Polycystin-1, the protein defective in a majority of patients with autosomal dominant polycystic kidney disease, is a ubiquitously expressed multi-span transmembrane protein of unknown function. Subcellular localization studies found this protein to be a component of various cell junctional complexes and to be associated with the cytoskeleton, but the specificity and nature of such associations are not known. To identify proteins that interact with the polycystin-1 C-tail (P1CT), this segment was used as bait in a yeast two-hybrid screening of a kidney epithelial cell library. The intermediate filament (IF) protein vimentin was identified as a strong polycystin-1-interacting partner. Cytokeratins K8 and K18 and desmin were also found to interact with P1CT. These interactions were mediated by coiled-coil motifs in polycystin-1 and IF proteins. Vimentin, cytokeratins K8 and K18, and desmin also bound directly to P1CT in GST pull-down and in vitro filament assembly assays. Two observations confirmed these interactions in vivo: (i) a cell membrane-anchored form of recombinant P1CT decorated the IF network and was found to associate with the cytoskeleton in detergent-solubilized cells and (ii) endogenous polycystin-1 distributed with IF at desmosomal junctions. Polycystin-1 may utilize this association for structural, storage, or signaling functions.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common genetic diseases in humans, affecting ~1 in 400 individuals and accounting for 8–10% of end stage renal disease (1). ADPKD is characterized by the formation and progressive expansion of cysts in ductal organs including both kidneys as well as by cardiovascular abnormalities. About 85% of ADPKD cases are caused by defects in polycystin-1 (2, 3), a transmembrane protein with a large extracellular region rich in protein-protein and protein-carbohydrate interaction modules. Although this composition is suggestive of a role in cellular and/or cell-matrix adhesion, the precise function(s) mediated by polycystin-1 remains unknown.

Polycystin-1 interacts with intermediate filaments. Genetic, immunochemical, and biochemical studies have suggested that polycystin-1 is involved in maintaining the structural integrity of cell-cell junctions in vascular and epithelial structures. In mouse models of ADPKD, mouse embryos lacking a functional polycystin-1 are uniformly edematous (4, 5) and die in utero of hemorrhages preceded by vascular leaks (4). Consistent with these data, polycystin-1 has been shown to distribute at apicolateral cell junctions in both epithelial and endothelial cells (6–9). Immunolocalization and/or cell fractionation studies found polycystin-1 at tight (10), adherens (7), and desmosomal (9) junctions, at focal contacts (11), and in the cytoplasm. A fraction has also been found in the Triton X-100-insoluble cell fraction, reflecting a strong association with cytoskeleton (12). Recently polycystin-1 was shown to be transported through a tubulin-dependent pathway to the lateral domain of cell membranes (13). The nature of the interactions utilized by polycystin-1 to localize at cell junctions associate with cytoskeleton or traffic to the cell surface is currently unknown. In this paper, we have identified a direct and specific interaction of polycystin-1 with the intermediate filament network. This interaction is mediated by the cytoplasmic tail of polycystin-1 binding directly to the IF proteins vimentin, cytokeratins 8 and 18, and desmin. Interaction was shown initially in a yeast two-hybrid screen of an epithelial cell library and confirmed both in vitro using GST pull-down and co-sedimentation assays and in whole cells using immunofluorescence analysis of both a membrane-anchored polycystin-1 cytoplasmic tail and the endogenous native protein. The functional significance of this association is discussed.

MATERIALS AND METHODS

DNA and Plasmid Constructs—The cDNAs and bacterial expression vectors for vimentin and desmin were kind gifts from the following sources: human vimentin cDNA, Dr. Elaine Fuchs (University of Chicago, Chicago, IL); pET11d-vimentin, Dr. Wallace Ip (University of Cincinnati College of Medicine, Cincinnati, OH) (14); and pET3a-desmin, Drs. Masakiv Inagaki and Hiroyasu Inada (Aichi Cancer Research Institute, Nagoya, Japan) (15). The cDNAs for cytokeratin K8 and K18 were amplified by reverse transcription-PCR from the human colon carcinoma HT29 cell line. The bacterial expression vectors for K8 and K18 were constructed by introducing NcoI and BamHI sites into the 5’ and 3’ ends of the cDNA by PCR and subsequently subcloning the cDNA into pET11d (Novagen). For mammalian expression constructs, cDNAs for vimentin, desmin, K8, and K18 were PCR-amplified with the introduction of an EcoRI site at the 5’ end and a BglII site at the 3’ end. The digested PCR fragments were then subcloned into the expression vector pCMV-5FLAG, which tags the FLAG epitope at the N terminus. Murine polycystin-1 and -2 cDNAs were amplified by reverse transcription-PCR from the human colon carcinoma HT29 cell line. The bacterial expression vectors for K8 and K18 were constructed by introducing NcoI and BamHI sites into the 5’ and 3’ ends of the cDNA by PCR and subsequently subcloning the cDNA into pET11d (Novagen). For mammalian expression constructs, cDNAs for vimentin, desmin, K8, and K18 were PCR-amplified with the introduction of an EcoRI site at the 5’ end and a BglII site at the 3’ end. The digested PCR fragments were then subcloned into the expression vector pCMV-5FLAG, which tags the FLAG epitope at the N terminus. Murine polycystin-1 and -2 cDNAs were amplified by reverse transcription-PCR. Murine cDNAs for the coiled-coil-containing proteins CD2AP (16), occludin (17), axin (18), conductin (19), and kinesin (20) were also amplified by reverse transcription-PCR. To construct plasmids for yeast two-hybrid analyses, DNA fragments were amplified by PCR with the introduction of an in-frame EcoRI site at the 5’ end. The PCR products were digested by EcoRI followed by phosphorylation of the 3’ blunted ends. The resulting fragments were then subcloned into...
the pLEXa or pB42AD vectors (CLONTECH) between EcoRI and XhoI (blunted) sites. The fusion construct sIg7P1CT was made by fusing murine P1CT from amino acids 4098–4293 to the end of CD7 transmembrane span section in the vector sIg7poly (kindly provided by Dr. Brain Seed, Massachusetts General Hospital, Boston, MA). pCD16.7 P1CT 115–226 was a kind gift from Dr. F. Walz (Beth Israel Hospital, Boston, MA). (21). GST-P1CC was constructed by subcloning the cDNA encoding amino acids 4173–4246 into pGEX-2T (Amerham Pharmacl Biotech) between the EcoRI and Smal sites. All PCR reactions were performed using Vent DNA polymerase (New England Biolabs). The oligonucleotide sequences for plasmid construction are available upon request.

**Yeast Two-hybrid Screen and Analysis**—mRNA from the collecting duct-derived Madin-Darby canine kidney (MDCK) cells was isolated using Dynabeads Oligo(dT)25 (Dynal). A yeast two-hybrid cDNA library was constructed from the mRNA using the SUPERSCRIP Choice System for cDNA Synthesis (Life Technologies, Inc.). The library contains 1.9 × 1010 independent clones. Murine P1CT from amino acids 4098–4293 was subcloned into pLEXa (pLEXa-P1CT) and used as bait to screen the library. This fragment is important for the function of polycystin-1, because mutations within P1CT have been shown to cause ADPKD (22, 23). Initial candidates were identified by examining leucine auxotrophy and by galactose-inducible β-galactosidase activity. False positives were eliminated by yeast mating assays according to the CLONTECH manual (PT540–11). To test interaction, a combination of a pLex-Bait construct and a pB42AD prey construct were co-transformed into yeast strain EGY48 (p8op-lacI). Transformants were grown on synthetic medium containing 2% galactose plus 1% raffinose but lacking uracil (Ura), histidine (His), and tryptophan (Trp). The culture was then either replicated onto a plate lacking leucine (Leu) to assess growth, or printed onto a filter paper for β-galactosidase analysis using X-gal as a substrate. To measure galactose-inducible β-galactosidase activity, a liquid assay was used; individual yeast colonies co-transformed with both bait and prey constructs were grown overnight in the synthetic medium lacking Ura, His, and Trp. The culture was then diluted 5-fold in fresh medium containing 2% galactose plus 1% raffinose and allowed to grow for another 5 h. After this induction period, the cell mass was measured as A600. The β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside as a substrate according to the CLONTECH manual and normalized to the cell mass (as A600/A600) for comparison.

**GST Pull-down Assay**—The bacterial strain BL21(DE3) was used for expression of GST or GST-P1CC. Each bacterial culture was induced by isopropyl-thio-β-D-galactopyranoside at 0.1 mm and allowed to express protein overnight at room temperature. The bacterial cells were lysed by sonication in 10 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, 1.5% sarkosyl, plus protease inhibitors. The supernatant, after centrifugation at 15,000 rpm for 30 min, was incubated with glutathione beads (Amerham Pharmacia Biotech). The beads were washed and then preabsorbed with 10 mm Tris-HCl, pH 7.4, 200 mm NaCl, 1 mm EDTA, and 5% BSA for 1 h. The beads were then washed once with a lysis buffer (20 mm Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, and 5 mm EDTA) and then loaded with 100 μl of cell lysates containing FLAG-tagged IF proteins. The beads were washed three times with 200 μl of lysis buffer, and the bound proteins were analyzed by Western blotting using the anti-FLAG antibody M2 (Sigma). For expression of FLAG-tagged IF proteins, HEK 293 cells in a 35-mm well plate were transfected with expression vectors for vimentin, desmin, or cytokeratin K8 plus K18. The cells were lysed 2 days later in 1 ml of lysis buffer, and the supernatant was used for GST pull-down experiments.

**Biotinylation**—The GST-P1CC fusion protein was expressed and isolated on glutathione beads as described above. The 8-kDa P1CC fragment was released from the beads by thrombin (Amerham Pharmacia Biotech) digestion and purified on a Q column (Amerham Pharmacia Biotech). The protein fractions were dialyzed into 50 mm HEPES, pH 8.0, N-Hydroxysuccinimide biotin (Pierce) was added to the P1CC at a biotin/P1CC molar ratio of 1.5. The reaction was allowed for 4 h at room temperature and stopped by 2 mm NH4Cl. Uncoated biotin was removed by filtration in a spin unit (molecular weight cut-off, 5,000; Millipore) and the biotin-conjugated protein was then dialyzed into the buffer containing 10 mm Tris-HCl, pH 8.3, 10 mm β-mercaptoethanol, 1 mm EDTA, and 0.1 mg/mL ETA. The resulting solution was centrifuged at 15,000 rpm for 15 min to remove any aggregates. 100 μg of IF proteins were then incubated with 0.15 or 0.015 μg of biotinylated P1CC (P1CC/Fm molar ratios of 0.01 and 0.001, respectively) in a total volume of 450 μl for 1 h at room temperature. Filament assembly was initiated by adding 50 μl of 0.4 mg/ml Tris-HCl, pH 7.0 (K8/K18) or 0.4 mg/ml Tris-HCl, pH 7.0, 0.5 m NaCl, and 10 mM MgCl2 (desmin or vimentin) to the assembly reactions. For controls, 7.5 μg of biotinylated P1CC was incubated alone or with 100 μg of BSA and then adjusted to the buffer as for vimentin and desmin. The assembly reactions were allowed for 2 h at room temperature. Filaments were pelleted by centrifugation at 30,000 × g for 2 h. The pellets were dissolved in 200 μl of 8 m urea in 10 mm Tris-HCl, pH 8.0. Five μg of pellet protein and 10 μl of a control sample were analyzed by SDS-PAGE followed by Western blotting using horseradish peroxidase-coupled streptavidin (Pierce) as a probe.

**Association with Cytoskeleton**—HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). 4 × 103 cells were plated in each well of a six-well plate 1 day before transfection. 2 μg of plasmid sIg7P1CT or its deletion construct and 9 μl of LipofectAMINE (Life Technologies, Inc.) were used for transfection of each well. The cells were lysed 2 days later in cytoskeleton stabilizing buffer containing 10 mm Pipes, pH 6.8, 100 mm NaCl, 3 mm MgCl2, 0.5% Triton X-100, 300 mm sucrose, and a protease inhibitor mixture (Roche Biochemicals) (27). The pellet was dissolved in an equal volume (200 μl/well) of Tris-HCl, pH 8.0, 6 m urea, 1 mm EDTA. 10 μl of lysate or pellet samples was analyzed by SDS-PAGE followed by Western blotting using the anti-FLAG antibody as a probe.

**Immunofluorescence**—To examine the subcellular distribution of the P1CT fusion construct, LLC-PK1 cells were transfected with plasmid pCD16.7PRD115–226 or its control pCD16.7. Twenty-four h after transfection, the cells were grown on coverslips in 4% serum medium with 10% FBS lacking calcium and/or cycloheximide. For some cells, nocodazole (Sigma) was used at a final concentration of 5 μg/ml for 1 h before fixation. For localization of endogenous polycys- tin-1, clone II MDCK cells were grown on coverslips in minimum essential medium with 10% FBS. The cells were fixed on ice for 40 min in a solution of 10% acetic acid and 50% ethanol, followed by incubation for 1 h at room temperature with a blocking solution containing 3% BSA, 2% FBS, and 0.01% Nonidot P-40. The cells were then incubated with 1:50 dilution of a rabbit anti-polycystin-1 LRR antibody (kindly provided by O. Ibraguimov-Beskrovnaya, Genzyme) (6) and mouse anti-desmin antibody (Becton Dickinson, 1:100). After washing, the coverslips were incubated with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Jackson Immunoresearch). For some cells, nocardazole (Sigma) was used at a final concentration of 5 μg/ml for 1 h before fixation. For localization of endogenous polycystin-1, clone II MDCK cells were grown on coverslips in minimum essential medium with 10% FBS. The cells were fixed on ice for 40 min in a solution of 10% acetic acid and 50% ethanol, followed by incubation for 1 h at room temperature with a blocking solution containing 3% BSA, 2% FBS, and 0.01% Nonidot P-40. The cells were then incubated with 1:50 dilution of a rabbit anti-polycystin-1 LRR antibody (kindly provided by O. Ibraguimov-Beskrovnaya, Genzyme) (6) and mouse anti-desmin antibody (Becton Dickinson, 1:100). After washing, the coverslips were incubated with fluorescein isothiocyanate-conjugated mouse anti-pan cytokeratins antibody (C-11, Sigma, 1:50) for 1 h and washed. The coverslips were mounted and examined by confocal microscopy.

**Quantitation of Polycystin-1 Association with Keratin Filaments**—To quantitate the association of polycystin-1 with keratin filaments, junctions showing clear individual filaments threading through the desmosomes were identified in micrographs. The number of filaments linked to polycystin-1 at desmosomes double-labeled with cytokeratin and desmoplakin was then counted.

**Calcium Switch Experiments**—For these experiments, MDCK cells grown on coverslips were first depleted of calcium by washing with Dulbecco’s modified Eagle’s medium with 10% FBS lacking calcium.
chondroitin sulfate (catalog number 21068; Life Technologies, Inc.) followed by an overnight incubation in the same medium. Calcium-containing normal Dulbecco’s modified Eagle’s medium with 10% FBS was then replenished. The cells were removed at different time points and processed for immunostaining.

RESULTS

Yeast Two-hybrid Screening of a cDNA Library for P1CT-interacting Proteins—To identify proteins that interact directly with polycystin-1, we carried out a yeast two-hybrid screen of an epithelial cell library as described (28) using P1CT (29) (Fig. 1A) as bait. Of 4.2 × 10⁶ yeast transformants, only 12 clones encoding six different proteins showed specific interaction with P1CT. These were identified by examining leucine auxotrophy and galactose-induced β-galactosidase activity and were confirmed by yeast mating assays. One of these interactants encoded the C-terminal 126 amino acids of vimentin (30). No β-galactosidase activity was detected above the negative control when the vimentin clone was co-transformed with an empty vector or the cytoplasmic tail of polycystin-2 (P2CT) (Fig. 1B), indicating that the interaction was specific. To evaluate the relative strength of interaction, we normalized the β-galactosidase activity to cell mass (A₄₉₀/A₆₀₀) and compared it with that of p53-large T-antigen interaction (used as a positive control). The strength of P1CT-vimentin interaction was ~60% of that between p53 and the large T-antigen. Of the six proteins identified in the screen, vimentin was the strongest interactor and was further characterized. One of the remaining five proteins (represented in three of the twelve clones) encoded 14-3-3 (31). The remaining four proteins are distinct from the known P1CT interactants such as polycystin-2 (32), RGS7 (regulator of G-protein signaling 7) (33), and PBP-1 (polycystin-1-binding protein-1) (34). Polycystin-2 is a multi-span transmembrane channel protein, and it is typically difficult to identify a transmembrane protein in a yeast two-hybrid screen. RGS7 and PBP-1 were identified from a B cell and a brain libraries, respectively. The potential relevance of the four clones in the function of polycystin-1 is being evaluated.

P1CT-Vimentin Interactions Are Mediated through the Coiled-coil Segments in Each Protein—Most of the sequence in the vimentin clone identified in the yeast two-hybrid screen was in a coiled-coil region. To determine whether the interaction between P1CT and vimentin was mediated by coiled-coil sequences present in both proteins, we tested the interaction of a set of deletion constructs for both P1CT and vimentin using yeast two-hybrid assays (Fig. 1C). Deletion of the coiled-coil sequence from either parental construct abolished the galactose-induced β-galactosidase activity and resulted in the failure of yeast transformants to grow in medium lacking leucine. In contrast, deletion of non-coiled-coil regions had no effect. We conclude that the interaction between P1CT and vimentin is mediated by the respective coiled-coil segments.

The coiled-coil motif of polycystin-1 was found previously to interact with polycystin-2 (32, 35), RGS7 (21), and PBP-1 (34). The identification of yet another coiled-coil protein interacting with the coiled-coil motif of P1CT raised the concern that P1CT may have a nonspecific affinity for coiled-coil sequences. To evaluate this possibility, we tested the ability of P1CT to interact with several additional coiled-coil proteins, some being components of various functional complexes where polycystin-1 was reportedly present. These included CD2AP, axin, occludin, kinesin, and conductin. CD2AP interacts with polycystin-2 (16) and with itself (Fig. 1D) through its coiled-coil region. However, it did not interact with P1CT. Axin, a protein involved in the Wnt/β-catenin signaling pathway, contains two coiled-coil regions, the C-terminal of which (from residue 831) is required for self-dimerization (36) and therefore serves as a positive control. Although axin self-interaction was evident, no interaction with P1CT was again detected. P1CT similarly did not interact with occludin, a tight-junction protein (17), and conductin (19), an axin homolog, and bound only weakly to kinesin (37), a motor protein associated with microtubules. Thus despite the abundance of coiled-coil proteins present at cell membranes, the fidelity of coiled-coil interactions is apparently maintained by specific sequences within P1CT. The ability of a coiled-coil region in one protein to interact with multiple partners has been previously observed in other systems. For example, the same coiled-coil motifs of syntaxin mediate interactions with different SNARE proteins (including syntaxin itself) at different steps of membrane fusion and protein trafficking (38). The multiple interactions mediated by the coiled-coil segment of P1CT may similarly serve sequential steps in a single pathway or different functions depending on the cell type.

P1CT Also Interacts with Epithelial and Muscle IF Proteins—Vimentin belongs to the intermediate filament protein family (39). IFs in different tissues are comprised of different IF proteins. All IF proteins share the same molecular architecture, each consisting of an N-terminal “head,” a highly conserved central coiled-coil rod, and a C-terminal tail (Fig. 2A) (40). The central coiled-coil rod is separated by a nonhelical linker into two regions, helix 1 and helix 2. In vivo, IF proteins are assembled into 10-nm insoluble filaments. IF tetramers are considered to be the building blocks for the higher order filament structure (41). A tetramer is formed from two anti-parallel strands of head-to-tail dimers, arranged in a staggered manner; its longitudinal elongation and lateral bundling give rise to the final IF structures. In this assembly scheme, one helix of the coiled-coil rod of an IF protein would be exposed at the end of a filament. Vimentin is mainly expressed in mesenchymal and endothelial cells (39). Other IF proteins, on the other hand, have different tissue-specific expression patterns; cytokeratins K8 and K18 are expressed in simple epithelial cells, such as those lining kidney tubules, and desmin is a muscle-specific IF protein. Because polycystin-1 is expressed in a wide variety of tissues, some lacking vimentin, and the deficiency of polycystin 1 affects endothelial as well as epithelial and muscle cell functions, we determined whether P1CT interacts with cytokeratins K8 and K18 and desmin. We generated three constructs for each IF protein (Fig. 2B); one encompasses the head domain and helix 1, the second contains helix 2 and the tail, and the third spans the whole protein. We examined the interaction of P1CT with each construct in yeast two-hybrid assays. With the exception of the helix 1 construct of cytokeratin 18 (K18 (1–232)), all single helix-containing constructs interacted with P1CT. The failure of P1CT to interact with K18 helix 1 was not an artifact, because the same K18 prey construct was able to interact with its in vivo partner K8 helix 1 (Fig. 2C). The results using full-length constructs were less consistent. Although K8 and K18 displayed weak interaction, yeast did not grow when transformed with the full-length desmin construct in pB42 vector. Full-length vimentin did not interact with P1CT but displayed strong interaction with itself. This differential reactivity of vimentin is likely the result of formation of mispaired IF protein oligomers when the full-length protein is expressed in the pLexA/pB42 system and has been previously noted with IF proteins analyzed in the yeast two-hybrid system (42).

In Vitro Interaction of P1CT with IF Proteins—To establish that P1CT interacts with the full-length IF proteins, we first carried out GST pull-down analysis (Fig. 3, A and B). A 77-amino acid peptide encompassing the coiled-coil region of P1CT (P1CC) (Fig. 1A) was expressed as a GST fusion protein in bacteria. The fusion protein was immobilized on glutathione

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beads and incubated with cell lysate. Proteins that bind to P1CC were analyzed by SDS-PAGE followed by Western blotting. To assign IF proteins unambiguously, we FLAG-tagged vimentin, desmin, and cytokeratins K8 and K18. We transfected HEK 293 cells with expression vector for FLAG-tagged vimentin or desmin. For cytokeratins, we co-transfected both K8 and K18 into the same cells. Fig. 3B shows the specific pull-down of K8, K18, vimentin, and desmin by GST-P1CC but
not by GST, indicating that P1CC interacted with the IF proteins. The amount of K8 is less than that of K18 in the K8/K18 panel, a reflection of the relative expression levels of the two proteins. The amount of K8 is less than that of K18 in the K8/K18 panel, a reflection of the relative expression levels of the two proteins.

In vitro filament assembly studies further confirmed the interaction of P1CT with IF (Fig. 3, C–E). IF assembly was done by stepwise dialysis of the recombinant proteins into a high pH/low salt buffer (39), under which condition the IF proteins exist mainly as tetrameric protofilaments. After the addition of biotinylated P1CC, followed by a 1-h incubation, filament assembly was initiated by adjusting pH and salt concentration, and the filaments were sedimented by centrifugation following by Western blotting. As shown in Fig. 3E, P1CC co-sedimented with vimentin, desmin, and cytokeratins K8/K18, and incorporation increased with higher concentration of P1CC in the assembly reaction. No P1CC was pelleted in the presence or absence of BSA under these conditions, indicating that the presence of P1CC in the IF co-sediment is not due to self-aggregation. Consistently, we found that the co-sedimentation of P1CC with K8/K18 was lower than with vimentin or desmin. This may be caused by different assembly kinetics for cytokeratins and/or by different assembly conditions (43). Increasing the P1CC concentration further did not significantly increase the incorporation, suggesting that binding was saturated (data not shown).

We also tested whether P1CC bound to preassembled IFs by incubation of IF with P1CC after filament assembly. No binding of P1CC to IFs was detected (data not shown). Together, these results indicated that binding of P1CC to soluble forms of IF proteins was required for the incorporation of P1CC into filaments in vitro. The fact that binding was saturable at a P1CC/IF molar ratio of 0.01 suggests that P1CC bound to unoccupied coiled-coil domains at ends of assembled filaments, consistent with the general filament assembly scheme (Fig. 2A).

The Coiled-coil Region of Polycystin-1 Mediates Its Association with the Cytoskeleton—The interaction of P1CT with IFs predicts that polycystin-1 would be in the Triton X-100 insoluble cytoskeleton fraction (27) as was previously reported (12). Our findings further suggested that this association is mediated by the coiled-coil region of polycystin-1. To test this, we...
made a set of three membrane-anchored P1CT constructs, each as a fusion protein of slg extracellular domain, CD7 transmembrane span, and a P1CT variant (Fig. 4). Each construct was tagged by a FLAG epitope at the C terminus. We transiently transfected the constructs into HEK 293 or HeLa cells. Two days later, the cells were lysed in the cytoskeleton stabilizing buffer. The distribution of each construct in the soluble lysate and in the cytoskeleton pellet was analyzed by Western blotting using the anti-FLAG antibody. Molecular mass markers (Bio-Rad) shown are those for ovalbumin (45 kDa) and bovine serum albumin (66 kDa).

The P1CT Fusion Construct Decorates Cytokeratin Filaments in LLC-PK1 Cells—To further substantiate P1CT-IF interaction, we set out to examine the cellular localization of the membrane-anchored P1CT fusion construct in transfected cells. This approach was previously used to validate the interaction of a desmoplakin fragment with IFs (25). We transfected the kidney epithelial cells LLC-PK1 with pCD16.7PKD115–226, a fusion construct containing the CD16 extracellular epitope, a CD7 transmembrane domain, and the C-terminal 112 amino acids of P1CT, which includes the coiled-coil domain (21). Cells transfected with a control CD16.7 vector showed a diffuse speckled pattern of perinuclear cytoplasmic and some surface membrane CD16 staining (Fig. 5). Strikingly, cells transfected with pCD16.7PKD115–226 invariably displayed a filamentous intracellular pattern of CD16 staining, decorating what could be cytokeratin filaments or microtubule networks. To distinguish between these two possibilities, we treated pCD16.7PKD115–226 expressing cells with nocodazole, a potent microtubule-disrupting reagent (44) that depolymerizes the microtubule network and causes deformation/collapse of IFs around nuclei, as a result of loss of connection of IF filaments to microtubules (45). Nocodazole treatment totally depolymerized microtubules, as assessed by immunofluorescence (data not shown). However, the filamentous distribution of CD16 remained and predictably showed a partial collapse of the filaments into a perinuclear ring, characteristic of the response of IFs to depolymerization of microtubules with which they associate (45). These data indicate that P1CT is capable of mediating the association with IFs when overexpressed in living cells.

Distribution and Kinetics of Association of Endogenous Polycystin-1 with Keratin Filament at Desmosomal Junctions—We next examined the subcellular distribution of endogenous polycystin-1 in relation to IFs and desmosomes. Because abnormal kidney function is an invariable feature of ADPKD, we examined the co-distribution of polycystin-1, cytokeratins, and desmoplakin in a cell line from this tissue. We initially carried out immunostaining on MDCK cells using monospecific antibodies directed against polycystin-1 and cytokeratins. Polycystin-1-dependent staining revealed discrete nodular junctional staining, together with some cytoplasmic staining; nuclear staining was also observed, a nonspecific feature previously found using this antibody (6). The antibody against cytokeratin revealed an extensive filamentous network that surrounded the nuclei and radiated into the desmosomal cell-cell junctions. Superimposing polycystin-1 and cytokeratin staining revealed a striking overlap at the cell junctions, which also overlapped with desmoplakin staining when all three antibodies were used simultaneously (Fig. 6). To quantify the extent of polycystin-1/cytokeratin co-localization, we counted the frequency of polycystin-1/junctional staining in areas of cell-cell contact where keratin filaments could clearly be identified converging on the cell membranes between neighboring cells. Of 232 desmosomes (double-labeled with cytokeratin and desmoplakin), 217 (93.5%) showed discrete foci of polycystin-1 staining. The kinetics of appearance of polycystin-1 at desmosomal junctions was also examined in cultured MDCK cells using calcium switch experiments. We found that desmoplakin was present in membrane junctions 30 min after replenishment of calcium in MDCK cultures, and at 120 min, both polycystin-1 and desmoplakin were present in desmosomes (data not shown). Similar kinetics were recently reported by Scheffers et al. (9).
Interaction required the coiled-coil motif of each partner. Second, P1CT interacted with full-length IF proteins in GST pull-down and in vitro filament assembly experiments. Third, membrane-anchored P1CT associated with the Triton X-100-insoluble cytoskeleton fraction through its coiled-coil segment. Finally, P1CT decorated IFs, and native polycystin-1 interacted with cytokeratins at desmosomal junctions in MDCK cells. These observations are strong evidence of a direct polycystin-1-IF interaction both in vitro and in vivo.

Membrane-anchored P1CT decorated the cytoplasmic IF network, whereas native polycystin-1 was localized in association with IF at desmosomes. This difference in subcellular distribution between a native protein and a recombinant fragment thereof has been observed previously. For example, the recombinant IF-interacting domain of desmoplakin has been shown to decorate cytoplasmic IFs, in contrast to the desmosomal localization of the intact protein (25). This difference in distribution may relate to the presence of additional domains in the native protein that may target it differently. Alternatively, overexpression of the recombinant fragment may saturate binding sites in IFs, which may only be available to the native protein under certain conditions.

The present data also show that P1CT interactions with IF subunits (Figs. 1, 2, and 3B) as well as with assembled IFs (Figs. 3E, 5, and 6). Because assembly of IF subunits into polymers is driven by the coiled-coil domain, the data raise the logical question of how P1CT can bind to assembled IFs in vivo. The assembled IF tetramer, schematically depicted in Fig. 2A, shows that the coiled-coil domains of IFs are exposed at the ends of the soluble tetramers; these are the expected sites of binding to P1CT. P1CT-IF interaction appears therefore to be different from other IF interactions such as those between vimentin and fimbrin (46); in the latter case the nonhelical domain of vimentin mediates the binding. An additional feature of IF that may help explain why P1CT can bind IF in vivo is the fact that IF assembly/disassembly is a dynamic process; helical domains are exposed as IFs continuously remodel in vivo. This has been clearly demonstrated with fluorescence recovery after photobleaching analysis of GFP-vimentin expressing cells where fluorescence recovery occurs rapidly in the middle of each vimentin filament without the translation of the gap in fluorescence to the ends of the filament (47). We suggest that the association of the polycystin-1 represents incorporation into the sides of the continuously remodeled intermediate filaments.

The localization of polycystin-1 at desmosomal junctions revealed in the present study is further supported by the congruent distribution of desmosomes and polycystin-1 in normal adult mouse kidney. In a systematic study of the expression of junctional proteins along the nephron, Piepenhagen and Nelson (48) found that the distribution of E-cadherin and α and β catenins is uniform in all nephron segments and that E-cadherin and α and β catenins are present along the entire length of the lateral cell membranes. In contrast, expression of the desmosomal marker desmoplakin is restricted to the distal nephron (beginning with the thick ascending loop of Henle, the distal convoluted and collecting tubules and collecting ducts), with the highest expression between the distal convoluted tubule and collecting ducts. Plakoglobin and cytokeratin K8, two other components of desmosomes, were strongly expressed in the same segments. The subcellular distribution of desmosomes was also segment-specific; desmoplakin was restricted to the apical-lateral membranes, co-localizing with ZO-1 in the distal convoluted tubules. In collecting tubules, desmoplakin was localized to both lateral and basal membranes. The expression profile of desmoplakin and cytokeratin matches that of polycystin-1, which in adult kidney is also found in the thick ascending loop of Henle, the distal convoluted tubule, and most abundantly in collecting ducts with a predominant lateral junctional distribution (8, 49).

The desmosome is a distinctive structure that tethers IFs to the plasma membrane and is most prominent in tissues exposed to mechanical stress (reviewed in Refs. 50 and 51). It is characterized by two parallel electron dense cytoplasmic plaques lying on either side of two adjacent plasma membranes that are separated by an intercellular electron-dense zipper-like midline. The latter is formed of desmosomal cadherins from apposing cells interacting homo- or heterotypically. Desmosomal junctions are the most specialized of vertebrate junctions and arose after adherens junctions in metazoan evolution; they also form after adherens junctions both during development and in cells that are induced to make junctions (reviewed in Ref. 51). In epithelial cells, desmosomal cadherins are linked to IFs mainly through plakoglobin and plakophilins (which, like β-catenin, are members of the armadillo family of nuclear and junctional proteins) and desmoplakins. Interaction among these proteins occurs in series: desmosomal cadherin-plakoglobin-plakophilin-IF, mirroring protein interactions at adherens junctions (classic cadherins-β-α-catenins-microfilaments), or laterally (e.g. cadherin-desmoplakin-IF; cadherin-plakophilin-IF). Connections also exist across junctions (e.g. E-cadherin-plakoglobin; desmosomal cadherin-β-catenin; plakoglobin-α-catenin), underscoring a close relationship between desmosomes and adherens junctions. In endothelial cells, which lack desmosomal cadherins, cadherins form hybrid junctional complexes linking to both IFs and microfilaments.

Our immunolocalization of endogenous polycystin-1 to desmosomes in epithelium and the supportive electron microscopy
data of Scheffer et al. (9) also brings the question of how polycystin-1–IF interaction is possible at desmosomes, given the relatively short length of the polycystin-1 C-tail and the potential dense barrier to cell surface provided by the plaque proteins. One possibility is that IF may penetrate the plaque en route to the plasma membrane as previously reported (50, 52, 53). It has also been shown that plakophilin, which interacts directly with IF proteins, is localized very close to the plasma membrane in the desmosomal plaque (54); an analogous situation may apply to polycystin-1. Alternatively, polycystin-1 may be localized at the periphery of desmosomes; IF converge at desmosomal plaques (55), and some may loop away toward the periphery, perhaps permitting additional interactions with other proteins such as polycystin-1.

The interaction of polycystin-1 with IF may serve several functions. First, expression of desmolakin, cytokeratin K8, and polycystin-1 in normal adult kidney is high in the distal half of the nephron where the degree of lateral membrane interdigitations between tubular epithelial cells is low. In this segment, paracellular conductances are low, and therefore the osmotic stress is high. An interdigitated lateral membrane and/or a well developed IF network and desmosomal junctions would render cells more resistant to deformation and other forms of physical stress. Polycystin-1 may contribute to the strengthening of lateral junctions, particularly in tissues where desmosomes are lacking as in endothelium. It is less likely that polycystin-1 is involved in initiating the formation of desmosomes because the phenotypes of mice with homozygous disruption of polycystin-1 or the desmosomal core protein desmolakin are different (embryonic lethality occurs earlier before kidneys begin to form, when desmolakin is disrupted (56)). Also, in calcium switch experiments, the appearance of polycystin-1 at lateral junctions follows that of desmolakin. This observation may account for the different phenotypes in the knockout animals (reviewed in Ref. 51) and suggests that polycystin-1 plays a maintenance rather than an initiation role at desmosomes.

Second, localization of polycystin-1 at desmosomes may be crucial for its signaling functions. P1CT, the region shown here to associate with IF, binds directly to P2CT, 14-3-3 (31), G-Box (15), and polycystin-1 in normal adult kidney is high in the distal half of the nephron where the degree of lateral membrane interdigitations between tubular epithelial cells is low. In this segment, paracellular conductances are low, and therefore the osmotic stress is high. An interdigitated lateral membrane and/or a well developed IF network and desmosomal junctions would render cells more resistant to deformation and other forms of physical stress. Polycystin-1 may contribute to the strengthening of lateral junctions, particularly in tissues where desmosomes are lacking as in endothelium. It is less likely that polycystin-1 is involved in initiating the formation of desmosomes because the phenotypes of mice with homozygous disruption of polycystin-1 or the desmosomal core protein desmolakin are different (embryonic lethality occurs earlier before kidneys begin to form, when desmolakin is disrupted (56)). Also, in calcium switch experiments, the appearance of polycystin-1 at lateral junctions follows that of desmolakin. This observation may account for the different phenotypes in the knockout animals (reviewed in Ref. 51) and suggests that polycystin-1 plays a maintenance rather than an initiation role at desmosomes.
