PKD2 Interacts and Co-localizes with mDia1 to Mitotic Spindles of Dividing Cells

ROLE OF mDia1 IN PKD2 LOCALIZATION TO MITOTIC SPINDLES*

Dana R. Rundle‡, Gary Gorbsky‡§, and Leonidas Tsiokas‡¶

From the ‡Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 and the §Molecular, Cell, and Developmental Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

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Mutations in pkd2 result in the type 2 form of autosomal dominant polycystic kidney disease, which accounts for ~15% of all cases of the disease. PKD2, the protein product of pkd2, belongs to the transient receptor potential superfamily of cation channels, and it can function as a mechanosensitive channel in the primary ciliary lumen of kidney cells, an intracellular Ca2+ release channel in the endoplasmic reticulum, and/or a nonselective cation channel in the plasma membrane. We have identified mDia1/Drf1 (mammalian Diaphanous or Diaphanous-related formin 1 protein) as a PKD2-interacting protein by yeast two-hybrid screen. mDia1 is a member of the RhoA GTPase-binding formin homology protein family that participates in cytoskeletal organization, cytokinesis, and signal transduction. We show that mDia1 and PKD2 interact in native and in transfected cells, and binding is mediated by the cytoplasmic C terminus of PKD2 binding to the mDia1 N terminus. The interaction is more prevalent in dividing cells in which endogenous PKD2 and mDia1 co-localize to the mitotic spindles. RNA interference experiments reveal that endogenous mDia1 knockdown in HeLa cells results in the loss of PKD2 from mitotic spindles and alters intracellular Ca2+ release. Our results suggest that mDia1 facilitates the movement of PKD2 to a centralized position during cell division and has a positive effect on intracellular Ca2+ release during mitosis. This may be important to ensure equal segregation of PKD2 to the daughter cell to maintain a necessary level of channel activity. Alternatively, PKD2 channel activity may be important in the cell division process or in cell fate decisions after division.

Autosomal dominant polycystic kidney disease affects 1 in 1,000 individuals primarily by the development of large, fluid-filled renal cysts that ultimately may lead to renal failure. Extraparenchymal manifestations such as cyst formation in the liver, pancreas, and spleen may also occur with autosomal dominant polycystic kidney disease as well as cranial aneurysms and secondary hypertension (1, 2). Positional cloning identified pkd2 as one of the genes mutated in ~15% of affected families (3–5). Polycystin-2 (PKD2) has significant sequence homology to the transient receptor potential channel proteins (6). PKD2 was found on male-specific sensory neurocilia in Caenorhabditis elegans (7) and later in the primary cilia of kidney epithelial cells (8, 9). The apparent function of nonmotile cilia is to act as a sensor of the extracellular environment and transmit this information to the cell body. Consistent with this idea, PKD2 has been shown to have an essential role in mediating Ca2+ entry in response to flow rate changes, suggesting that it may be part of the mechanosensing machinery residing in the primary ciliary lumen of terminally differentiated epithelial cells (10). However, in addition to its expression in ciliary structures, PKD2 expression has been reported in the endoplasmic reticulum of LLC-PK1 cells (11), in the plasma membrane of Madin-Darby canine kidney and mouse inner medullary collecting duct (IMCD) cells (12, 13), and in apical membranes of human term syncytiotrophoblasts (14). The pool of PKD2 expressed in the endoplasmic reticulum is believed to function as an intracellular Ca2+ release channel, whereas plasma membrane-anchored PKD2 functions as a nonselective cation channel. Whereas all of these functions are likely to represent physiological functions, its role as a mechanosensor is more likely to be associated with the cystic phenotype seen in pathogenic mutants of PKD2. This idea is supported by recent findings that loss-of-function mutations in proteins required for ciliary formation and function often result in cystic diseases (8, 7, 15–17). Alternatively, PKD2 may have a role in cell division during development regulating the differentiation process of kidney tubular cells. Loss of PKD2 in pathogenic mutants may account for the less differentiated phenotype of cystic epithelial cells, thus indirectly affecting ciliary function. Ciliary function of PKD2 in terminally differentiated cells may be more closely related to disease progression (18, 19). These findings highlight the fact that PKD2 is a multifunctional protein with several possible roles in cell physiology and kidney development; the exact mechanisms regulating these apparently divergent cellular functions and subcellular localizations are largely unknown.

mDia1 (mammalian diaphanous 1) was originally identified as a RhoA-interacting protein in a yeast two-hybrid screen using activated RhoA as bait (20), and it was later shown to function as a downstream effector of RhoA in signal transduction and cytoskeletal organization (21–23). mDia1 is also referred to as Drf1 (diaphanous-related formin 1) because it has...
homology to formin, the limb deformity (ld) gene product. Formin has a proline-rich domain and a 130-amino acid region that have been named FH1 and FH2 domains, respectively (24). A third region, the FH3 domain, is an amino-terminal domain that is the least conserved FH domain among formin family members (25). Diaphanous-related formin homology proteins such as mDia1, mDia2, and yeast Bni1p contain all three FH (24) as well as an amino terminus GTPase binding domain (Rhod binding domain) (20) and a carboxyl terminal interaction domain termed Dia autoregulatory domain (DAD domain) (26). The DAD domain loops around to bind the N terminus of mDia in the Rhod GTPase binding domain to hold mDia1 in a closed and inactive state. When Rho protein binds mDia1, the DAD domain releases the N terminus to form an active mDia1 to expose its internal domains for interactions with effector molecules (27, 26). The FH1 domain binds profilin, an actin-binding protein (20), and Src homology 3 domains containing proteins such as Src (28) and IRS5p3/BMI2p (29). The FH2 domain has been reported to be involved in coordinating microtubules and the actin cytoskeleton during cell division (30). mDia1 contains a 173-amino acid region in the C terminus of its FH3 domain that is necessary for mDia1 localization to mitotic spindles in HeLa cells (31). A similar FH3 domain function has been seen in Schizosaccharomyces pombe when FH3 domains from Fus1 or Cdc12 formin-related proteins were fused to GFP, and GFP was subsequently localized to the projection tip of mating cells. These results suggest that FH3 domains may serve to specifically localize formin-related proteins to specific subcellular sites (25). The *diaphanous* subfamily appears to primarily function in cytokinesis and in cytoskeletal rearrangement and stabilization. Consistent with its role in actin polymerization in hair cells of the ear, naturally occurring mutations in mDia1 cause nonsyndromic deafness in humans (32). mDia1 was also recently found to be essential for mechanotransduction in response to integrin activation in SV-80 human fibroblast cells (33) but is not involved in the regulation of integrin-mediated cell adhesion in Jurkat cells (34). In conclusion, mDia1 appears to serve several basic cellular functions ranging from mechanosensation to cytokinesis through a possible effect on cytoskeletal organization.

In the present study, we identified and characterized an interaction of PKD2 and mDia1 using a yeast two-hybrid screen and co-immunoprecipitation experiments. The interaction was further supported by indirect immunofluorescence and confocal microscopy of the endogenous proteins in mitotic cells. RNAi experiments in mitotic HeLa cells have shown a concomitant loss of PKD2 from the mitotic spindle in cells lacking mDia1, and we have demonstrated diminished intra-cellular Ca²⁺ release in these cells. Our results suggest that a novel function of mDia1 is to bind the PKD2 channel protein possibly contained in an ER vesicle, facilitate its movement to mitotic spindles during cell division, and modulate intracellular Ca²⁺ release during mitosis.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Constructs HA-PKD2, HA-PKD2ΔI (1–643), slg, slg7-PKD2/Ile⁶⁵⁷–Val⁶⁶⁰, and slg7-PKD2/Val⁶⁶³–Val⁶⁶⁰ were previously described in Ref. 6. GFP-mDia1 and GFP-ΔNsmDia1 were obtained from Dr. Shu Narumiya, GFP-Xc252 mDia1 was made by converting amino acid residues Glu¹⁹⁰ to a stop codon by site-directed mutagenesis using QuickChange™ (Stratagene). PKD2-myc was constructed by replacing the stop codon of human PKD2 with the sequence corresponding to a Myc epitope.

**Yeast Two-hybrid Screen**—The L40 yeast strain containing both his and lacZ reporters under the control of LexA binding sites was sequentially transformed with a bait plasmid in pLexA containing a portion of the C-terminal cytoplasmic region of human PKD2 (Ser⁶⁵¹–Val⁶⁶⁰; accession number NM_002979) and a whole mouse day 9.5 embryonic library in pVP16. From 4 × 10⁶ independent clones screened, several clones were identified that contained inserts interacting with PKD2 Ser⁶⁵¹–Val⁶⁶⁰. All inserts were frame with the DNA binding domain of LexA, and their interaction with PKD2 was further verified by reintroducing the rescued plasmids back to the L40 yeast strain that was originally used in the screen.

**Cell Culture**—LLC-PK1 cells were maintained in M199 medium, HeLa cells were maintained in RPMI 1640, HEK293T cells were grown in Dulbecco’s modified Eagle’s medium, and Madin-Darby canine kidney and IMCD cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium. All media were supplemented with 10% fetal bovine serum except LLC-PK1, which required 3% fetal bovine serum. All cell culture media were from Invitrogen. Cells were cultured at 37°C in a 5% CO₂ atmosphere. Cells used for confocal microscopy were plated on glass coverslips or 24-well plates and allowed to adhere. HeLa cell coverslips were coated with CellTak (BD Biosciences) per manufacturer’s instructions for some confocal microscopy experiments. Cells grown for cilia staining were allowed to grow until confluent.

**Antibody Production and Characterization**—Antibodies to PKD2 were made by generating a glutathione S-transferase (GST) fusion protein expressing amino acids Ile⁶⁵⁷–Arg⁷⁴² of human PKD2 (GST-PKD2/Ile⁶⁵⁷–Arg⁷⁴²). GST-PKD2/Ile⁶⁵⁷–Arg⁷⁴² was injected into rabbits or chickens to generate polyclonal antibodies (26). Parts of the antibodies were initially determined by ELISA using a purified fragment of PKD2/Ile⁶⁵⁷–Arg⁷⁴² fused to maltose-binding protein (MBP) (MBP-PKD2/Ile⁶⁵⁷–Arg⁷⁴²). Rabbit or chicken PKD2 antibody purified by affinity-purified using MBP-PKD2/Ile⁶⁵⁷–Arg⁷⁴² and following the Glutathione-Agarose method (Pierce) according to the manufacturer’s instructions.

Antibody titers were determined by ELISA using purified MBP-PKD2/Ile⁶⁵⁷–Arg⁷⁴². The specificity of the antibodies was evaluated by immunoblotting lysates of transfected HEK293T cells with an expression plasmid for HA-tagged wild type PKD2 (HA-PKD2) or a truncation mutant lacking the antigenic region (HA-PKD2ΔI) (36). Isolated microtubules from each 15-cm dish were resuspended in 200 μl of Laemmli sample buffer, and 30 μl was run on SDS-PAGE, subjected to Western blot, and probed with a polyclonal α-Myc antibody at 1:500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal antibody to GFP at 1:500 (Santa Cruz Biotechnology), or a monoclonal anti-β-tubulin antibody at 1:5,000 (Sigma; Clone 2.1). Horseradish peroxidase-conjugated secondary antibodies were used at 1:10,000 followed by enhanced chemiluminescence detection (Pierce, Biosignal).

**Immunoprecipitation, in Vitro Binding, and Western Blotting**—Cells were lysed in 1 ml/10-cm dish of 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% sucrose, and Complete protease inhibitors (Roche Applied Science). Lysates were cleared by centrifugation at 12,000 × g for 20 min, and protein assay (BCA; Pierce) was done on the supernatants. Co-immunoprecipitation of PKD2 and mDia1 (Transduction Laboratories) was performed in the HEK293T cell lysate from a 15-cm dish, whereas all other co-immunoprecipitations were from a volume less than or equal to a single 10-cm dish of cleared cell lysate. GST isoform control antibody was from Santa Cruz. After an 8-h incubation with primary antibody, 30 μl of 50% G-agarose beads was added for 2 h to capture immune complexes. The beads were washed five times with lysis buffer and then mixed with Laemmli buffer for SDS-PAGE. For immunoblotting, mDia1 antibody and rabbit affinity-purified PKD2 antibody were both used at 1:500 under nonreducing or reducing conditions. The antibodies were used at 1:10,000 followed by enhanced chemiluminescence detection after Western blotting. Immunoprecipitations using IgG-tagged constructs (slg, slg7, slg7-PKD2/Gln⁷⁴³–Val⁷⁵⁶, or slg7-PKD2/Ile⁶⁵⁷–Val⁷⁶⁰) were done as described earlier (6, 33). GFP-tagged mDia1 fusion proteins were detected with a rabbit polyclonal antibody against GFP at 1:5,000. A polyclonal rabbit affinity-purified PKD2 antibody was used to detect porcine PKD2 (26). For Western blotting of PKD2 in LLC-PK1 cells, we designed two PKD2-specific constructs, PKD2-N522 and PKD2-N322, PKD2-N522 was targeted specifically porcine PKD2, whereas PKD2-N322 was targeted only porcine and human PKD2. These constructs were made in our previously described DNA vector, pUB/HIRNAI vector (36). Porcine PKD2 cDNA sequences were determined based on a partial pig EST sequence tag clone (GenBank™ number AY 915 886). The PKD2-N522 sequence was 5′-gatccgccgtcttggtgtagaagtggctcaaggaactgca-cagaaagttgggaa-3′, whereas the PKD2-N322 sequence was 5′-gat-ccgccgtcttggtgtagaagtggctcaaggaactgca-cagaaagttgggaa-3′. Both sequences represent the sense strand of the double-stranded DNA.
mDia1-dependent Localization of PKD2 to Mitotic Spindles

RESULTS

Identification of mDia1 as an Interacting Partner of PKD2—To better understand the function and regulation of PKD2 we employed a yeast two-hybrid screen using human PKD2 C-terminal residues Ser825 to Val968 as bait. This region of PKD2 was chosen because it would enable us to identify interacting proteins with the region of PKD2 that is mostly deleted in the longest truncation pathogenic mutant described to date, PKD2(Ser825–Val968) was cloned into the pLexA vector and used to screen a mouse embryonic day 9.5 library fused to the activation domain of VP16. Several clones that contained inserts interacting with PKD2(Ser825–Val968) were identified from the 4 × 10⁶ independent clones screened. All inserts were in-frame with the DNA binding domain of LexA, and their interaction with PKD2 was further verified by reintroducing the rescued plasmids back to the L40 yeast strain originally used in the screen. One of the clones matched amino acids Leu143 to Leu260 of mouse p140 Diaphanous (p140Dia, mDia1, accession number NM_007858). The PKD2-interacting region of mDia1 lies in the C-terminal portion of an already described functional domain of mDia1 that is called the RhOa binding domain and partially overlaps with the N terminus of the mDia1 FH3 domain.

Production and Characterization of PKD2-specific Antibodies—To confirm the interaction between PKD2 and mDia1, we generated polyclonal antibodies to PKD2 and used a commercially available monoclonal antibody to mDia1. We selected a region of PKD2 composed of amino acids Ile679–Arg742 as antigen for the production of polyclonal antibodies in chicken and rabbit. Rabbit or chicken immune serum was affinity-purified by passage over a column of immobilized MBP-PKD2(679–742). Antibody characterization by immunoblotting is shown in Fig. 1. Specifically, we have expressed HA-PKD2 that should be recognized by the polyclonal antibodies and a truncated form, HA-PKD2(1–643), which does not contain the antigenic site. Cell lysates containing the tagged proteins were analyzed by Western blot followed by detection with chicken anti-PKD2 (Fig. 1A) or rabbit anti-PKD2 (Fig. 1B). In both cases, the antibody did not recognize HA-PKD2(1–643) (Fig. 1, A and B, lane 1) but did recognize a 110-kDa protein (Fig. 1, A and B, lane 2), which corresponds to the expected molecular weight of HA-PKD2. The rabbit antibody also recognized an smaller molecular weight protein to a much lesser extent (Fig. 1B, lane 2), and we would suggest that this is a proteolytic fragment of the larger HA-PKD2 molecule because it was not detected in HA-PKD2(1–643)-transfected lysates (Fig. 1B, lane 1). Relatively equal expression of HA-PKD2(1–643) and HA-PKD2 proteins in the same lysates is shown in Fig. 1, C and D. The specificity of the rabbit affinity-purified antibody to PKD2 was further confirmed by indirect immunofluorescence staining of IMCD (Fig. 1E) and LLC-PK1 (Fig. 1F) cells, where PKD2 is known to co-localize with stabilized tubulin in ciliated cells. These results indicate that we have generated two polyclonal antibodies suitable for PKD2 detection by Western blotting.

In Vivo Interaction of PKD2 and mDia1 in Native Cells—To determine whether native PKD2 and mDia1 are associated in mammalian cells, we performed co-immunoprecipitation experiments in native human embryonic kidney cells (HEK293 cells) because they have been shown to express a significant amount of endogenous PKD2 (37), and mDia1 was expected to be present in HEK293 cells due to its ubiquitous expression in mammalian tissues (20). Fig. 2A shows that immunoprecipitation of mDia1 with a commercially available monoclonal antibody to mDia1 immunoprecipitated PKD2 from HEK293 cell lysates (Fig. 2A, lane 1). PKD2 was not immunoprecipitated when an isotype-matched monoclonal antibody (anti-GST) was used as a

Ca²⁺ Fluorescence Spectroscopy—HeLa cells in 10-cm dishes were treated with 40 ng/ml nocodazole for 12 h prior to mitotic shake off. Mitotic cells were harvested and washed twice in Ca²⁺-loading buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, 0.1% bovine serum albumin, 15 mM HEPES, 0.025% pluronic acid) containing 40 ng/ml nocodazole. Cells were loaded with 2 μM Indo AM (Molecular Probes). ToPro3 (Molecular Probes) was included in the next to the last wash at 2 μM to visualize DNA. Coverslips were mounted with ProLong (Molecular Probes). ToPro3 was excited with a HeNe633 laser, and antibody staining was visualized with laser excitation from argon-488 and krypton-568 lasers and viewed on a Leica TCS NT Microscope. Images were processed with Leica Lite software.

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control (Fig. 2A, lane 2). The specificity of our antibody for endogenous PKD2 in HEK293 cell lysates is evident in Fig. 2A, lane 3, where it is shown to detect a single band with a molecular mass of ~110 kDa. We also confirmed the immunoprecipitation of endogenous mDia1 with the mDia1 antibody (Fig. 2B, lane 1) and its expression in HEK293 lysates (Fig. 2A, lane 3) by stripping the membrane shown in Fig. 2A and reprobing for mDia1. These results confirmed the yeast two-hybrid results and further provided in vivo evidence that mDia1 is an interacting protein with PKD2, suggesting that the two proteins exist in a native complex in HEK293 cells. Although we were able to sufficiently immunoprecipitate endogenous mDia1 to detect an interaction with PKD2, the commercial mDia1 antibody is not recommended for immunoprecipitation, and this technical limitation required a large amount of HEK293 cell lysate for our immunoprecipitation experiments. This was also evident by the small amount of immunoprecipitated mDia1 (Fig. 2B, lane 1). In light of this, we have used our PKD2 antibody as an immunoprecipitating antibody in Nalm 6 (pre-B cell line) and in mIMCD3 (mouse kidney inner medullary collecting duct) lysates. Endogenous mDia1 was detected in these immunoprecipitants (data not shown), indicating that the mDia1-PKD2 interaction is common to more than one cell type.

**In Vitro Interaction between PKD2 and mDia1**—To determine whether the C-terminal cytoplasmic tail of PKD2 was sufficient to mediate the interaction with mDia1 and to test whether the longest truncation pathogenic mutant of PKD2 could still interact with mDia1, we IgG-tagged the entire C-terminal cytoplasmic tail (sIg.7-PKD2/Ile679–Val968 or a truncation of the C-terminal tail of human PKD2 terminated at residue Glu871 (sIg.7-PKD2/Gln743–Glu871). It should also be noted that these constructs were designed to be forwarded to the secretory pathway and eventually inserted in the plasma membrane by the addition of the leader sequence of CD45 and the transmembrane segment of CD7 (6). Next, we co-transfected HEK293T cells with control sIg.7, sIg.7-PKD2/Gln743–Glu871, or sIg.7-PKD2/Ile679–Val968 with full-length GFP-tagged mDia1 (GFP-mDia1) or an N-terminal deletion mutant of GFP-mDia1 (GFP-ΔN3mDia1) that lacks the PKD2 binding site based on our yeast two-hybrid results. Protein A was used to capture the IgG fusions and their interacting proteins from transfected cell lysates. The results shown in Fig. 2C are a Western blot of the Protein A pull-downs using an antibody to GFP to detect the presence of GFP-mDia1 or GFP-ΔN3mDia1. Fig. 2C, lanes 1 and 2, serve as controls to indicate that non-specific binding between slg.7 control and GFP-mDia1 or GFP-ΔN3mDia1 did not occur; nor was there specific binding between GFP-mDia1 or GFP-ΔN3mDia1 with the slg.7-PKD2/Gln743–Glu871 (lanes 4 and 6). Specific binding occurred when slg.7-PKD2/Ile679–Val968 and GFP-mDia1 were present to-
gether in the lysate (Fig. 2C, lane 3), with the middle band being at the expected molecular weight for GFP-mDia1. We also observed a band of GFP reactivity in lane 5, indicating that GFP-ΔN3mDia1 may associate with sIg.7-PKD2/Ile679–Val968. This could occur via a homotypic interaction between GFP-ΔN3mDia1 with unlabeled, endogenous mDia1, since mDia1 has been reported to form multimers in vitro when an mDia1 N terminus construct was shown to bind to a separate mDia1 C terminus construct (27). The molecular weight of the GFP-immunoreactive band in lane 6 did not correspond to any of the GFP-tagged constructs or to the in vitro proteolytic fragment evident in lane 3 (see below), making it likely to be an artifact.

**In Vitro Proteolytic Cleavage of GFP-mDia1**—We also note a smaller size GFP-reactive band associating with sIg.7-PKD2/Ile679–Val968 (Fig. 2C, lane 3). The size of this band is nearly identical to GFP-ΔN3mDia1, but it could not represent GFP-ΔN3mDia1, because the only GFP-tagged construct transfected in these cells was full-length GFP-mDia1. A recent publication (28) has shown that in vitro proteolytic digests can generate stable, active C-terminal fragments of both mDia1 and mDia3. These results prompted us to reason that mDia1 could undergo proteolytic cleavage to generate an N-terminal fragment capable of interacting with PKD2 because the PKD2-interacting domain remains intact. Because the C-terminal tail of mDia1 has been shown to interact with its N terminus, we tested whether deletion of the 52 most C-terminal residues (GFP-mDia1/C-52) could affect the proteolytic cleavage of wild type mDia1. We chose to delete the 52 C-terminal residues of mDia1-dependent Localization of PKD2 to Mitotic Spindles

![Fig. 2. Biochemical interaction of mDia1 and PKD2 and in vitro proteolytic cleavage of mDia1. A, in vivo interaction between mDia1 and PKD2. Native HEK293 cell lysates were immunoprecipitated with a mouse monoclonal (IgG, anti-mDia1 (lane 1) or an irrelevant isotype-matched antibody to GST (lane 2). Immunocomplexes or crude lysate (lane 3) were probed with polyclonal antibody to PKD2 (1:5000). B, the membrane from A was stripped and reprobed with anti-mDia1 (1:5000) (lower panel). C, in vitro interaction between mDia1 and PKD2. HEK293T cells were transfected with the indicated combination of expression plasmids, and sIg.7 fusion proteins were captured by Protein A. The presence of GFP-tagged proteins in immunocomplexes or in lysates (input) was detected with anti-GFP (1:1000). Lanes 1 and 7, slg.7 and GFP-mDia1; lanes 2 and 8, slg.7 and GFP-ΔN3mDia1; lanes 3 and 9, slg.7-PKD2/Ile679–Val968 and GFP-mDia1; lanes 4 and 10, slg.7-PKD2/Gln743–Glu871 and GFP-mDia1; lanes 5 and 11, slg.7-PKD2/Ile679–Val968 and GFP-ΔN3mDia1; lanes 6 and 12, slg.7-PKD2/Gln743–Glu871 and GFP-ΔN3mDia1. D, in vitro cleavage of GFP-mDia1. Cell lysates containing GFP-ΔN3mDia1 (lane 1), C-terminally truncated GFP-ΔC52mDia1 (lane 2), GFP-mDia1 (lane 3), and GFP (lane 4) were transfected into HEK293T cells and detected with anti-GFP (1:1,000). The bands in lanes 1, 2, and 4 and the upper band in lane 3 represent the correct molecular weights for GFP-ΔN3mDia1, GFP-ΔC52mDia1, GFP-mDia1, and GFP, respectively. The lower band in lane 3 is detected by GFP at a molecular weight similar to GFP-ΔN3mDia1, although this lysate is from cells transfected with the full-length GFP-mDia1. IP, immunoprecipitation; IB, immunoblot.
mDia1, because a naturally occurring mutation truncating these residues results in deafness (32). We have analyzed cell lysates transfected with either GFP-ΔN3mDia1 (Fig. 2D, lane 1), GFP-mDia1ΔC-52 (Fig. 2D, lane 2), the entire GFP-mDia1 molecule (Fig. 2D, lane 3), or GFP alone (Fig. 2D, lane 4) by Western blotting and detection with GFP antibody. The correct molecular weight for GFP-ΔN3mDia1 is shown in lane 1. The GFP-reactive band in the second lane represents mDia1ΔC-52 and is slightly smaller than the full-length GFP-mDia1, which is the higher molecular weight band shown in lane 3. GFP is a 27-kDa protein and is shown in lane 4. However, in Fig. 2D, lane 3, there is a smaller band that runs at a molecular weight almost identical to GFP-ΔN3mDia1, and this band is also present in the cell lysates shown in Fig. 2C, lanes 7, 9, and 10. We have consistently seen this smaller GFP-reactive protein band in lysates containing full-length GFP-mDia1, and we have considered that this may represent a form of mDia1 that has undergone a proteolytic cleavage only when in the closed conformation, since we did not see this band in the slightly shorter mDia1ΔC-52-containing lysates. The C terminus 52-amino acid truncation shortens the C-terminal intramolecular interaction domain (39), presumably leaving the molecule in an open position that is not susceptible to proteolysis. This could generate a functional mDia1 isoform lacking the FH1 and FH2 domains but maintaining the N-terminal Rho binding domain and FH3 domain that overlaps the PKD2 binding region.

Co-localization of PKD2 and mDia1 in Dividing Cells—To determine the subcellular localization of PKD2 and mDia1, we first employed indirect immunofluorescence and confocal microscopy on porcine kidney LLC-PK1 cells, since we have found PKD2 to be present on their nonmotile cilia (Fig. 1, A and F), in keeping with the published findings of others who have reported PKD2 on kidney epithelial cell cilia (8, 9). We theorized that cilia might be a possible site for interaction between PKD2 and mDia1, since cilia are microtubule-based structures and mDia1 and mDia2 have both been reported to interact with microtubules (31, 32). None of our efforts to demonstrate the presence of mDia1 on cilia by confocal microscopy were successful. Because mDia1 has already been reported to localize to the mitotic spindles of HeLa cells (31), we looked to see if this could be reproduced in conjunction with PKD2 immunoreactivity on LLC-PK1 mitotic spindles (Fig. 3A). Fig. 3A shows co-localization of endogenous PKD2 (green, left) and mDia1 (red, middle) in LLC-PK1 cells (merged, right). Fig. 3B shows LLC-PK1 cells similarly treated with normal rabbit and mouse IgG as nonspecific binding controls. These data suggest that mitotic spindles are a possible site of interaction for mDia1 and PKD2 in vivo.

Whereas the localization of mDia1 to the mitotic spindles of dividing HeLa cells has been shown (31), the finding that PKD2 localizes in these cellular structures is novel. Therefore, we examined PKD2 subcellular localization during cell division in other cell lines. Fig. 4A indicates that PKD2 immunoreactivity (left, green) overlaps with that of α-tubulin (middle, red) on the microtubule-based spindles of mitotic LLC-PK1 cells. We repeated this in human cervical carcinoma HeLa cells (Fig. 4, B and C) and mouse IMCD cells (Fig. 4D) to demonstrate that this is a specific localization of PKD2 in dividing cells in general and not restricted solely to LLC-PK1 cells. We have also analyzed LLC-PK1 cells with our chicken polyclonal antibody to PKD2 (green) and with ToPro3 DNA staining; this did not generate any observable fluorescence. Characteristic PKD2 immunoreactivity on spindle microtubules was evident with this second antibody to PKD2.

To directly that confirm PKD2 is in contact with mitotic spindles, we isolated mitotic spindles from HeLa cells transiently...
PKD2-myc in supernatants following microtubule isolation. Lane 1 shows expression of PKD2-myc in crude HEK293T cell lysates as a molecular weight reference. B, lane 1, GFP-mDia1 in crude HEK293T cell lysates as a molecular weight reference; lanes 2 and 3, GFP-mDia1 in nocodazole- and taxol-stabilized mitotic HeLa preparations.

**Fig. 5.** Association of transfected PKD2 and mDia1 with purified spindles from HeLa cells. A, mitotic spindle preparations from HeLa cells transfected with PKD2-myc and stabilized with either nocodazole (lanes 2, 4, and 6) or taxol (lanes 3, 5, and 7) were immunoblotted (IB) with rabbit (Rb) anti-Myc (lanes 1, 2, 3, 6, and 7) or mouse (Ms) anti-β tubulin (lanes 4 and 5). Lanes 6 and 7 represent expression of PKD2-myc in supernatants following microtubule isolation. Lane 1 shows expression of PKD2-myc in crude HEK293T cell lysates as a molecular weight reference. B, lane 1, GFP-mDia1 in crude HEK293T cell lysates as a molecular weight reference; lanes 2 and 3, GFP-mDia1 in nocodazole- and taxol-stabilized mitotic HeLa preparations.

transfected with Myc-tagged PKD2 (PKD2-myc) in the presence of taxol, a microtubule-stabilizing drug, or in the presence of the microtubule-stabilizing drug nocodazole. A PKD2-myc control (Fig. 5A, lane 1) and aliquots of the isolated spindles were subjected to Western blot and detected with antibody to Myc (Fig. 5A, lanes 2 and 3) or to β-tubulin (Fig. 5A, lanes 4 and 5). The levels of PKD2-myc in cell lysates from the spindle preparations were also detected with Myc antibody (Fig. 5A, lanes 6 and 7) to show that PKD2-myc expression levels were similar in both preparations. Although the β-tubulin level was less in the nocodazole-stabilized preparation (Fig. 5A, lane 4 versus lane 5), PKD2-myc immunoreactivity in both nocodazole- and taxol-stabilized spindle preparations appeared similar. Fig. 5B shows mitotic spindle preparations from HeLa cells transfected with GFP-mDia1 in the presence of nocodazole or taxol (Fig. 5B, lanes 2 and 3, respectively), subjected to Western blot, and detected with antibody to GFP. Lane 1 is an aliquot of HEK293 cell lysate expressing GFP-mDia1 for molecular weight comparison. These results indicate that mDia1 and PKD2 may be associated with tubulin in pericentriolar material contained in the nocodazole-stabilized preparations, as well as with mitotic spindle microtubules in the taxol-stabilized spindle preparations. A second possibility is that PKD2 and Dia1 could be associated with a nonmicrotubule component of the spindle that remains in the presence of nocodazole and taxol.

To provide additional evidence that our PKD2-specific antibodies detected a PKD2-specific signal on mitotic spindles, we knocked down endogenous PKD2 in LLC-PK1 cells by RNAi and tested whether the spindle-specific signal would be eliminated or reduced. We generated two RNAi silencing constructs for PKD2, with one construct (PKD2KD2-3) designed to specifically silence porcine PKD2 and the other (PKD2KD2-3) designed to silence both porcine and human PKD2. Fig. 6A demonstrates the effectiveness of these constructs in HEK293T cells expressing human HA-PKD2 with PKD2 running as a 110-kDa size band and multimers of PKD2 at ~200 kDa. Fig. 6A, lane 1, indicates the control expression level of HA-PKD2, and lane 2 shows a reduction in HA-PKD2 because the PKD2KD2-2 silencing construct is capable of silencing both pig and human PKD2. Lane 3 does not show a reduction in HA-PKD2, since PKD2KD2-2 is a pig-specific knockdown construct and should not silence human HA-PKD2. These results show that PKD2KD2-3 can reduce but not completely abolish expression of HA-PKD2 in a transient transfection assay. Slow PKD2 protein turnover may account for the protein detection in transient transfection-based assays. Next, we co-transfected each of the PKD2 silencing constructs with GFP-tagged tubulin (GFP-tubulin) into LLC-PK1 cells. GFP-tubulin was used to mark transfected cells and to label mitotic spindles in dividing cells. Fig. 6, B and C, show that significant silencing of endogenous PKD2 occurs in co-transfected cells, as judged by PKD2 immunoreactivity (red, middle) on the mitotic spindles, thereby providing strong supporting evidence that our antibodies detected endogenous PKD2 in mitotic spindles.

**mDia1-dependent Localization of PKD2 to the Mitotic Spindles**—Since we had shown that PKD2 physically associates and co-localizes with mDia1 in the spindles and mDia1 has been shown to organize and stabilize microtubules, we tested whether PKD2 expression in the spindles requires mDia1 activity. Therefore, we elected to knock down mDia1 in HeLa cells and test whether PKD2 could still be detected in the spindles. We generated two mDia1-inactivating constructs (pSUPER-retro-mDia1KD1 and pSUPER-retro-mDia1KD2) and a control construct containing irrelevant DNA to mouse I-mfa (pSUPER-retro-KD control) in the pSUPER-retro vector. We prepared vesicular stomatitis virus-G recombinant retroviruses with these constructs that would allow permanent silencing of human mDia1 in cells infected with the mDia1-silencing constructs and no effect with the irrelevant DNA. Pools of infected HeLa cells were selected in the presence of 1 μg/ml puromycin and analyzed for PKD2 localization in the spindles by indirect immunofluorescence and confocal microscopy. The results of these experiments are shown in Fig. 7. Efficient silencing levels of endogenous mDia1 in cells infected with mDia1KD1 or mDia1KD2 virus are shown in Fig. 7A. Fig. 7, B and C, represents the complete stack of confocal images and shows that both silencing constructs result in diffuse, indistinct, and non-specific PKD2 staining throughout the cytosol but no defined staining on mitotic spindles (green, left). However, tubulin staining (red, middle) remains well defined. The merged image (right) reflects the overlay of well defined tubulin staining (red) on spindles with diffuse and cytosolic PKD2 staining (green). Fig. 7D shows that there is no effect on PKD2 localization to mitotic spindles in HeLa cells by the silencing vector control DNA. These results suggest that mDia1 activity is required for the localization of PKD2 to the mitotic spindles.

**Fluorescence Spectroscopy of HeLa and HeLa mDia1KD1 Cells**—Fig. 8A shows intracellular Ca²⁺ release assays in Ca²⁺ buffer in response to 10 μM histamine in mDia1 knockdown

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cells (HeLa mDia1KD1, red) and in HeLa cells (black). The inset bar graph representing normal HeLa cells (black, n = 4) and HeLa mDia1KD1 cells having reduced mDia1 expression (red, n = 4) shows that there is no significant difference in histamine responsiveness in these cell types. Additionally, the inset confocal images indicate that PKD2 immunoreactivity (green) is concentrated in a perinuclear position at the endoplasmic reticulum in both cell types. This suggests that in nonmitotic normal or mDia1 knockdown HeLa cells, the localization of PKD2 has remained concentrated at the endoplasmic reticulum, and these findings suggest that PKD2 channel activity or ER localization is not altered by decreased mDia1 expression in nonmitotic cells. Previous studies by others have generated profiles of intracellular Ca\(^{2+}\) release in normal and mitotic HeLa cells (40, 41), which have enabled us to assess the quality of our mitotic cell preparations by analyzing the reproducibility of their earlier results with our own. Fig. 8B represents a typical Ca\(^{2+}\) release assay in mitotic HeLa and HeLa mDia1KD1 cells in contrast to nonmitotic HEla cells in the presence of Ca\(^{2+}\) and in response to 10 \(\mu\)M histamine. As expected, normal interphase HeLa cells (green) were responsive to histamine by exhibiting a rapid intracellular Ca\(^{2+}\) release followed by a prolonged period of extracellular Ca\(^{2+}\) entry that remains above base-line values. Normal mitotic HeLa cells (black) given an identical amount of histamine released intracellular Ca\(^{2+}\) followed by little or no extracellular Ca\(^{2+}\) entry, whereas mitotic mDia1 knockdown cells had decreased intracellular calcium release under these conditions. These findings are consistent with the earlier studies and verify that we have obtained suitable preparations of interphase and mitotic cells. The mitotic HeLa mDia1KD1 cells (red) responded to histamine in a manner similar to mitotic HeLa (black) cells, with the exception that each assay reflected a decrease in overall intracellular Ca\(^{2+}\) release in the cells with decreased mDia1 expression. This difference, as measured by peak increase over baseline values, approaches a decrease of ~50% for each experiment. To determine the histamine response in the absence of extracellular Ca\(^{2+}\), we repeated this assay with wild type HeLa and HeLa mDia1 knockdown cells in buffer containing EGTA. Fig. 8C represents one of these assays, with a bar graph to the right showing the results of three assays. The mitotic HeLa knockdown (red) cells have a 60% decrease (0.05 ± 0.004) in Ca\(^{2+}\) release versus normal mitotic HeLa cells (black, 0.083 ± 0.007). These findings suggest that Ca\(^{2+}\) release is occurring with little or no Ca\(^{2+}\) entry during mitosis and that mitotic mDia1 knockdown cells do not release Ca\(^{2+}\) as efficiently as wild type mitotic HeLa cells. These results imply that mDia1 can modulate intracellular Ca\(^{2+}\) release in mitotic cells, although our assay cannot determine whether this is a direct or indirect effect only on PKD2.

**DISCUSSION**

Using a yeast two-hybrid screen, we have identified a protein-protein interaction between the C-terminal tail of human PKD2 and the N terminus of mDia1. The interaction was verified by co-immunoprecipitation experiments in vivo in na-
tive cell lines and in vitro in transfected cells. Our results suggest that the interaction is more prevalent in the mitotic spindle of dividing cells and that loss of mDia1 activity results in the loss of PKD2 from the spindle. These results prompt us to conclude that mDia1 facilitates the localization of PKD2 to the spindle during cell division. We propose that the interaction is likely to have physiological significance, because it occurs in cells under physiological conditions. In addition, the interaction is likely to be conserved between mammalian homologs of PKD2 but not in C. elegans and Drosophila PKD2 homologs, since the homology at the very C terminus is quite low among different organisms. Similarly, PKD2L or the disease-causing mutant PKD2(871X) would not be expected to interact with mDia1, since they lack all or most of the mDia1 binding site. Conversely, the mDia1-related proteins, mDia2 and mDia3, do not have significant homology to mDia1 in the PKD2 binding region. The implication of these results is 2-fold. First, PKD2 may be symmetrically divided between mother and daughter cell, and second, PKD2 activity may be essential during or immediately following cell division.

The PKD2 binding site (amino acids 143–260) is in the mDia1 RhoA binding domain (amino acids 63–260) that overlaps with the mDia1 FH3 domain (amino acids 157–456), which contains a 173-amino acid region that binds polymerized tubulin (31). This suggests that PKD2 could compete with or block RhoA binding to generate a unique open and functional form of mDia1 still capable of interacting with its effector molecules via its coiled-coil, FH1, and FH2 domains. Thus, PKD2 may activate mDia1 independently of RhoA, and this may have implications for mDia1 functions that do not require RhoA. A second scenario is that PKD2 keeps mDia1 in a closed, nonfunctional form in terms of FH1 and FH2 domain interactions but still permits mDia1 to transport PKD2 to the spindle. The close proximity of tubulin and PKD2 binding sites would presumably be present in either the open or closed configuration of mDia1, and we are currently evaluating the necessity of an open mDia1 conformation in PKD2 binding. This possibility is supported by our in vitro data showing that an in vitro proteolytically cleaved N-terminal form of mDia1 binds PKD2. This fragment of mDia1 should represent an active form, since it lacks a portion of the inhibitory C-terminal autoregulatory domain. The existence of a similar fragment has not been shown in vivo, and we consider it possible that this may occur in vitro due to the influence of GFP molecule fused to the N

![Fig. 7. mDia1-dependent localization of PKD2 to the spindle of HeLa cells. A, down-regulation of endogenous mDia1 by RNAi in HeLa cells. Native HeLa cell lysates (lane 1) and lysates of HeLa cells infected with a retrovirus carrying mDia1KD1 (lane 2) or mDia1KD2 construct (lane 3) were immunoprecipitated with mouse (Ms) anti-mDia1 and immunoblotted (IB) with the same antibody. The arrow points to the correct molecular weight for mDia1, whereas the lower band is a complex of mouse IgG heavy and light chains. B and C, representative mitotic HeLa cell infected with a retrovirus carrying mDia1KD1 (B) or mDia1KD2 (C) inactivating construct was stained with rabbit (Rb) anti-PKD2 (green) or mouse anti-α-tubulin (red). DNA staining is shown with ToPro3 (blue). D, HeLa cells transfected with the silencing control construct showing PKD2 staining (left, green), tubulin (middle, red), and their colocalization (right, yellow) on mitotic spindles of dividing cells.](http://www.jbc.org/content/106/30/29736/F7.large.jpg)
terminus of the protein. We are currently investigating whether such cleavage occurs in vivo and whether it depends on the presence of an intact C terminus. The functional implication of mDia1 cleavage is that it may generate a part of the molecule that would still be capable of mediating some of the functions of full-length mDia1 independently of RhoA binding.

Our results indicate that PKD2 localizes to the spindle during cell division. This was supported by indirect immunofluorescence staining of endogenous PKD2 in several cell types using two independent antibodies, RNAi, and biochemical evidence showing PKD2 association with purified spindles in transfected cells. Based on these results, we conclude that the mitotic spindle is a physiological subcellular localization of PKD2 during cell division. However, we presently do not have evidence for the physiological role of PKD2 localization at the mitotic spindle. We would like to propose two not necessarily mutually exclusive possibilities. The simpler one would be that PKD2 is proportionally divided between mother and daughter cells through symmetric loading on the spindle. Second, PKD2 channel activity is required during or immediately following cell division. There is precedence for both of these possibilities, since there are a number of proteins that can be defined as chromosomal passenger proteins or spindle-associated proteins. For example, survivin is necessary for chromosome alignment and spindle checkpoint assembly arrest (42), PRC1 is a microtubule-associated protein that maintains the midzone (43), and p23 transiently associates with mitotic spindles during cell cycle with a currently unknown function (44). The Drosophila transmembrane Axs protein co-localizes with ER proteins and is in a structure that ensheaths spindles during meiosis, and loss of Axs function impairs meiotic progression (45). Therefore, it is not necessarily unique to find a resident ER transmembrane protein bound to microtubule spindles during cell division. It remains possible that PKD2 is taken to the spindle microtubules primarily to ensure its coordinated segregation into the mother-daughter cells so that Ca\(^{2+}\) homeostasis can be maintained in the new cell.

The idea that PKD2 has a role in cell division by regulating Ca\(^{2+}\) homeostasis is an attractive one and is supported by previous findings that PKD2 can function as an intracellular Ca\(^{2+}\) release channel. A previous study has examined mitotic Chinese hamster ovary cells for positioning of the IP\(_3\) receptor (IP\(_3\)R) and endoplasmic reticulum Ca\(^{2+}\) stores and found that Ca\(^{2+}\) stores localized to the future cleavage cortex prior to the onset of cleavage furrow formation, and the IP\(_3\)R protein was associated with microtubule bundles (46). We have verified that the ER-resident IP\(_3\) receptor protein co-localizes with tubulin on the mitotic spindles of HeLa cells by indirect immunofluorescence (data not shown). Another study has shown that microinjection of Ca\(^{2+}\) store-enriched microsomes into dividing newt eggs induced a new cleavage furrow at ~70% of the injection sites via an IP\(_3\)R-induced Ca\(^{2+}\) release (47). Taken together, these findings suggest that ER-resident proteins such as the IP\(_3\)R and now the PKD2 calcium channel are positioned with Ca\(^{2+}\) stores in dividing cell...
mDia1-dependent Localization of PKD2 to Mitotic Spindles

The fact that HeLa cells containing mDia1 knockdown constructs continued to grow well in culture is not an entirely unexpected result, since mouse embryonic stem cells that are deficient or null for mDia1 continue to proliferate and show an increased level in the Drl2/p134Dia2 protein (51). Therefore, mDia2 may be performing the role of mDia1 in cytokinesis in our mDia1 knockdown HeLa cell lines, since it is normally present in HeLa cells (28). A second consideration is that protein silencing does not necessarily yield a complete cessation of protein expression, and this could also allow cytokinesis to continue, particularly if a second formin protein could be assisting in a redundant fashion.

However, our confocal images of HeLa mDia1 knockdown cells do not indicate that a second protein is capable of quantitatively bringing PKD2 to the mitotic spindles, since the loss of PKD2 staining is almost complete in some HeLa cells and having varying levels of reduction in others, consistent for a population of cells. Therefore, our results suggest that mDia1 has an additional function beyond its well documented role as a cytoskeletal organizing protein in that it partitions PKD2 and perhaps other proteins to mitotic spindles.

In conclusion, mDia1 is generally considered to be a cytoskeletal organizing protein capable of forming actin stress fibers and regulating the formation and orientation of stable microtubules. We now suggest that a novel function for mDia1 is to facilitate PKD2 localization to mitotic spindles, possibly by binding the C terminus of PKD2 that protrudes into the cytoplasm from the membrane of a subset of ER vesicles and to modulate intracellular Ca$^{2+}$ release in mitotic cells. In terms of an autosomal dominant polycystic kidney disease, some mutations in PKD2 could permit equal distribution among dividing cells via mDia1, although truncation mutations of PKD2 unable to interact with mDia1 would generate mother-daughter spindles largely devoid of PKD2 and presumably having less intracellular Ca$^{2+}$ release. Other PKD2 loss of function mutations may segregate evenly during cell division but result in subsequent cellular abnormalities resulting in cyst formation due to impaired PKD2 activity.

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