Genomic Organization and Functional Analysis of Murine PKD2L1*

Received for publication, October 8, 2004, and in revised form, November 12, 2004
Published, JBC Papers in Press, November 17, 2004, DOI 10.1074/jbc.M411496200

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Mutations in genes that encode polycystins 1 or 2 cause polycystic kidney disease (PKD). Here, we report the genomic organization and functional expression of murine orthologue of human polycystin-2L1 (PKD2L1). The murine PKD2L1 gene comprises 15 exons in chromosome 19C3. Coexpression of PKD2L1 together with polycystin-1 (PKD1) resulted in the expression of PKD2L1 channels on the cell surface, whereas PKD2L1 expressed alone was retained within the endoplasmic reticulum (ER). This suggested that interaction between PKD1 and PKD2L1 is essential for PKD2L1 trafficking and channel formation. Deletion analysis at the cytoplasmic tail of PKD2L1 revealed that the coiled-coil domain was important for trafficking by PKD1. Mutagenesis within two newly identified ER retention signal-like amino acid sequences caused PKD2L1 to be expressed at the cell surface. This indicated that the coiled-coil domain was responsible for retaining PKD2L1 within the ER. Functional analysis of murine PKD2L1 expressed in HEK 293 cells was undertaken using calcium imaging. Coexpression of PKD1 and PKD2L1 resulted in the formation of functional cation channels that were opened by hypo-osmotic stimulation, whereas neither molecule formed functional channels when expressed alone. We conclude that PKD2L1 forms functional cation channels on the plasma membrane by interacting with PKD1. These findings raise the possibility that PKD2L1 represents the third genetic locus that is responsible for PKD.

Polycystic kidney disease (PKD) is a common autosomal dominant Mendelian disorder that affects one out of every 1,000 people (1). The clinical features of PKD include progressive development of multiple bilateral cysts in the kidneys, which causes renal failure in 50% of patients. Positional cloning analysis revealed two loci, namely polycystin-1 (PKD1, mapped to chromosome 16p13.3) and polycystin-2 (PKD2, mapped to chromosome 4q21–23) (2–5). Mutations of PKD1 and PKD2 are responsible for 85 and 10% of PKD cases, respectively. Overall, ~95% of genetically responsive PKD loci have been clarified; the remaining loci have not been identified.

When expressed with PKD1, PKD2 forms a calcium-permeable nonselective cation channel. PKD2 has also been reported to release calcium from intracellular stores (6–8). Computer-based homology screening revealed four other homologs of PKD (9). Among these clones, polycystin-2L1 (PKD2L1, initially named PKDL) is interesting because it is homologous to PKD2. Mice that lack PKD2L1 develop polycystic kidney disease with a lethal phenotype (10). Human PKD2L1 forms cation channels when expressed in Xenopus oocytes, but it has not been examined in a eukaryotic expression system to date (11).

Because of the existence of a genetically modified mouse model and the possibility that PKD2L1 is an additional PKD locus, we identified murine PKD2L1 cDNA and analyzed the functions of the corresponding protein. We found that PKD2L1 had two endoplasmic reticulum (ER) retention signal-like sequences at its C terminus and was localized to the ER when expressed alone. Coexpression of PKD2L1 with PKD1 resulted in the formation of cation channels in human embryonic kidney 293 (HEK 293) cells.

**EXPERIMENTAL PROCEDURES**

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis—Total RNA was isolated from the brain of a C57BL/6 mouse using a RNasey kit (Qiagen Inc., Valencia, CA). The reverse transcription reaction was performed using a first strand cDNA synthesis kit (Invitrogen, Life Technologies) in a volume of 25 μl at 42 °C for 45 min. For the reverse transcription-polymerase chain reaction (RT-PCR) reaction, PKD2L1-specific sequences were amplified for 34 cycles using primers PKD2L1IS (5′-ATGATTAGTCTGGGACCCCAAGA-3′) and PKD2L1AS (5′-GGACGGATTACCGTCTCCCTCCGCAA-3′), which correspond to the murine PKD2L1 sequences1MNSMSPKN5 and 783VWNLNPS266, respectively. As a control, murine β-actin cDNA was amplified for 30 cycles with primers B1A1 (5′-CACTGTTG-GAGATGGAGAACG-3′) and B1A2 (5′-CACCTCTGAGGTCAGTGCAC-3′), which amplify a 185-bp fragment. Comparative RT-PCR reactions were carried out under the same conditions for 34 PCR cycles. Control RT-PCR reactions in this case were carried out for 28 cycles with the aforementioned primers for β-actin.

Cell Culture—Human embryonic kidney 293 (HEK 293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum. HEK 293 cells were transfected with an expression vector (pQBi25/50) carrying the murine PKD2L1 gene cloned into the N-terminal portion of enhanced green fluorescent protein (EGFP). This facilitated the visual identification of the subcellular localization of PKD2L1 by EGFP fluorescence (Qbiogene, Inc., Carlsbad, CA) and/or pDsRed2-ER (Clontech, Palo Alto, CA). Human PKD1 was expressed with the FLAG epitope in a pCI expression vector (Promega, Madison, WI) with a pIRESpuro3 expression vector (Clontech). Selection was by puromycin and transient expression was achieved using Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA).

For the deletion analysis, we used Not1-2 (5′-AAGCGGCCGCACAACATCTCTTCCAGAACACA-3′) and EcoRI-4 (5′-GACGAATGGAGAA-3′). This facilitated the visual identification of the subcellular localization of PKD2L1 by EGFP fluorescence (Qbiogene, Inc., Carlsbad, CA) and/or pDsRed2-ER (Clontech, Palo Alto, CA). Human PKD1 was expressed with the FLAG epitope in a pCI expression vector (Promega, Madison, WI) with a pIRESpuro3 expression vector (Clontech). Selection was by puromycin and transient expression was achieved using Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA).
C-terminal end of PKD2L1. Mutations were introduced with a Takara LA PCR in vitro mutagenesis kit (Takara, Ohtsu, Japan). The oligomeric DNA primers SacI-K569A (5'-GCAAGAAGTCCTGTCCGCACTCCTGGCTAAGCATCTTGTCCAGCAGGAGTCTTGTCC-3') and NdeI-D575A (5'-AGAACGACTACCTAGGAGTCTTGTCCAGCAGGAGTCTTGTCC-3') were used to introduce mutations at K569A and D575A, respectively. To establish clones that stably expressed PKD2L1 with/without mutations, G418 (0.5 mg/ml) selection was used according to the manufacturer's instructions (Invitrogen).

Fluorescence Resonance Energy Transfer Assay—A fluorescence imaging system (IXFLA Fluorescence Microscopy System, Olympus, Tokyo, Japan) was used for the FRET analysis, as described previously (12). ECFP or EYFP was introduced into the N terminus of PKD2L1 (see above), thereby creating fusion proteins. Both types of chimeric DNA plasmid were expressed transiently in HEK 293 cells. To obtain a FRET image, the fluorescence intensity of the EYFP emission (510–550 nm) with ECFP excitation (435–441 nm) was divided by that of ECFP emission (460–480 nm) with ECFP excitation (435–441 nm), according to the Aquacosmos FRET protocol (Hamamatsu Photonics, Hamamatsu, Japan). As a control, the fluorescence intensity of EYFP emission (510–550 nm) with EYFP excitation (490–510 nm) was measured.

Immunoprecipitation—Immunoprecipitation was carried out using a protein G immunoprecipitation kit (Sigma). The cell pellet was resuspended in 1.0 ml of lysis buffer (20 mM sodium phosphate, 150 mM sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, pH 7.2) and complete TM protease inhibitor mixture (Roche Applied Science) at a concentration of ~1 mg/ml, and was set on ice for 1 h before being centrifuged at 10,000 × g at 4 °C for 15 min. The cleared lysate was incubated with 2 μg of a monoclonal antibody directed against the FLAG tag (which was at the C terminus of PKD1) at 4 °C for 1 h (6). Protein G-Sepharose (50 μl) was added to each sample, and the samples were incubated for an additional hour at 4 °C. The immunoprecipitate was washed five times with 1 ml of IP buffer and was then eluted with 60 μl of Laemmli buffer. The eluted products (15 μl) were incubated for a second hour at 4 °C. The immunoprecipitate was evaluated using unpaired Student's t-test. The significance was calculated at a probability of less than 0.05.

RESULTS

Molecular Cloning—Our cloning strategy was based on a detailed search for homologues of full-length murine PKD2 in an expressed sequence tag (EST) data base using the program BLASTN (NCBI; www.ncbi.nlm.nih.gov/blast/). Four individual clones (accession numbers BB594469, BB645918, BE914496, and BG175110) were identified. All of them shared overlapping cDNA sequences and were found to be partial sequences of a single gene. With the combined sequences of these four EST clones, we identified three additional murine cDNA clones (accession numbers AF271381, BC046386, and AK046772). AP271381 and AK046772 were thought to be full-length clones of a single gene with different C-terminal sequences, suggesting that they are splice variant forms.

RT-PCR Identification of Transcripts of the Murine PKD2L1 Gene—Murine brain total RNA was reverse-transcribed and amplified by PCR using primers PKD2L1S and PKD2L1AS, which were designed from the EST and mouse genomic DNA sequences. One product (2,280 bp) was subcloned into the pZero-2-vector (Invitrogen), and sequenced. This product was an orthologue of the human PKD2L1 (9). We attempted to amplify AK046772 (possibly a splice variant of PKD2L1), but no products were obtained. We also attempted to amplify the full-length cDNA of PKD2L1 with PCR-based rapid amplification of cDNA ends (RACE), but failed to detect the appropriate product. Because AK046772 contains sequences that are identical to mouse genomic sequences with stop codons, we speculate that this clone was derived partially from murine genomic DNA.

Tissue-specific expression profiles of the identified murine PKD2L1 gene were examined using RT-PCR. The upper panel in Fig. 1A shows the expression of PKD2L1 in various tissues. In mouse, there was moderate expression in the kidney, retina, brain, colon, intestine, and aorta, and lower levels of expression in the testis, dorsal root ganglion, pancreatic β islets, and adrenal gland. The aforementioned pattern of expression of PKD2L1 in mouse concurs with the results of similar studies of human tissues (9).

Developmental Regulation of PKD Genes—The level of expression of PKD1 and PKD2 is high during embryonic development of the kidney, and declines thereafter (14, 15). In the early stage, only PKD2 is expressed in the ureteric bud and the uninduced metanephros. Subsequently, PKD2 is expressed diffusely at all stages of nephron development and persistent expression of PKD2 transcripts and protein is observed throughout nephrogenesis. By contrast, high levels of PKD1 expression first appear in the differentiated proximal tubules. Proximal tubule expression of the PKD1 and PKD2 genes gradually decreases, and PKD1 expression is restricted to the distal tubules in fetal nephrogenesis.

These previous findings led us to analyze the expression of PKD2L1 transcripts during kidney development. The results of our comparative RT-PCR analysis of PKD2L1 transcript expression are presented in Fig. 1B. The highest level of expression of PKD2L1 was in the fetal kidney, while there was moderate expression in the newborn kidney and a relatively low level of expression in the adult kidney. These data suggest that the expression of PKD2L1 decreases as the kidney develops.

The deduced amino acid sequences of murine PKD2L1 are presented in Fig. 1C. PKD2L1 has seven transmembrane domains, the last six of which are similar to the voltage-dependent calcium channel α1 subunit (9). PKD2L1 also contains one putative EF-hand-like domain (a putative calcium-binding structure) and one coiled-coil domain in the C-terminal cytoplasmic tail. We discovered two ER retention signal-like sequences that we named ER1 and ER2, which will be discussed below. A computer algorithm (DNAsis; Hitachi, Japan) predicted that there was one tyrosine kinase phosphorylation site, eight protein kinase C phosphorylation sites, eight glycosylation sites, and twelve casein kinase II phosphorylation sites.

Comparison of the Amino Acid Sequences of Murine PKD2L1 with Those of Other Mammalian PKD2L1 Proteins—Fig. 2 shows the alignment of the deduced amino acid sequences of all known PKD2L1 proteins. The fact that the rat orthologue has an insertion of 50 amino acids in the middle of a transmembrane domain suggests that this might be a splice variant that forms non-functional channels.

Structure of the Murine PKD2L1 Gene—The structure of the murine PKD2L1 gene was characterized using the program BLAST (NCBI, www.ncbi.nlm.nih.gov/blast/). One genomic
DNA clone, which was derived from mouse chromosome 19C3 (accession number NW-000148.1), had a sequence that was identical to the DNA sequence of the PKD2L1 gene. A detailed analysis of the exons and most of the introns in the identified clone indicated that the coding region of the gene extended over 29 kb (Fig. 3A). The PKD2L1 gene contained 15 translated exons and existed as a single copy locus in the haploid mouse genome; this is largely similar to human PKD2L1, although the differences in the C-terminal amino acid sequence in mouse and human PKD2L1 resulted in an additional exon in the human orthologue (16). All the exon-intron borders followed the GT-AG rule (Fig. 3B).

Fig. 3A indicates the insertion site of a transgene within intron 2 of the PKD2L1-deficient (Krd) mouse. A portion of the mutated sequence was published previously (10). Because one end of the insertion site of the transgene was not published, we have not yet analyzed the exact genomic modifications of this allele in the Krd mutant.
Computer-based analysis revealed a complex organization of the transmembrane channel segments of PKD2L1 relative to other channel-forming transient receptor potential (TRP) channels, such as murine TRPM4 (12). Four of the six channel pore-forming transmembrane domains of PKD2L1 were extended to two exons: S1 was in exons 5 and 6; S3 was in exons 6 and 7; S4 was in exons 7 and 8; and S6 was in exons 9 and 10. The remaining two transmembrane domains were encoded by single exons.

Subcellular Localization and Formation of Multimeric Channel Complex by PKD2L1—The subcellular localization in HEK 293 cells of transiently expressed murine PKD2L1 was examined by visualizing PKD2L1 that contained N-terminally fused EGFP (Fig. 4A). Murine PKD2L1 did not integrate into the plasma membrane. Therefore, to analyze the subcellular localization of the protein, we coexpressed PKD2L1 using the DsRed-ER plasmid, which contained an ER-targeting sequence and enabled us to locate the ER using DsRed (see Fig. 4B, panel...
Fig. 3. Physical map of the murine PKD2L1 gene (A) and the nucleotide and derived amino acid sequences of the translated exons (B). Insertion site of the transgene in the intron 2 is indicated by the arrow and adjacent intron sequences (A). The intron donor and acceptor sites are shown in lowercase letters. The translated amino acid sequence is shown above the nucleotide sequence of the coding exons in single letter code, and both sequences are numbered on the right. Only nucleotides that encode a protein are numbered. Amino acids within transmembrane domains are underlined. The sequences of all the exons and the adjacent sequences were derived from the sequence of mouse chromosome 19C3 (accession number NW_000319).
FIG. 4. Subcellular localization of PKD2L1. A, typical expression of EGFP fused to PKD2L1 and transiently expressed in HEK 293 cells. The EGFP fluorescence (left panel) and the corresponding phase-contrast image (right panel) are shown. The cell surface is indicated by a broken line (orange). Scale bar, 10 µm. B, coexpression of ECFP-fused PKD2L1 and DsRed-ER. Panel i, phase contrast image. Panel ii, DsRed fluorescence. Panel iii, ECFP fluorescence. Panel iv, overlay of the images in panels i–iii. Scale bar, 30 µm. Arrows indicate ECFP- and DsRed-positive cells. C, FRET images produced by the adjacent localization of PKD2L1. A representative result of the FRET ratio images of PKD2L1 is shown. ECFP and EYFP were introduced into the N terminus of PKD2L1 as independent constructs. Both constructs were transiently expressed in HEK 293 cells. Panel i, phase contrast image. Panel ii, EYFP fluorescence. Panel iii, ECFP fluorescence. Panel iv, overlay of the images in panels i–iii. ECFP, EYFP, and the FRET signal are represented as cyan, yellow, and orange, respectively. Scale bar, 30 µm. The arrow indicates a FRET-positive cell.

Next, we analyzed the formation of multimeric channel complexes with FRET, which has been used to analyze the formation of TRPC4 (17) and TRPM4 (12) channels. For the FRET analysis, two different fusion protein constructs for ECFP and EYFP were prepared. ECFP or EYFP was N-terminally fused to PKD2L1. If the distance between the two different protein molecules was less than 10 nm, a FRET signal would be detected (17). The coexpression of these two plasmids resulted in an apparent increase in the emission fluorescence signal at 500 nm due to FRET at an excitation wavelength of 436 nm (Fig. 5A,

FIG. 5. Trafficking of PKD2L1 by PKD1. A, typical result of the coexpression of EGFP-fused PKD2L1 and/or PKD1 is shown. EGFP was fused to the N terminus of PKD2L1 and was transiently expressed in HEK 293 cells. Confocal images of EGFP fluorescence (upper panel) and corresponding phase-contrast images (lower panel) are presented. Expression of PKD2L1 alone resulted in the EGFP signal being localized to an intracellular organelle. Coexpression of PKD2L1 and PKD1 caused the EGFP-fused PKD2L1 to move to the cell surface, whereas no such effect was observed after the coexpression of PKD2L1 and PKD1 with a pathogenic mutation (R4227X). Scale bar, 10 µm. B, coimmunoprecipitation of PKD1 and PKD2L1. HEK 293 cells were co-transfected with FLAG-tagged PKD-1 (PKD1-F) and/or EGFP-PKD2L1. Immunoblots of cell lysates (left) and immunoprecipitation products (IP) with an anti-FLAG antibody (right) are presented. The images on the top and bottom indicate the results of immunoblots of HEK 293 cells probed with an anti-FLAG and an anti-EGFP antibody, respectively. PKD1 was present in the IP product of samples transfected with PKD1. EGFP-PKD2L1 was present in both of the samples transfected with PKD2L1, but was present only in the IP product of the samples that expressed both PKD1 and PKD2L1 (lane 2).

FIG. 6. Deletion analysis revealed a region in the C terminus of PKD2L1 that was essential for interaction with PKD-1. Constructs of deletion mutants (A) and corresponding results with coexpression of PKD1 (B). The EGFP signals (upper panels) and corresponding phase contrast pictures (lower panels) are presented. With constructs 1 and 2, coexpression of PKD2L1 and PKD1 resulted in movement of EGFP-fused PKD2L1 to the cell surface. With construct 3 (which had an intact coiled-coil domain and 20 additional amino acids at its C-terminal), EGFP-fused PKD2L1 was trafficked partially to the cell surface. The construct with only the initial portion of the coiled-coil domain failed to produce any trafficking of EGFP-fused PKD2L1. Panel ii). Expression of PKD2L1 and PKD1 that carried a pathogenic mutation (R4227X) prevented trafficking of PKD2L1 to the cell surface (Fig. 5A, panel iii).

ii). This coexpression analysis revealed that PKD2L1 and DsRed were colocalized at the ER (Fig. 4B, panels iii and iv).

PKD1 and PKD2 interact, we surmised that PKD1 might also interact with PKD2L1. To test this, PKD2L1 was coexpressed with/without PKD1 that did/did not have a pathogenic mutation (R4227X). Expression of PKD2L1 alone resulted in less EGFP-fused PKD2L1 on the cell surface (Fig. 5A, panel i), whereas coexpression of PKD1 and PKD2L1 caused PKD2L1 to be expressed at the cell surface (Fig. 5A,
Changes in intracellular calcium concentrations. A, representative images of HEK 293 cells that expressed both PKD2L1 and PKD1. Exposure of these cells to a calcium-free extracellular solution (blue bar) caused a small decrease in intracellular calcium concentrations ([Ca\(_{2+}\)]), whereas hypo-osmotic stimulation (black bar) caused a marked increase in [Ca\(_{2+}\)]. B, representative traces of changes in [Ca\(_{2+}\)], in HEK 293 cells that expressed PKD2L1 and PKD1 (panel i), PKD2L1 alone (panel ii), and PKD1 alone (panel iii). Panel iv, statistical analysis of the changes in [Ca\(_{2+}\)], in response to calcium-free extracellular solution and hypo-osmotic stimulation (Hypo). *, p < 0.05 (n = 17 cells).

We further examined the trafficking of PKD2L1 by PKD1 and tested the hypothesis that the entire PKD1 protein is required for the functional expression of PKD2L1. We coexpressed a unique series of FLAG-tagged full-length complementary DNA constructs of human PKD1 (with/without R4227X mutation) and PKD2L1 in HEK 293 cells (Fig. 5B). As shown in Fig. 5B, PKD1 and EGFP-fused PKD2L1 were co-immunoprecipitated after coexpression in HEK 293 cells; this was not the case for the R4227X mutant of PKD1. These data suggested that the full-length PKD1 and PKD2L1 proteins associated within HEK 293 cells to form a heteromeric complex, as is the case for PKD1 and PKD2. In the cell lysate (Fig. 5B, left panel), there were no bands that corresponded to PKD1 (~400 kDa; Fig. 5B, lanes 2–4). This was probably caused by a low transfer rate for the relatively large molecule, and the low level of expression of PKD1.

Identification of the PKD2L1 Domain That Interacted with PKD1—To identify the domain in PKD2L1 that was responsible for the interaction between PKD2L1 and PKD1, we designed serial deletion mutant constructs of PKD2L1 (Fig. 6A) and then examined the trafficking of PKD2L1 by PKD1 (Fig. 6B). Because the interaction domain of PKD2 is located near the coiled-coil region of the C-terminal region, we deleted only amino acids in PKD2L1 that were in close proximity to the putative coiled-coil region. Coexpression of PKD2L1 constructs 1 and 2 together with PKD1 resulted in the localization of EGFP-fused PKD2L1 to the cell surface (Fig. 6B, panels i and ii), whereas construct 3 produced a less intense EGFP signal at the cell surface and a moderately intense signal within the cytoplasm (Fig. 6B, panel iii). Coexpression of construct 4 and PKD1 produced only marginal trafficking of PKD2L1 (Fig. 6B, panel iv). Collectively, our data suggested that the C-terminal sequence of PKD2L1 (specifically, the coiled-coil domain) is responsible for the interaction between PKD2L1 and PKD1.

Formation of Functional PKD2L1 Channels—The formation of PKD2L1 channels was examined using calcium imaging. To confirm the expression of PKD2L1 channels, cDNAs were subcloned into EGFP-containing expression vectors, which were expressed transiently in HEK 293 cells. As a negative control, the plasmid backbone was transfected. A typical recording of changes in [Ca\(_{2+}\)], in HEK 293 cells in which PKD1 and PKD2L1 was coexpressed is presented in Fig. 7A. The removal of extracellular calcium had little effect on [Ca\(_{2+}\)] (data not shown). The aforementioned increase in [Ca\(_{2+}\)], whereas hypo-osmotic stimulation (200 mOsm) produced a transient increase in [Ca\(_{2+}\)], in cells that expressed both PKD1 and PKD2L1 (Fig. 7A and B, panel i). The transient nature of this increase was consistent with the existence of a channel regulatory mechanism. Hypo-osmotic stimulation in the absence of extracellular calcium produced no detectable increase in [Ca\(_{2+}\)] (data not shown). The aforementioned increase in [Ca\(_{2+}\)], in response to hypo-osmotic stimulation was not observed in HEK 293 cells transfected with either PKD2L1 or PKD1 alone (Fig. 7B, panels ii and iii, respectively) or the plasmid backbone (data not shown). Statistical analysis confirmed that hypo-osmotic stimulation produced a significant increase in [Ca\(_{2+}\)] (Fig. 7B, panel iv).

Endoplasmic Reticulum Retention Signals in PKD2L1—We next examined which amino acid sequences were responsible...
for the subcellular localization of PKD2L1, i.e. which amino acid sequences caused PKD2L1 to be retained with the ER. There were two ER retention signal-like sequences (KDEL-like) at the C-terminal of PKD2L1 (Fig. 1C). These sequences were located between the final transmembrane sequence and the EF hand region, and we named the sequences ER1 (KEEL) and ER2 (KDQL in murine PKD2L1). ER1 was fully conserved in all three species in which PKD2L1 has been described, whereas ER2 was conserved only partially among the same species (Fig. 8A). Because the amino acid sequence of the KDEL is usually not invariable, we surmised that ER1 and ER2 might be ER-retention signals. To determine whether this was the case, we introduced a mutation of EGFP-fused PKD2L1 at K569A (in ER1) or D575A (in ER2) and carried out an expression analysis in HEK 293 cells (Fig. 8B). The EGFP signal in cells that expressed the K569A mutant was localized to the cell surface; by contrast, the EGFP signal in cells that expressed the D575A mutation was retained within the cell (panel ii). These results suggested that ER1, rather than ER2, was responsible for the retention of PKD2L1 within the ER.

Endoplasmic Reticulum Retention Signals and PKD2L1 Channel Formation—To confirm the formation of functional PKD2L1 channels, we established stable cell lines that expressed PKD2L1 and various mutants of PKD2L1, and analyzed the channel function (Fig. 9). The results of the Western blot analysis of EGFP-fused PKD2L1 expression is presented in Fig. 9A. Hypo-osmotic stimulation of cells that expressed PKD2L1 with the K569A mutation produced a transient increase in [Ca$^{2+}$]i (Fig. 9B, panel ii), which resembled the response of cells that coexpressed PKD2L1 and PKD1 (see above and Fig. 7). No response was detected in cells that expressed unmutated PKD2L1 (Fig. 9B, panel i). A proportion of the cells that expressed the D575A mutation (four of nine cells) exhibited a relatively small transient increase in [Ca$^{2+}$]i in response to hypo-osmotic stimulation (Fig. 9B, panel iii). Overall, the results of the mutation analysis suggested that ER1 plays the major role in the retention of PKD2L1 within the ER.

DISCUSSION

In the present study, we examined the genetic organization and functional expression of murine PKD2L1. PKD2L1 was expressed widely in excitable and nonexcitable cells. We clarified the structure of the murine PKD2L1 gene. EGFP-fused PKD2L1 was found to be localized to the ER. Coexpression of PKD1 and PKD2L1 caused the latter to be localized to the cell surface rather than to the ER, which indicated that PKD1 traffics PKD2L1. Calcium imaging revealed that PKD2L1...
forms a calcium-permeable cation channel. Finally, we discovered two ER retention signal-like sequences in PKD2L1, and demonstrated that these sequences were related to the subcellular localization of PKD2L1 to the ER.

Our analysis of the genomic structure of the murine PKD2L1 gene revealed a complex organization of the transmembrane channel domains. We found that PKD2L1 contained fifteen translated exons that extended over 29 kb. Four of the six channel-forming transmembrane domains of the PKD2L1 gene were extended to two exons. This is contrary to most pore-forming transmembrane domains, such as TRP channels and the \( \alpha_1 \) subunits of voltage-dependent calcium channels, all of which are encoded within single exons. The fact that the rat orthologue has a 50 amino acid insertion within the transmembrane domain suggests that PKD2L1 might not function as a cation channel in this species.

Evidence suggests that PKD2 interacts directly with PKD1 (18–20). This direct interaction is consistent with the clinical observation that patients with mutations of either PKD1 or PKD2 develop an identical phenotype of renal and extrarenal disease (although a milder form of the disease results from mutations of PKD2) (20, 21). Evidence for the involvement of a third genetic locus in PKD includes the existence of diseases that resemble PKD and for which genetic linkage to PKD1 or PKD2 has been excluded (22–25).

The fact that coexpression of PKD1 and PKD2L1 in the present study resulted in the formation of functional channels at the cell surface was interesting in light of the similarity of PKD molecules to voltage-dependent calcium channel \( \alpha_1 \) subunits and TRP channels. The heterodimeric interaction between PKD1 and PKD2 at their C-terminal cytoplasmic tails (18, 19) led us to speculate that PKD2L1, which has the highest homology to PKD2 among the members of the PKD family, might have similar features. Indeed, we found that PKD1 trafficked PKD2L1 molecules from the ER to the cell surface. This observation might explain the lethal phenotype of PKD2L1-deficient (10) and PKD2-deficient (26) mice. Furthermore, the extreme hypocellularity in electroretinograms and the abnormal retinal structure of PKD2L1-deficient mice resembles the phenotype of the original Drosophila TRP mutants (27). Because the cytoplasmic C-terminal fragment of PKD1 regulates calcium-permeable cation channels, it is likely that the interaction between PKD1 and PKD2L1 (or PKD2), and the trafficking of PKD2L1 by PKD1, might be essential to the formation of functional cation channels. Presumably, the lack of such interactions (i.e. the absence of trafficking) would lead to the development of PKD.

To identify the domain of PKD2L1 that was involved in the interaction between this protein and PKD1, we evaluated the trafficking of PKD2L1 by PKD1 using several deletion mutants of PKD2L1. Our data suggested that the coiled-coil domain of PKD2L1 was responsible for the interaction between PKD1 and PKD2L1. Our data are consistent with the idea that the subcellular localization of PKD2L1 and the interaction between PKD2L1 and PKD1 are determined by the ER retention signal-like sequences that we identified in the present study (primarily ER1, with ER2 playing a less important role).

We found in the present study that PKD2L1 formed functional cation channels that responded to hypo-osmotic stimulation. Apart from the demonstration by Chen et al. (11) of the formation of nonselective cation channels by PKD2L1 expressed in Xenopus oocytes, no other functional analysis of
PKD2L1 has been reported. We based our functional study of PKD2L1 channels on studies of the mechanosensing properties of PKD1 and PKD2 channels (28), which raises the possibility that PKD2L1 channels might also be mechanosensitive; this idea has yet to be tested.

PKD2-deficient mice have cardiac defects (26). Because PKD2-like channels have been reported to exist within the heart (29), and the expression pattern of PKD2 during heart development is relatively stable (15), PKD2 channels might be involved in cardiac development. However, PKD2L1 is thought to be expressed in the epicardium and ventricular blood vessels to a greater degree than in heart muscle (30), and no phenotype in the cardiovascular system has been observed in Krd mice. Therefore, further studies are required to clarify the physiological importance of PKD2L1, and to determine the role of PKD2L1 in cardiac development.

In the present study, we found a relatively low level of PKD2L1 expression in the fetal kidney. If expression of PKD2L1 is also down-regulated during development in humans, stably expressed PKD2 channels might compensate for the lower abundance of PKD2L1 channels after birth, and would result in no apparent phenotype in hemizygous offspring. It is extremely difficult to identify the loci that are responsible for PKD: because PKD2L1 mutations are lethal when homozygous, few patients are available for study. Based on the results of this study, we propose that PKD2L1 is an excellent candidate for the third locus that is responsible for PKD. Further studies are required to determine whether this is, in fact, the case.

Acknowledgments—We thank Dr. Gregory G. Germino for providing PKD1 clones and Ryo Morita, Toshihiro Kuremoto, and Akiyuki Wakita for technical help.

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J. Biol. Chem. 2005, 280:5626-5635.
doi: 10.1074/jbc.M411496200 originally published online November 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411496200

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