger blood vessels that

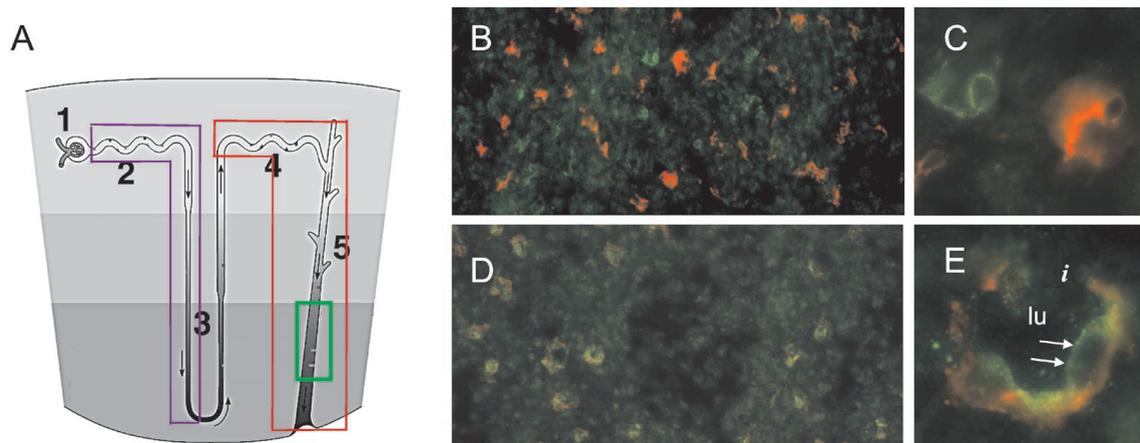


Figure 3. PCL colocalizes with aquaporin-3 (AQP3) to the inner medullary collecting ducts in the mouse kidney. (A) Schematic representation showing where AQP1 (blue box), AQP3 (red box), and PCL (green box) are expressed in the nephron. (B) Double-labeling indirect immunofluorescence on adult mouse kidney transverse sections using anti-PCL (green) and anti-AQP1 (red). (C) Higher magnification of panel B. (D) Anti-PCL (green) and anti-AQP3 (red). (E) Higher magnification of panel D. *lu*, lumen; *arrows*, apical PCL staining; *I*, intercalated cell that was negative for both PCL and AQP3 expression. Magnifications: $\times 20$ in B and D; $\times 63$ in C and E.

were marked by using an anti-PECAM antibody (data not shown). No expression was detected in the myocytes or in any of the smaller blood vessels. Other cell types found to express PCL included spermatocytes in the testis (Figure 5E) and reticular cells in the red pulp of the spleen (Figure 5F).

Discussion

In this study, we generated a polyclonal PCL-specific anti-serum and used it to define the tissue and cellular localization of PCL. Immunostaining of the developing mouse kidney showed that expression of PCL was first detectable at E16 in the inner medulla, which coincides with the maturation of inner medullary collecting ducts. Higher levels of PCL were found in the adult kidney, where its expression in the inner medulla was further supported by detection of *Pkd* mRNA from this region. Unlike PC1 and PC2, PCL was detected in only one type of nephron segment, as demonstrated by co-localization experiments with AQP3.

This study suggests that PCL's direct involvement in ADPKD is unlikely. The restricted localization of PCL to the inner medullary collecting ducts is not compatible with the wide distribution of cystic lesions in ADPKD, which involves all nephron segments. However, a secondary role for PCL in cystogenesis should not be excluded, because the functional relationships between PCL and PC1 and/or PC2 have not yet been determined. PC1 and PCL appear to colocalize in some of the same structures of the adult mouse kidney (12–14), including the apical region of the collecting ducts. In contrast, PC2 is weakly expressed in collecting ducts and is found in the basolateral compartment in mice (15–17). Protein mislocalization due to missorting, is a common feature of ADPKD during cystogenesis (28,29), and it is possible that the mistargeting of either PCL and/or PC2 could favor their interaction. For example, a recent study has demonstrated that a known human mutation in *PKD2* (R742X), which removes most of the carboxyl-terminal tail, results in its protein product being targeted

to the plasma membrane instead of to the intracellular membranes, where the wild-type protein resides (23,30). Therefore, it is possible that PCL could associate with PC1 and/or PC2 in a physiologic or pathologic situation and thus contribute indirectly to cyst formation in ADPKD.

A common feature of PC1, PC2, and PCL in localization studies was the intracellular granular/punctuate staining in the inner medullary collecting ducts. Whether these proteins are located in the same intracellular structures remains to be determined. Colocalization studies of polycystins may have significant functional relevance because it has been shown that polycystins can interact with each other *in vitro* (10,11), and in fact, PC2 function at the cell membrane appears to depend on association with PC1 (25).

The possibility that PCL may play an indirect role in ADPKD was also suggested by preliminary results that showed an increase in PCL expression in the cystic kidneys of transgenic *Pkd1^{del34}* mice (18,31; N. Basora and J. Zhou, unpublished results). These mice have been genetically modified to delete exon 34 of the *Pkd1* gene, mimicking many known human truncation mutations. Mice that are homozygous for this mutation develop severe kidney disease, and in a significant number of the older heterozygote mice, kidney cysts are readily visible (31). The reasons for this apparent increase in PCL expression are unknown but may occur in response to altered ion absorption in the diseased kidneys.

PCL functions as a Ca^{2+} -regulated nonselective cation channel that is permeable to Ca^{2+} , Na^+ , and K^+ ions when expressed in *Xenopus* oocytes (21). Expression of PCL in the apical region of tubular epithelia in the inner medullary collecting ducts suggests that PCL could play a role in Na^+ reabsorption, because extracellular Ca^{2+} and K^+ levels are very low in the tubular fluid of this segment. Na^+ reabsorption in inner medullary collecting ducts was reported to be mediated by amiloride-sensitive nonselective cation channels that are

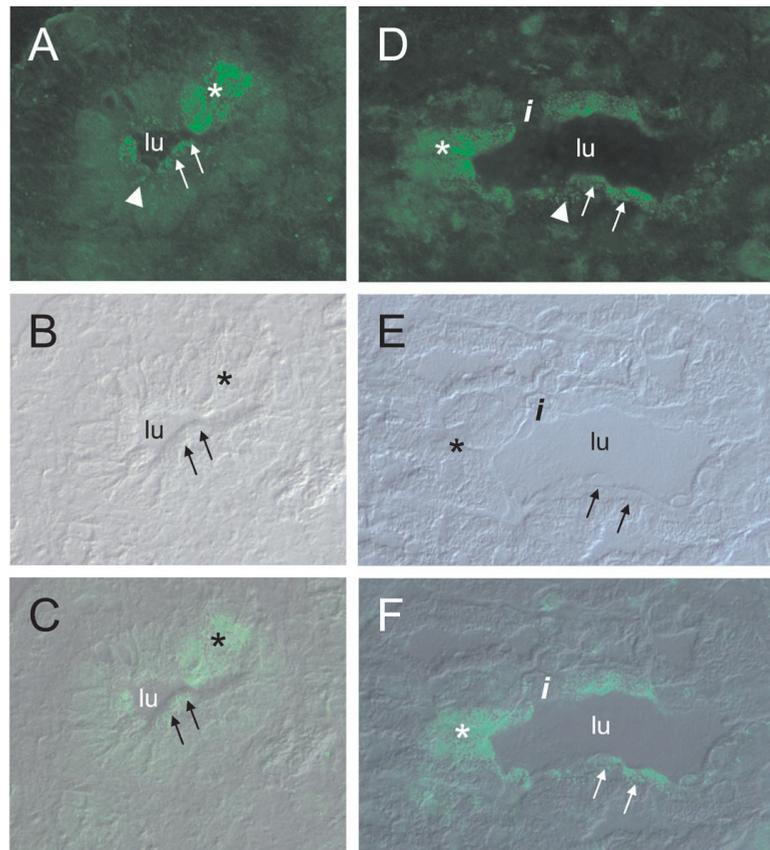


Figure 4. PCL is located in the apical region of tubular epithelia. (A) PCL expression at E16 in the apical region (arrows). Punctuate intracellular staining can also be seen (arrowheads). (B) Nomarski optics image of the same field as in panel A. (C) Overlay of images in panels A and B. (D) PCL expression of the adult kidney. (E) Nomarski optics and (F) an overlay of the two images in panels D and E. *lu*, lumen; *, principal cell that has been cut across the apical plane (as if viewed from the top); *i*, intercalated cell. Magnification: $\times 40$ in A through F.

inhibited by atrial natriuretic peptide via its second messenger cGMP (32) and perhaps also by epithelial Na^+ channels (ENaC) that are not cGMP-sensitive (33,34). These channels have single-channel conductances of 28 pS and 4–6 pS, respectively. The PCL channel is pH-sensitive but does not appear to be regulated by protein kinase A- and C-dependent phosphorylation events, nor is it cAMP sensitive (21). The presence of PCL channels with a large single-channel conductance of 137 pS in inner medullary collecting ducts may provide the nephron with an alternative way for fine-tuning extracellular fluid ionic composition and volume. Future studies of the regulation of PCL channel are required to more thoroughly understand the mechanism of volume regulation in this nephron segment.

It is interesting to note that PCL was found in a variety of cell types, including epithelial, neuronal, and endothelial cells. The subcellular distribution of PCL expression appeared to be different in these cell types. In polarized epithelial cells, such as in the kidney and the epicardium, staining was clearly predominant in or near the plasma membrane. In retinal neurons, however, the staining pattern of PCL was distinctly intracellular. The reasons for these differences in distribution are currently unknown, but they could suggest diverse func-

tions of PCL in specific cell types or diverse targeting of PCL as a result of alternative splicing (35). Similar tissue-specific differences in subcellular distribution have been reported for PC2 (16,17,36).

PCL may be a candidate gene for other unmapped human genetic cystic disorders, particularly autosomal dominant medullary cystic kidney disease (MCD) (37). This condition resembles familial juvenile nephronophthosis (FJN) and is characterized by renal cysts at the corticomedullary junction or medulla and by salt wasting (38). The main distinction between MCD and FJN is the age of onset and their mode of inheritance. MCD occurs much later in life and is dominant; FJN is recessively inherited. The gene loci for FJN and MCD have been mapped to human chromosomes 2q13 (29) and 1q21 (38,39), respectively, but several studies (38,39) have shown genetic heterogeneity for the MCD locus, which suggests the existence of unidentified loci.

In summary, by generating a specific antiserum, we were able to demonstrate expression and localization of a newly identified polycystin, PCL, in mouse tissues. These data in combination with evidence of PCL channel activity are crucial for elucidating the physiologic role of this novel cation channel and may shed light into the function of other polycystins.

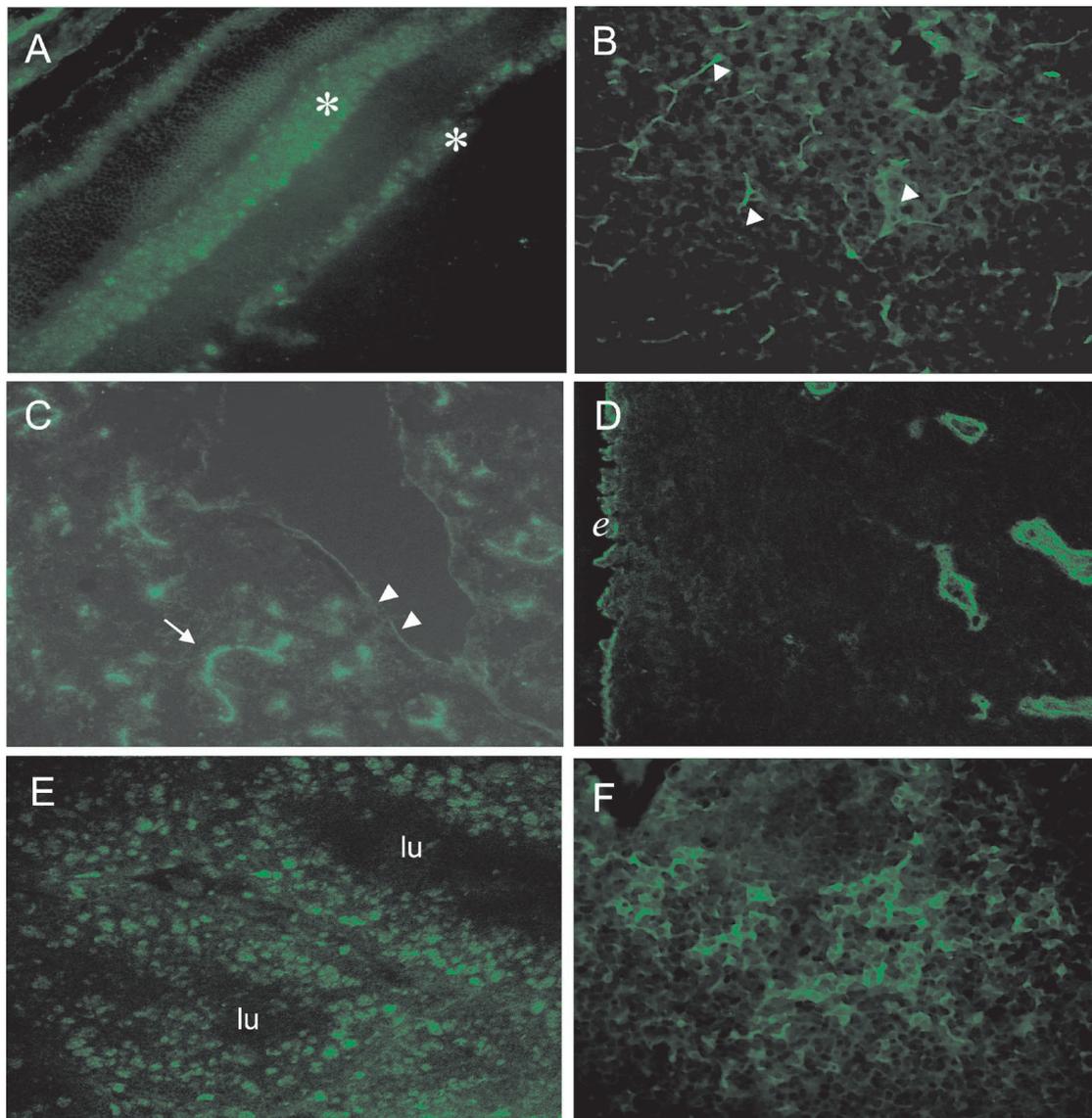


Figure 5. PCL expression in various adult mouse tissues. Indirect immunofluorescence using anti-PCL antiserum on adult mouse (A) retina (* indicates the inner nucleated and ganglion cell layers), (B) liver (arrowheads indicate the bile canaliculi), (C) pancreas (arrow and arrowheads indicate the positively stained smaller and larger pancreatic ducts [weaker staining], respectively), (D) heart (*e* indicates epicardium), (E) testis (*lu* indicates lumina of an individual seminiferous tubule), and (F) spleen. Magnification: $\times 20$ in all.

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