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In Vivo Interaction of the Adapter Protein CD2-associated Protein with the Type 2 Polycystic Kidney Disease Protein, Polycystin-2*

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We identified a developmentally regulated gene from mouse kidney whose expression is up-regulated in metanephrogenic mesenchyme cells when they are induced to differentiate to epithelial cells during kidney organogenesis. The deduced 70.5-kDa protein, originally named METS-1 (mesenchyme-to-epithelium transition protein with SH3 domains), has since been cloned as a CD2-associated protein (CD2AP). CD2AP is strongly expressed in glomerular podocytes, and the absence of CD2AP in mice results in congenital nephrotic syndrome. We have found that METS-1/CD2AP (hereafter referred to as CD2AP) is expressed at lower levels in renal tubular epithelial cells in the adult kidney, particularly in distal nephron segments. Independent yeast two-hybrid screens using the COOH-terminal region of either CD2AP or polycystin-2 as bait identified the COOH termini of polycystin-2 and CD2AP, respectively, as strong interacting partners. This interaction was confirmed in cultured cells by co-immunoprecipitation of endogenous polycystin-2 with endogenous CD2AP and vice versa. CD2AP shows a diffuse reticular cytoplasmic and perinuclear pattern of distribution, similar to polycystin-2, in cultured cells, and the two proteins co-localize by indirect double immunofluorescence microscopy. CD2AP is an adapter molecule that associates with a variety of membrane proteins to organize the cytoskeleton around a polarized site. Such a function fits well with that hypothesized for the polycystin proteins in renal tubular epithelial cells, and the present findings suggest that CD2AP has a role in polycystin-2 function.

The development of the mouse metanephric kidney provides a model system in which embryonic mesenchymal cells convert into polarized, well differentiated epithelial cells. Kidney morphogenesis starts on embryonic day 11 when an epithelial structure, the ureteric bud, bulges from the Wolffian duct and invades the metanephric mesenchyme. Through a series of reciprocal interactions the mesenchyme-to-epithelium transition leads to the formation of highly specialized, functionally diverse cell types that are characteristically patterned along the nephron to regulate waste removal and salt and water balance. This complex process is initiated by an inductive signal involving the leukemia inhibitory factor or other members of the interleukin-6 cytokine family and a mesenchymal growth factor (fibroblast growth factor 2, transforming growth factor α, or fibroblast growth factor 9), which together trigger aggregation of the cells (1). This developmental system is ideal for discovering the variety of "epithelial" genes that are activated (*e.g.* cytokeratins and basement membrane collagens) and of "mesenchymal" genes that are inactivated (*e.g.* vimentin and interstitial collagens) as a consequence of the mesenchyme-to-epithelium transition (*cf.* Ref. 2).

In autosomal dominant polycystic kidney disease (ADPKD),[1] the mature kidney tubular epithelial cells lose their differentiated function and morphology (3). The kidney develops expanded, fluid-filled cystic structures lined by flattened epithelial cells. The pathophysiological changes leading to, or resulting from, cyst formation include increased cell proliferation and apoptosis, abnormal fluid secretion, interstitial inflammation, and matrix accumulation (*cf.* Refs. 4–6).

Two genes associated with mutations causing human ADPKD have been cloned. Mutations in PKD1 (7–10) or PKD2 (11) are responsible for most cases of ADPKD (12). Although the disease is inherited as an autosomal dominant trait, evidence now indicates that cyst formation is a focal process resulting from somatic mutations on the normal allele ("second hits") of the respective disease gene (13, 14). Polycystin-1, the protein product of *PKD1*, is a transmembrane glycoprotein with a large extracellular NH_2-terminal domain thought to be involved in cell-cell or cell-matrix interactions (7–10). Polycystin-2, the *PKD2* gene product, shares homology with PKD1, with the voltage-activated Ca^{2+}/Na^{+} channels (11), and the *trp* family of calcium channels (15). Recently, one of two proteins with close

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF149092.

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[1] The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; CD2AP, CD2-associated protein;SH3 domain, Src homology domain 3; METS-1, mesenchyme-to-epithelium transition protein with SH3 domains; kb, kilobase pair; bp, base pair; RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

structural similarity to polycystin-2 (16–18) has been found to function as a calcium-regulated cation channel (19).

Expression studies of polycystin-2 have indicated that it is part of the epithelial gene repertoire activated at the time of nephron maturation and elongation (20, 21). Mice lacking *Pkd2* (and *Pkd1*) exhibit a histologically intact nephrogenic zone but form cystic structures rather than mature elongating tubules in the inner cortical regions after embryonic day 15 (22). It has been suggested that polycystin-1 and polycystin-2 are part of a common signaling pathway, but the mechanism of their signaling remains elusive (23). The discovery of interacting partners for the polycystins represents an effective means of improving the understanding of the function of these disease genes.

Here we show that CD2AP (24, 25) is strongly up-regulated during kidney differentiation. CD2AP physically interacts with polycystin-2 in cells in culture and partially co-localizes with polycystin-2 in kidney tubules. CD2AP likely functions in the polycystin signaling pathway as an adapter molecule mediating association of polycystin-2 with multimeric intracellular and membrane complexes in renal tubular epithelia.

EXPERIMENTAL PROCEDURES

Cloning METS-1/CD2AP—The 1.7-kb 3′-end of the CD2AP transcript (clone B102; Ref. 26) was identified by differential screening of an embryonic day 17 mouse kidney cDNA library as described previously (26, 27). Briefly, duplicate filters were hybridized with [α-^{32}P]dCTP-labeled first strand cDNA probes representing embryonic day 11 uninduced metanephrogenic mesenchyme or 17-day embryonic kidney. The filter hybridized with the 17-day kidney probe was stripped and reprobed with an adult kidney cDNA probe. The clones that showed differential expression were isolated, and the inserts were recovered in pBluescript SK(−) by the Stratagene *in vivo* excision protocol. Longer CD2AP cDNA clones were obtained by screening of an adult mouse kidney cDNA library (M. Zerial, EMBL, Heidelberg, Germany) and the 5′-end was cloned by 5′-rapid amplification of cDNA ends (5′-RACE System, Life Technologies, Inc.). Filter hybridizations were performed overnight at 42 °C in 50% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, containing the labeled probe at 1×10^6 cpm/ml. Filters were washed in 0.5× SSC, 1% SDS at 50 °C and exposed with intensifying screens at −70 °C overnight. The primers used for 5′-RACE were as follows: first round, 5′-CAC TGG GTC CAC ACC ACC TC-3′, 5′-GTG CAT CTA CAA TAC ACA AC-3′, 5′-CTA AGT TCG TCC ACG G-3′; second round, 5′-CTA CTG GAA GTT CTT GTC CG-3′, 5′-CTC CAA ATC CAA TTC CTC GG-3′, 5′-CTT TGG CTG TGC AAC TGA TC-3′.

The 1914-bp open reading frame of CD2AP was obtained in three overlapping fragments, one from cDNA-library screenings and two from 5′-RACE, that lacked restriction sites suitable for ligation. Therefore, the full-length open reading frame was cloned by reverse transcription-polymerase chain reaction with gene-specific primers from embryonic day 17 mouse kidney total RNA. The primers used were as follows: 5′-primer, 5′-GCC GCC GGA TCC ATG GTT GAC TAT ATT-3′; 3′-primer, 5′-GCC GCC CTC GAG TCA AGA CAA CAG AAC AGC-3′. The GenBank™ accession number for the sequence, originally named METS-1, is AF149092.

RNA Isolation and Northern Blotting—Two micrograms of poly(A)-enriched RNA were resolved in 1.6% agarose-formaldehyde gel and blotted onto GeneScreen Plus™ membrane (DuPont). The 1.7-kb 3′-end of the CD2AP cDNA was labeled with [^{32}P]dCTP by random priming and hybridized as described previously (27).

In Situ Hybridization—Tissue samples of the developing kidney were fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin. The sense and antisense cRNA-probes (550-bp long 3′-end fragment of CD2AP cDNA subcloned into pGEM3) were labeled with [^{35}S]UTP (Amersham Pharmacia Biotech) by the SP6/T7 run-off transcription method (Riboprobe II Core System, Promega). The hybridization was performed essentially as described (27).

Yeast Two-hybrid Screening—We used three different bait constructs of CD2AP for the yeast two-hybrid analysis, one from the NH_2 terminus and two from the COOH terminus covering amino acids 1–167, 353–637, and 423–637, constructs N, C1, and C2, respectively. The fragments were amplified by polymerase chain reaction from the CD2AP cDNA and subcloned as *Xho*I-*Bam*HI inserts into pLexA (28) to generate LexA fusion proteins. The primers used were construct N 5′-primer, 5′-TTT CTC GAG CGT TGA CTA TAT TGT GGA GTA TGA C-3′; construct N 3′-primer, 5′-GCC GCC GGA TCC TCA GGA CTC TAA TTC TTT CAC-3′; construct C1 5′-primer, 5′-GCC GCC CTC GAG CGA CCT GTC AGC TGC AGA GAA GAA AGC-3′; constructs C1 and C2 3′-primer, 5′-GCC GCC GGA TCC TCA AGA CAA CAG AAC AGC-3′; construct C2 5′-primer, 5′-GCC GCC CTC GAG CGC AGC CAA AAT TAA TGG AGA AGT TCC-3′. The adult mouse kidney MATCHMAKER cDNA library (catalogue number ML4002AB, CLONTECH) in the pGAD10 vector was used as prey. The screening was performed essentially as described (29). Briefly, the library and the bait constructs were transformed into yeast strain L40 that was then cultured in synthetic, minimal selection medium (SD medium) lacking Trp, Leu, and His. The positive clones on selective media were further confirmed by monitoring expression of the *lacZ* reporter gene. No autonomous activation of the *HIS3* or *lacZ* reporter genes was observed with any of the bait constructs. The inserts of *lacZ*-positive clones were amplified by polymerase chain reaction with pGAD10 vector-specific primers and subcloned into pGEM-T (Promega) for sequencing.

Production of the Antisera—CD2AP (residues 6–574) was expressed as a His_6-tagged fusion protein in *Escherichia coli*, purified, and used for immunizing rabbits. The specific antibody reactivity in Western blotting (Fig. 3*A*) and immunofluorescence was blocked by preincubating the working dilution of the antiserum with the purified recombinant protein at 1 and 10 μg/ml, respectively. The preimmune serum was negative.

A polycystin-2 rabbit antiserum (YCC2), generated against residues 687–962, has been characterized elsewhere (20, 30). An independent polycystin-2 rabbit antiserum (R223) was raised against a GST-tagged fusion protein carrying amino acid residues 747–968 of the mouse protein (GenBank™ accession number AF014010). In Western blotting, R223 detects a single band migrating at 110 kDa (not shown), corresponding to the size of polycystin-2 (11). A mouse monoclonal antibody, YCE2, directed against residues 687–754 of PKD2 specifically recognized polycystin-2 on Western blots.[2]

Transient Transfections, Immunohistochemistry, and Confocal Microscopy—For preparation of frozen sections, kidneys were rapidly frozen in Tissue-Tek® O.C.T. Compound (Sakura). The sections were fixed in 3.5% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. The primary antisera and the tetramethylrhodamine B isothiocyanate-conjugated secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) were diluted in PBS containing 0.5% saponin and 5% fetal calf serum.

The CD2AP full-length open reading frame (amino acids 1–637) was subcloned into pCDNA3.1 (Invitrogen). The full-length PKD2 construct (TM4-FL) and the LLC-PK_1 cell line stably expressing PKD2 in pCDNA3.1 have been previously described (30). Stable LLC-PK_1 cells (LLC-PK_1/TM4) expressing full-length PKD2 were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. LLC-PK_1/TM4 cells in 6-well culture plates were transfected with 2 μg of expression plasmid containing full-length CD2AP using Cytofectene (Bio-Rad). M-1 cells, which express native polycystin-2 and CD2AP, were transiently transfected with TM4-FL. Twenty four hours after transfection, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and incubated with polyclonal anti-CD2AP and/or with monoclonal YCE2 anti-polycystin-2 antibody. Immunofluorescence was visualized by subsequent incubation with anti-rabbit-IgG-AlexaFluor (488 nm) and/or anti-mouse IgG-AlexaFluor (594 nm) (Molecular Probes). Fluorescence microscopy was performed with a Zeiss Axiophot microscope and confocal microscopy with a Zeiss LSM 410 inverted laser scanning microscope.

In Vitro Binding Assay—In vitro binding assays were performed essentially as described previously (31). A bacterially expressed Pkd2-GST fusion protein containing amino acids 747–968 of mouse polycystin-2 (GenBank™ accession number AF014010) or GST alone were purified using glutathione-Sepharose. The protein samples were resolved by SDS-polyacrylamide gel electrophoresis (10% gel) and transferred to polyvinylidene difluoride (Bio-Rad) membranes. Filters were blocked with 5% nonfat dry milk in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20, 1 mM $CaCl_2$ (TBSC buffer). GST-CD2AP (amino acids 331–637) was biotinylated with sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's instructions and added in TBSC at 1 μg/10 ml. After extensive washes in TBSC the filters were incubated with streptavidin-conjugated alkaline phosphatase (Amersham Pharmacia Biotech), 0.2% gelatin in TBSC, washed, and developed using the Amersham Pharmacia Biotech enhanced chemiluminescence (ECL) system.

[2] Y. Cai and S. Somlo, manuscript in preparation.

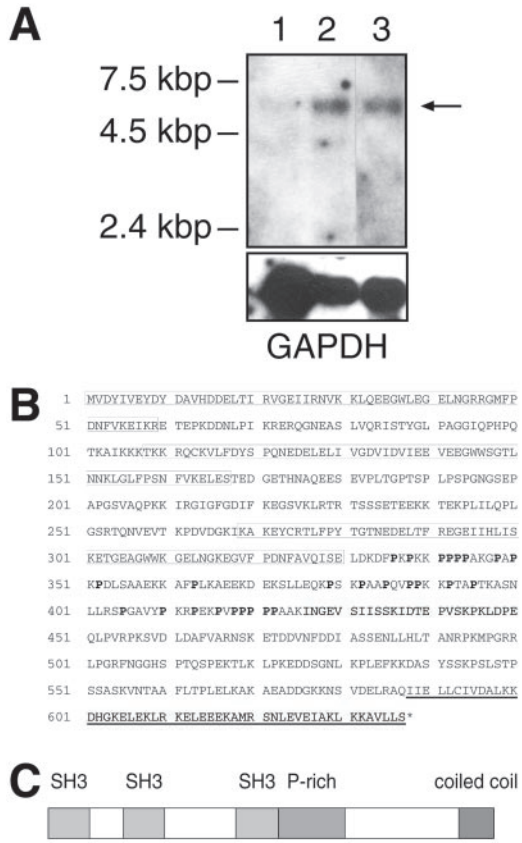

FIG. 1. **Expression of CD2AP in the developing kidney and sequence characteristics of the protein.** *A,* Northern blot of CD2AP. *Lane 1,* embryonic day 11 undifferentiated mouse metanephric mesenchyme; *lane 2*, embryonic day 17 kidney; *lane 3*, adult mouse kidney. The CD2AP mRNA (*arrow*) is strongly up-regulated during kidney differentiation and maturation. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *B,* the deduced amino acid sequence of CD2AP (originally named METS-1; GenBank™ accession number AF149092). SH3 domains are *boxed*, prolines in the proline-rich region are in *boldface type,* and the predicted coiled-coil region is *underlined. C,* schematic presentation of the structural features of CD2AP.

Co-immunoprecipitation—Co-immunoprecipitations, using lysates of M-1 cells, were performed as described (32), except that Nonidet P-40 (Roche Molecular Biochemicals) was used instead of CHAPS. The CD2AP antiserum or preimmune serum were used for immunoprecipitation, and the presence of polycystin-2 in the immunoprecipitates was monitored by Western blotting using the YCC2, R223, and YCE2 polycystin-2 antibodies followed by detection with ECL. Co-immunoprecipitations were also performed with the YCC2 antiserum and monitored by Western blotting with the CD2AP antiserum.

RESULTS

Cloning of METS-1/CD2AP—To search for genes induced in the developing mouse kidney, we carried out a differential cDNA library screening (26, 27). From the original screening of 10,000 plaque-forming units, we obtained 36 clones that were differentially expressed either between embryonic day 11 metanephric mesenchymes and embryonic day 17 kidneys or between embryonic day 17 and adult kidneys. METS-1 (mesenchyme-to-epithelium transition protein with SH3 domains)/CD2AP (clone B102; Ref. 26) was identified as a gene showing strong up-regulation between embryonic days 11 and 17 (Fig. 1*A*). The ~5.4-kb cDNA contains a 1914-bp open reading frame with a 66-bp 5′-untranslated region and an ~3.4-kb 3′-untranslated region. CD2AP is predicted to encode a cytosolic protein of 70.5 kDa (Fig. 1, *B* and *C*). The NH_2 terminus of the protein contains three Src homology domain 3 (SH3 domains) known to mediate protein-protein interactions by binding to proline-rich motifs (33–35). A proline-rich region follows, in turn, the third SH3 domain of CD2AP, providing potential recognition sites for SH3 domains. A region rich in serine and threonine residues, which could be subject to phosphorylation, lies between the second and third SH3 domains. The COOH-terminal half of the protein is predicted to be globular, and the last 50 amino acids display high propensity for coiled-coil structure as predicted by the Coils and Paircoil programs (36).

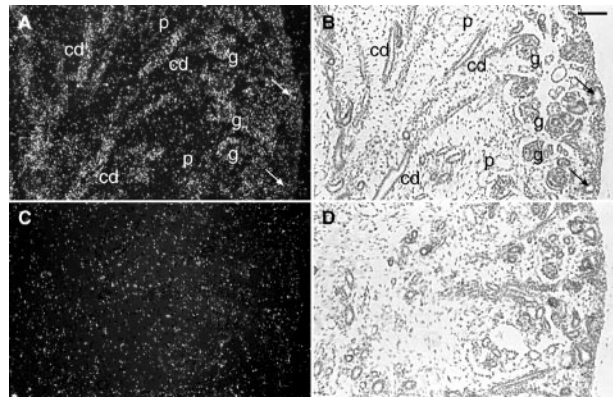

FIG. 2. **Localization of the CD2AP transcript in the 17-day embryonic mouse kidney by *in situ* hybridization.** *A* and *C* are dark field images, and *B* and *D* are the corresponding bright field images. Strong expression of CD2AP (*A* and *B*) is seen in the collecting ducts (cd), in which the medullary, more mature branches show a significantly higher level of expression than the sprouting tips (*arrows*). The transcript is also abundant in glomeruli (*g*). Proximal tubules (*p*) show weak or negligible signal. No signal was seen in the sense-strand controls (*C* and *D*). *Bar,* 90 μm.

CD2AP Localization in the Kidney—The expression pattern of CD2AP was studied by *in situ* hybridization in the embryonic day 17 mouse metanephric kidney, which contains all stages of nephron and collecting duct differentiation (Fig. 2) (37). At the mRNA level, early epithelializing structures show distinct expression of CD2AP. The medullary, more mature branches of collecting ducts, and the glomeruli contain a high level of the transcript, whereas the expression in the ingrowing tips of the ureteric bud is significantly lower (Fig. 2).

We examined the CD2AP protein expression pattern in embryonic day 17 and adult mouse kidney (Fig. 3). The CD2AP antiserum recognizes a single ~80-kDa protein in the kidney (Fig. 3*A*). The immunoblotting (Fig. 3*A*) and immunocyto/histochemical (not shown) signal was competed by fusion protein, and the preimmune serum was negative. The distribution of the CD2AP protein in the embryonic day 17 kidney is similar to that of the mRNA. Strong expression of CD2AP is seen in the mature, medullary branches of collecting ducts and in mature glomerular podocytes (Fig. 3*B*). Although it is already present at low levels in the undifferentiated mesenchyme cells, CD2AP expression becomes more abundant in early epithelializing structures (Fig. 3, *B* and *C*). In the adult kidney, CD2AP retains very strong expression in glomerular structures and fairly strong expression in cortical collecting ducts (Fig. 3*D*). There is weaker but positive immunoreactivity for CD2AP in distal nephron segments including those staining positive for Tamm-Horsfall protein, whereas proximal tubular staining is very low or absent (Fig. 3, *D* and *E*).

CD2AP Interacts with Polycystin-2 in Reciprocal Yeast Two-hybrid Screens—The presence of several potential protein-binding motifs in CD2AP prompted us to search for potential interacting partners by a yeast two-hybrid screen using different parts of the protein as baits. Screening of an adult mouse kidney library with the COOH-terminal region of CD2AP (Ala^{423}–Ser^{637}) resulted in the identification of five clones all encoding the cytosolic COOH terminus of the Pkd2 protein, polycystin-2 (11, 38). The longest cDNA fragment contained

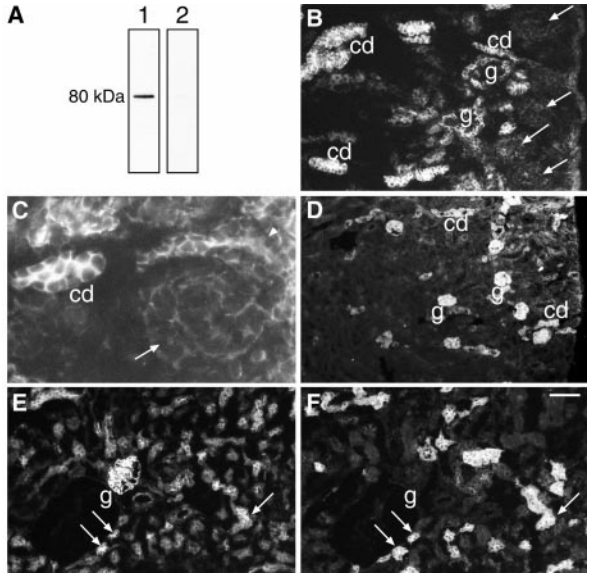

FIG. 3. **Characterization of the CD2AP antibody and immunohistochemical localization of the CD2AP protein in the mouse kidney.** *A,* immunoblotting. *Lane 1,* in 17-day embryonic kidney the CD2AP antibody recognizes a single band that migrates at ~80 kDa. *Lane 2,* the reactivity is blocked by preincubating the antibody with the CD2AP recombinant protein. *B–F,* immunofluorescence microscopy. In embryonic day 17 kidney (*B*), CD2AP protein is found at high levels in the glomerular podocytes (*g*) and collecting ducts (*cd*). In the cortical region of the kidney, CD2AP expression is induced in the early stages of epithelial differentiation, such as vesicles and comma- and S-shaped bodies (*arrows*). At higher magnification (*C*) an S-shaped body (*arrow*) is positive for CD2AP. The tip (*arrowhead*) of the collecting duct (*cd*) exhibits a weaker signal than the more medullary part (*cd*). In the adult mouse kidney (*D–F*), CD2AP expression in the cortex (*D*) is strongest in glomeruli (*g*) and collecting ducts (*cd*) with weaker staining in a subset of distal tubular segments. Serial sections of adult mouse kidney medulla stained for CD2AP (*E*) and anti-Tamm-Horsfall antigen (*F*) show CD2AP staining in Tamm-Horsfall-positive nephron segments (*arrows*). Proximal tubules are negative in the adult. *Bar*, 50 μm in *B* and *E–F*; 15 μm in *C*; 100 μm in *D*.

Gly^{747}–Val^{968} of polycystin-2, the shortest Val^{836}–Val^{968}, and the common overlapping region of all positive polycystin-2 clones was amino acids Val^{836}–Val^{968}. Conversely, a yeast two-hybrid screen using the COOH terminus of PKD2 (amino acids Ile^{680}-Val^{968}) as bait and a mouse embryonic day 9–10 library as prey identified a clone encoding amino acids Ser^{527}–Glu^{615} of CD2AP among a number of positive clones.[3] The sensitivity and specificity of the latter screen is corroborated by the fact that it also identified the COOH termini of Pkd1 and Pkd2, both known to interact with the COOH terminus of Pkd2 (39, 40).

CD2AP Associates with Polycystin-2 in Mammalian Cells—The physical interaction between CD2AP and polycystin-2 was verified by *in vitro* binding assays. Biotinylated GST-CD2AP specifically bound to a bacterially expressed GST fusion protein containing the polycystin-2 COOH terminus (Fig. 4*A*). The mouse kidney-derived cortical collecting duct epithelial M-1 cell line expresses both CD2AP and PKD2 endogenously. To test whether direct interaction occurs between the native CD2AP and PKD2 proteins *in vivo*, M-1 cell lysates were subjected to immunoprecipitation using the CD2AP and polycystin-2 antisera, respectively (Fig. 4, *B* and *C*). Western blotting of the CD2AP precipitates using the anti-polycystin-2 antisera YCC2 and R223 (not shown) and monoclonal antibody YCE2 (shown) revealed that the CD2AP antiserum co-immunoprecipitated native polycystin-2 (Fig. 4*B*). Conversely, the YCC2

[3] Y. Maeda and S. Somlo, unpublished observations.

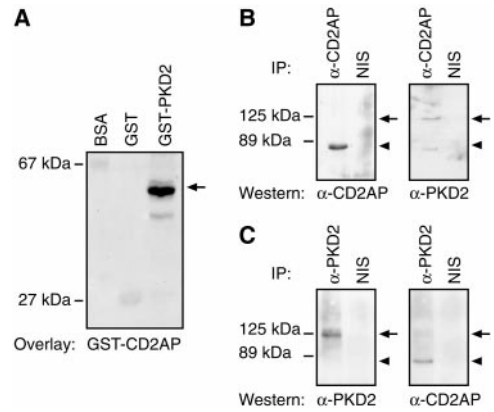

FIG. 4. **Association of CD2AP and polycystin-2.** *A, In vitro* binding assay. Two micrograms of bovine serum albumin (*BSA*), GST, or the cytosolic COOH terminus of mouse polycystin-2 (Gly^{747}–Val^{968}) fused with GST (GST-PKD2) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The biotinylated COOH terminus of CD2AP, GST-CD2AP (Leu^{331}–Ser^{637}), binds to a 51-kDa band (*arrow*), corresponding to the size of the GST-PKD2 fusion protein. *B* and *C,* co-immunoprecipitation from wild type M-1 cells, a mouse renal collecting duct-derived cell line. *B,* immunoprecipitation (*IP*) with CD2AP antiserum (α-CD2AP) and non-immune serum (*NIS*); immunoblotting with CD2AP antiserum (*left*) and YCE2 anti-polycystin-2 antibody (*right*). The *left panel* confirms the specific immunoprecipitation of CD2AP (*arrowhead*). The *right panel* shows the co-immunoprecipitated ~110-kDa band (*arrow*) detected by YCE2. The weak band at ~80 kDa was a nonspecific cross-reacting band. The ~110-kDa band, but not the lower band, was independently recognized by the polyclonal anti-polycystin-2 antisera YCC2 upon reprobing of the same blot (not shown) and by the R223 and YCC2 antisera in independent experiments (not shown). *C,* immunoprecipitation by YCC2 (α-PKD2) and non-immune serum (*NIS*). Immunoblotting with YCC2 confirms specific immunoprecipitation of polycystin-2 (*left, arrow*). Immunoblotting with CD2AP antiserum reveals specific co-immunoprecipitation of CD2AP (*right, arrowhead*).

anti-polycystin-2 antiserum was found to co-immunoprecipitate native polycystin-2 and CD2AP from the same cell line (Fig. 4*C*). This is the first interaction between a polycystin protein and a putative binding partner that is demonstrable by reciprocal immunoprecipitation studies using endogenously expressed, native proteins. Taken together, these findings of physical association between CD2AP and polycystin-2 in renal tubule-derived epithelial cells strongly support the occurrence of direct interaction between these native proteins *in vivo*.

CD2AP Co-localizes with Polycystin-2—We examined the spatial distribution of CD2AP and polycystin-2 in cells to determine if their expression patterns are permissive for functional interaction. Immunofluorescent cell staining of wild type M-1 cells revealed that native CD2AP is expressed in a diffuse, fine reticular and perinuclear cytoplasmic pattern (Fig. 5*A*). This pattern is similar to that described for polycystin-2 transfected into a wide range of cell lines (30), including M-1 cells (Fig. 5*A*). This native pattern of CD2AP expression was recapitulated in LLC-PK_1 cells transiently transfected with the full-length CD2AP cDNA (Fig. 5*B*). Indirect double immunofluorescence confocal microscopy on LLC-PK_1 cells stably expressing full-length polycystin-2 (30) and transiently transfected with full-length CD2AP showed strong co-localization of the proteins in a cytoplasmic reticular pattern (Fig. 5*B*). In a few cells extending cytoplasmic processes on the tissue culture dish, both proteins showed very similar patterns of redistribution within the cells (Fig. 5*C*). The association of native proteins in cells is supported by their co-localization in epithelial cell lines.

DISCUSSION

The first stages of mesenchyme-to-epithelium transition leading to differentiation of the excretory nephrons involve

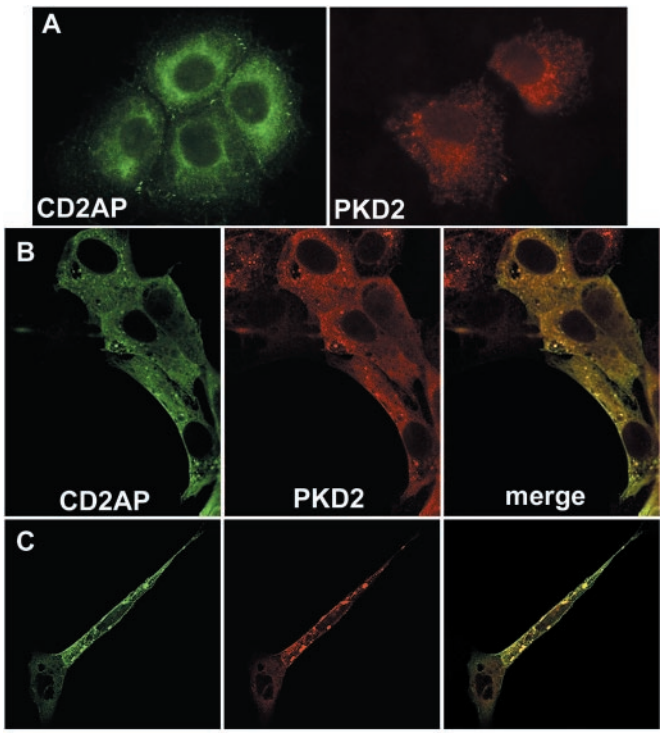

FIG. 5. **Co-localization of CD2AP and polycystin-2.** *A,* the cellular expression pattern of endogenous CD2AP in M-1 cells in culture (*left panel*) is cytoplasmic in diffuse, fine reticular pattern. This is similar to that reported for cells transfected with full-length polycystin-2 (*right panel,* M-1 cells transiently expressing PKD2) (30). *B,* double indirect immunofluorescence confocal images of LLC-PK_1 cells stably expressing full-length human PKD2 and transiently overexpressing full-length mouse CD2AP reveal co-localization of the two proteins in those cells expressing the CD2AP construct. Occasionally, individual cells with cytoplasmic extensions showed some redistribution of both proteins (*C*). The co-localization of the proteins was maintained under these circumstances.

changes in cell adhesiveness and morphology associated with modifications in cell adhesion proteins, extracellular matrix components, cytoskeletal organization, and cytoskeleton-membrane interactions (37). In search for novel developmentally regulated genes, we identified CD2AP as a cDNA up-regulated during the mesenchymal-to-epithelial transition in mouse kidney development. CD2AP is present at low levels in undifferentiated mesenchyme cells but is strongly up-regulated when these cells start to aggregate and differentiate. During subsequent maturation of nephrons, the different cell lineages show highly differential expression of CD2AP. The transcript is partially down-regulated in the excretory tubules, whereas a high level of expression is retained in the glomerular podocytes and the collecting ducts. CD2AP is distinctly more abundant in the medullary, more mature branches of the collecting ducts than in the ingrowing tips where differentiation is still in progress. Abnormalities in kidney development in the absence of CD2AP were not reported in CD2AP null mice (41), but progressive renal lesions primarily affecting the glomerulus occurred in the 1st month postnatally. Lack of CD2AP confers a disease susceptibility in the postnatal kidney that is a model for congenital nephrotic syndrome. The glomerular lesion in null mice is associated with proximal tubular dilatation thought to be secondary to albuminuria (41). The knockout model suggests that CD2AP has a role in maintaining the differentiated organization of the filtering nephron rather than in transducing early inductive signals between the epithelium and the mesenchyme.

The same is largely true of polycystin-2. Although there is weak expression of PKD2 in the ureteric bud, there is marked up-regulation of the protein in maturing nephron segments (20, 21). Targeted inactivation of *Pkd2* does not disrupt the inductive signals in the nephrogenic zone of the developing kidney but does interfere with the proper maturation of elongating excretory tubules (22). The central role of polycystin-2 in maintaining the differentiated state of the nephron is highlighted in mice whose normally formed kidneys undergo cystic degeneration after focal loss of Pkd2 expression along the nephron in adult life (14). Although the absolute requirement in tubular maturation does not seem to apply to CD2AP as it does for Pkd2, this does not preclude a significant role for CD2AP in the maintenance of renal tubular structure. The tubular maturation and maintenance roles of polycystin-2 may be mediated by discrete protein associations. Thus, polycystin-2 may function in the elongation of normal tubules through one set of regulated interactions and may mediate the maintenance of this structure through another set of interactions. The occurrence of early tubular dilatation in the CD2AP null mice may represent an early manifestation of this effect. Since the rate of cyst growth is unknown, cyst formation may be a relatively slow process and may not manifest in the short life span of CD2AP null mice (41).

The data presented here strongly support the *in vivo* association of CD2AP with polycystin-2. The physical association is proven by reciprocal yeast two-hybrid screens and reciprocal co-immunoprecipitation of the native proteins from renal epithelial cell lines. This is the first instance in which a putative binding partner for a polycystin protein can be verified by reciprocal co-immunoprecipitation of full-length, endogenously expressed protein (15, 39, 40, 42–44).

CD2AP is an adapter protein without predicted transmembrane spans. Yet its endogenous immunofluorescence pattern in cells shows a finely reticular pattern most consistent with an ER membrane distribution. Polycystin-2 is known to be an ER membrane protein in cultured cells (30), and CD2AP co-localizes with PKD2 by double indirect immunofluorescence confocal analysis in transfected cells. Immunohistochemical studies of polycystin-2 expression in the adult kidney have shown that polycystin-2 is strongly expressed in distal nephron segments (20, 45, 46). We have found that CD2AP is also expressed in these segments. The tissue co-localization of the two proteins, however, is not complete. CD2AP is also found in structures lacking polycystin-2, such as glomeruli. This does not preclude association with polycystin-2 since CD2AP is an adapter molecule known to be shared by different signaling cascades in different cell types (24, 25, 41).

Polycystin-1 and polycystin-2 interact through their COOH-terminal domains (39, 40) and participate in a larger macromolecular complex (47–49). They are thought to act in a common signal transduction pathway (23) in which polycystin-1, with numerous potential extracellular interaction domains including 16 Ig-like PKD1 repeats, likely mediates specialized cell-cell or cell-matrix adhesions (10). It may do so by regulating the activity of a polycystin-2-containing channel complex (11). CD2AP is an adapter molecule that has also been found to interact with CD2 and nephrin, both immunoglobulin superfamily member proteins involved in forming specialized cell adhesions (41). Furthermore, CD2AP has been implicated in regulation of the spatial and temporal assembly of signaling complexes that link membrane proteins to the cytoskeleton, including the focal adhesion complex protein $p130^{CAS}$ (25). CD2AP-containing complexes cause membrane receptor clustering and cytoskeletal polarization (24). In keeping with such a role, recent studies on epithelial cells from ADPKD cysts have found that basolateral, but not apical, trafficking of proteins is dysregulated. Lack of an intact polycystin complex leads to

disruption of E-cadherin-dependent cytoarchitecture, including the protein assemblies such as the sec6/8 exocyst crucial for basolateral protein delivery (47). By analogy, we propose that in the kidney tubule, CD2AP acts as an adapter protein mediating association of polycystin-2 with multimeric intracellular complexes. These complexes may include polycystin-1, cytoskeletal components (48, 49), components of the basolateral membrane targeting machinery (47), or signaling complexes such as those mediating Pkd2 induced AP-1 activation (50).

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