The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton

Anna Rachel Gallagher*, Anna Cedzich†, Norbert Gretz†, Stefan Somlo‡, and Ralph Witzgall*§

*Institute for Anatomy and Cell Biology I, University of Heidelberg, Im Neuenheimer Feld 307, 69120 Heidelberg, Germany; †Medical Research Center, Klinikum Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1–3, 68167 Mannheim, Germany; and ‡Section of Nephrology, Yale University School of Medicine, Boyer Center for Molecular Medicine, Room 136C, 295 Congress Avenue, New Haven, CT 06536-0812

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Despite the recent positional cloning of the *PKD1* and *PKD2* genes, which are mutated in the great majority of patients with autosomal-dominant polycystic kidney disease (PKD), the pathogenic mechanism for cyst formation is still unclear. The finding, that the PKD1 and PKD2 proteins interact with each other through their COOH termini, suggests that both proteins are part of the same protein complex or signal transduction pathway. Using a yeast two-hybrid screen with the PKD2 protein, we isolated the PKD2-interacting protein Hax-1. The specificity of the interaction was demonstrated by the fact that PKD2L, a protein closely related to PKD2, failed to interact with Hax-1. Immunofluorescence experiments showed that in most cells PKD2 and Hax-1 colocalized in the cell body, but in some cells PKD2 and Hax-1 also were sorted into cellular processes and lamellipodia. Furthermore we demonstrated an association between Hax-1 and the F-actin-binding protein cortactin, which suggests a link between PKD2 and the actin cytoskeleton. We speculate that PKD2 is involved in the formation of cell-matrix contacts, which are dysfunctional without a wild-type PKD2 protein, thus leading to cystic enlargement of tubular structures in the kidney, liver, and pancreas.

polycystin-2 | cortactin | focal adhesion | cell-matrix contact | cell–cell contact

With an incidence of ≈1:1,000 autosomal-dominant polycystic kidney disease (ADPKD) is one of the most common monogenetic human diseases (1). The positional cloning of two genes, which are mutated in the vast majority of patients with ADPKD, has opened new opportunities for a better understanding of cyst formation (2, 3). Whereas the PKD1 protein contains a number of motifs suggesting it is involved in cell–cell or cell–matrix interactions (4, 5), the homology between PKD2 and the α_1-subunits of voltage-activated calcium channels has led to the hypothesis that PKD2 influences calcium fluxes (3), but so far no experimental evidence has been presented for both assumptions. Meanwhile it could be demonstrated that PKD1 and PKD2 interact with each other through their COOH termini (6, 7), indicating that both proteins are part of the same protein complex or signal transduction pathway. The picture becomes even more complex by the recent identification of two PKD2-related proteins, the first of which has been named PKD2L (8, 9) and PKDL (10), the second PKD2L2 (9). The genomic location of the genes coding for PKD2L and PKD2L2 makes it unlikely that one of them is involved in renal cyst formation.

The *PKD2* gene encodes a protein of 968 aa with six putative transmembrane domains, both the NH_2 and COOH terminus have been suggested to extend into the cytoplasm (3). So far the characterization of the PKD2 protein has focused on its COOH terminus, which contains a calcium-binding EF-hand and a coiled–coil domain and represents the interface for the interaction with PKD1 (6, 7). Other domains in the COOH terminus of PKD2 are responsible for the association with TRPC1 (11), a member of the family of store-operated calcium channels, and for the homodimerization of PKD2 (6, 7). Very little, however, is known about the rest of the PKD2 protein. In this report we describe that loop 5 of PKD2 mediates interaction with Hax-1, an actin cytoskeleton-associated protein. This interaction may link PKD2 to cell–matrix contacts and therefore could explain many of the abnormalities found in polycystic kidneys.

Materials and Methods

Yeast Two-Hybrid Screen. A fragment coding for the region between transmembrane segments 5 and 6 of human PKD2 (3) was subcloned into the bait plasmid pPC97 (12) to create pPC97/PKD2, L5. MaV103 yeast cells were transformed with the bait construct by using standard protocols (13) and further characterized by testing for protein expression and self-activation of the bait before screening a mouse embryonic day 13–14 cDNA library in the prey plasmid pPC86. Approximately 7×10^5 transformants were first screened for histidine prototrophy in the presence of 50 mM 3-aminotriazole (Sigma) and consequently assayed for activation of the *URA3* and *LacZ* genes. A second two-hybrid screen was carried out with the same bait but with a human adult kidney cDNA library (a kind gift from Mike Brasch, Life Technologies, Rockville, MD).

Cloning of Full-Length Hax-1 and Construction of Hax-1 Mutants. The murine Hax-1 clone isolated from the two-hybrid screen was lacking 93 codons at the 5′ end, so the full-length cDNA was generated by using a PCR-based strategy. Hax-1 mutants also were generated by PCR and cloned into the prey plasmid pPC86. The authenticity of all PCR-derived constructs was confirmed by sequencing.

Expression Constructs, Cell Culture, and Transfection Protocols. Full-length and partial PKD2 and Hax-1 cDNAs were cloned into the expression vectors pcDNA3 (Invitrogen), pUHD10–3 (a kind gift from Hermann Bujard, Zentrum, für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany) and pEBG (a kind gift from Tom Force, Massachusetts General Hospital, Charlestown).

COS-7 cells were transiently transfected by the DEAE-dextran method (13). HtTA-1 cells (HeLa cells containing a tetracycline-controlled transactivator; ref. 14) were stably transfected by using a calcium phosphate protocol (15). Forty-eight hours after the transfection, cells were plated onto 10-cm Petri dishes and selected with hygromycin (300 μg/ml; Calbiochem) or puromy-

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Abbreviations: PKD, polycystic kidney disease; HA, hemagglutinin; GST, glutathione *S*-transferase.

§To whom reprint requests should be addressed. E-mail: ralph.witzgall@urz.uni-heidelberg.de.

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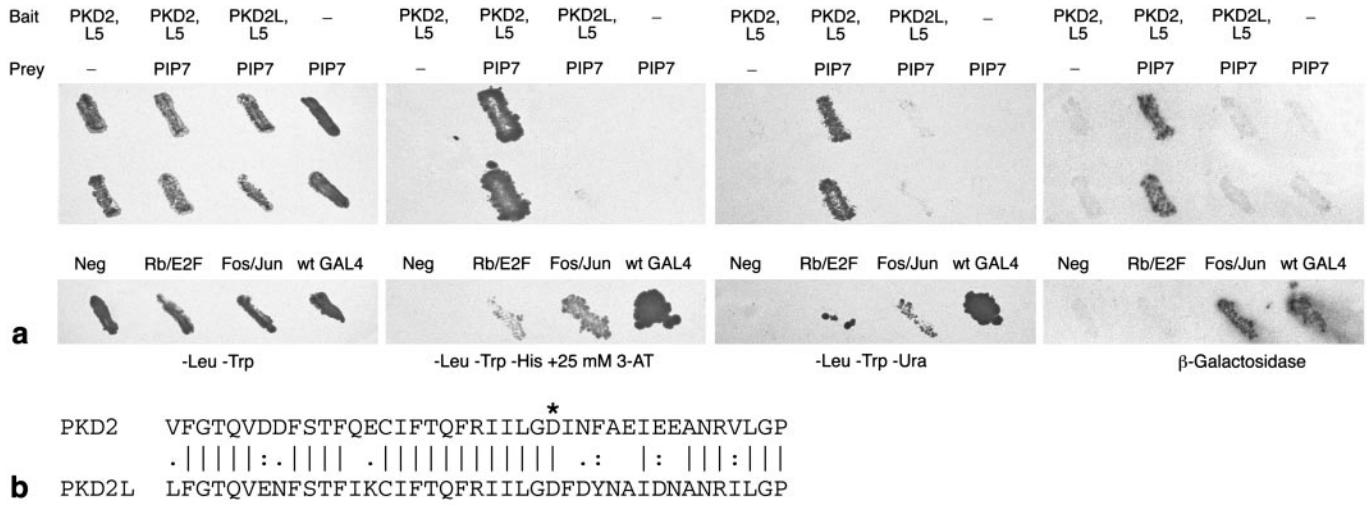

Fig. 1. Two-hybrid assays for the interaction between PKD2 and Hax-1. (*a*) Using three different assays it can be seen that Hax-1 interacts strongly and specifically with PKD2. Both in an assay for histidine and uracil prototrophy and in a β-galactosidase assay the region between the fifth and sixth transmembrane domain of PKD2 (loop 5; PKD2, L5) scores positive when expressed together with a truncated Hax-1 protein (PIP7). Loop 5 of the closely related PKD2L protein, however, fails to interact with Hax-1 (PKD2L, L5). The bottom panels show the negative and positive controls either containing no bait and no prey (Neg), a bait/prey combination of the retinoblastoma protein (Rb) and E2F (Rb/E2F), a bait/prey combination of Fos and Jun (Fos/Jun), and the wild-type GAL4 protein (wt GAL4). The master plates without additional selection are shown on the left. (*b*) Sequence comparison of loop 5 in human PKD2 and PKD2L. The negatively charged (aspartic acid) residue, which is considered to be important for cation selectivity in Ca^{2+} channels, is indicated by *.

cin (0.5 μg/ml; Calbiochem). Approximately 2 weeks later, resistant colonies were isolated and tested for expression of PKD2 and Hax-1.

Coimmunoprecipitation Studies. Cells were lysed in a buffer containing 1% Triton X-100, 0.05% SDS, 150 mM NaCl, 10 mM Tris·HCl (pH 7.5), 2 mM EDTA (pH 8.0), 10 mM sodium orthovanadate, 1 μg/ml of leupeptin, and 1 mM PMSF. After the protein concentration was determined according to an improved Bradford assay (16), cell lysates corresponding to 500 μg of protein were incubated with either 20 μl of swollen glutathione-agarose beads or antibody-coated protein A Sepharose beads for 4 h at 4°C. The beads were washed twice with lysis buffer, and the precipitated proteins were analyzed by Western blot analysis. The following primary antibodies were used: the mouse mAb 12CA5, which is directed against an epitope of the influenza virus hemagglutinin (HA) protein (cell culture supernatant diluted 1:30), a mouse monoclonal anti-glutathione *S*-transferase (GST) antibody (diluted 1:2,000; Sigma), and a mouse mAb directed against an epitope of the c-Myc protein (diluted 1:1,000; Roche Molecular Biochemicals). The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG Fab (Sigma), was used at a dilution of 1:10,000.

Immunocytochemistry and Lectin Staining. Cells were plated on glass coverslips and allowed to grow for an additional 2 days. After fixation the cells were permeabilized with 0.05% Triton X-100, 1× PBS, 2% BSA. The following primary antibodies were used: the rabbit polyclonal anti-PKD2 antibody YCC2 (17) (diluted 1:4,000), supernatant from the anti-HA-epitope hybridoma 12CA5 (diluted 1:30), a mouse monoclonal anti-GST antibody (diluted 1:1,000; Sigma), a rabbit polyclonal anti-GST antibody (diluted 1:200; Santa Cruz Biotechnology), a rabbit polyclonal anti-cortactin antibody (18) (diluted 1:1,000; a kind gift from Xi Zhan, American Red Cross, Rockville, MD), a rabbit polyclonal antibody against Sec61 β, a marker of the endoplasmic reticulum (diluted 1:100; a kind gift from Martin Pool and Bernhard Dobberstein, Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany), a mouse mAb against the Golgi 58K protein, a marker for the Golgi apparatus (diluted 1:40; Sigma), and a mouse mAb against Mn-superoxide dismutase, a marker for mitochondria (diluted 1:100; Bender MedSystems, Vienna). The Golgi apparatus was demonstrated alternatively with FITC-conjugated wheat germ agglutinin (diluted 1:100; Sigma).

Specific binding was demonstrated with FITC-conjugated anti-mouse IgG from Cappel (diluted 1:150), Cy3-conjugated rat anti-mouse IgG from Dianova (Hamburg, Germany) (diluted 1:300), and Cy3-conjugated goat anti-rabbit IgG from Dianova (diluted 1:300).

Results

Identification of a PKD2-Associated Protein by a Yeast Two-Hybrid Screen. A bait consisting of the region between transmembrane domains 5 and 6 of human PKD2 was used to screen a mouse embryonic day 13–14 cDNA library. Of approximately 7×10^5 colonies, only one clone with a 700-bp cDNA insert, PIP7, led to the activation of the *HIS3*, *URA3*, and *LacZ* reporter genes (Fig. 1*a*). Sequence analysis of PIP7 revealed that it was murine Hax-1, formerly isolated in a yeast two-hybrid screen with HS1, a substrate for nonreceptor tyrosine kinases (19). To corroborate the specificity of the interaction between PKD2 and Hax-1, we also tested loop 5 of PKD2L (8–10), which is highly conserved to the corresponding region in PKD2 (Fig. 1*b*), for interaction with PIP7. Hax-1 did not interact with PKD2L (Fig. 1*a*), which indicates that the COOH-terminal end within loop 5 of PKD2 is necessary for the specific interaction with Hax-1.

To define which region within Hax-1 was necessary for the interaction with PKD2, a number of deletion mutants were constructed and assayed by using the two-hybrid system. Plasmids encoding the mutants were transformed into yeast expressing loop 5 of PKD2 and tested for their ability to grow on histidine-free medium in the presence of 25 mM 3-aminotriazole; in addition, a liquid β-galactosidase assay was performed. A mutant comprising the last 102 aa of Hax-1 still interacted with PKD2 (Fig. 2). The subsequent screening of a human adult kidney cDNA library with loop 5 of PKD2 also identified two interacting clones as human HAX-1, one of them encoded a COOH-terminal fragment comprising amino acids 184–279 (data not shown). This supports the hypothesis that the inter-

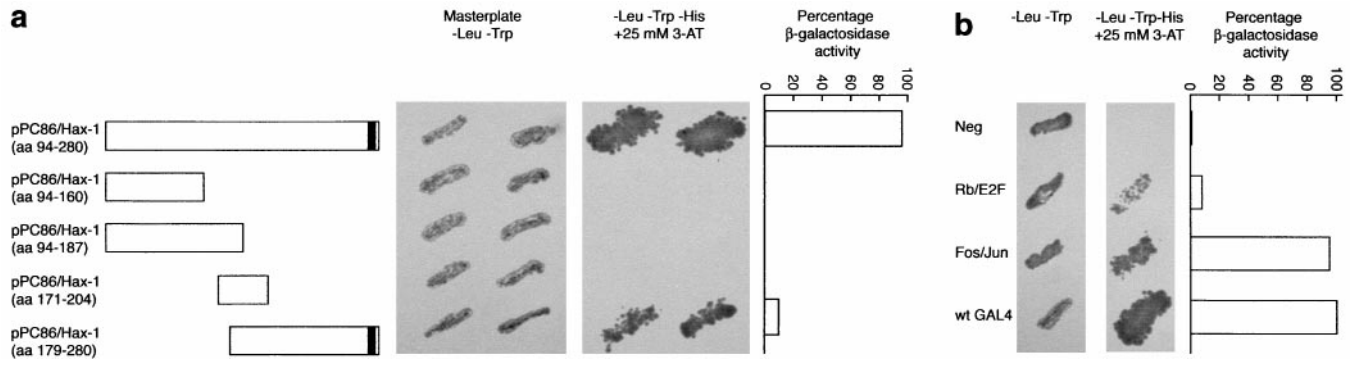

Fig. 2. The COOH terminus of Hax-1 mediates interaction with PKD2. The yeast two-hybrid system was used to determine the region in Hax-1 necessary for the interaction with loop 5 of PKD2. An interaction was assayed by prototrophy for histidine in the presence of 25 mM 3-aminotriazole and by a liquid β-galactosidase assay (the β-galactosidase activity achieved with the wild-type GAL4 protein was set at 100%). Although the last 102 aa of Hax-1 are sufficient for the association with PKD2, this interaction is not as strong as the one seen with the longer Hax-1 fragment. Therefore additional amino acids are probably necessary as part of the domain interacting with PKD2 or to maintain the appropriate conformation of Hax-1 necessary to interact with PKD2. The putative transmembrane domain of Hax-1 is depicted as a vertical bar at the COOH terminus.

acting region between Hax-1 and PKD2 is located at the COOH terminus of Hax-1.

Hax-1 Associates with PKD2 in Mammalian Cells. To confirm the interaction between Hax-1 and PKD2, coimmunoprecipitation experiments were carried out in stably transfected HeLa cells inducibly expressing full-length HA-tagged human PKD2 and constitutively expressing a fusion protein between GST and murine full-length Hax-1. Immunoprecipitation of PKD2 also resulted in the pull-down of Hax-1, proving an interaction between the two proteins also in mammalian cells (Fig. 3). Further evidence that Hax-1 and PKD2 interact *in vivo* was provided by immunofluorescence studies. Cells in which only the HA-tagged PKD2 protein or the GST/Hax-1 fusion protein were expressed showed an intracellular reticular staining pattern (Fig. 4 *a* and *b*), double-staining for markers of various cytoplasmic organelles demonstrated that both PKD2 and Hax-1 are associated with the endoplasmic reticulum, but not with the Golgi apparatus nor the mitochondria (Fig. 4 *c–t*). In the cells where PKD2 and Hax-1 were coexpressed, PKD2 in most cases colocalized with Hax-1 in the cell body, but both proteins also could be detected in cell processes and lamellipodia (Fig. 5).

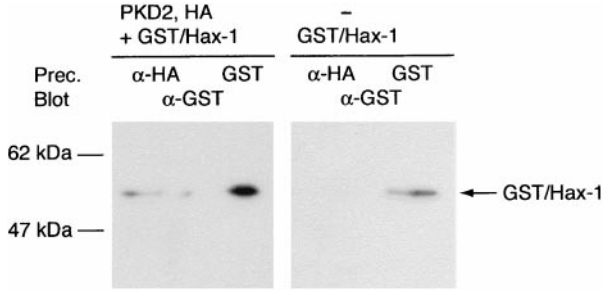

Fig. 3. Coprecipitation of PKD2 and Hax-1 in mammalian cells. Protein extracts from stably transfected HeLa cells inducibly expressing a HA epitope-tagged PKD2 protein and constitutively expressing a GST/Hax-1 fusion protein were incubated with the anti-HA epitope antibody 12CA5 (Prec.). Immunoprecipitated proteins were analyzed by Western blot with an anti-GST antibody (Blot). The GST/Hax-1 fusion protein is precipitated only in extracts from those cells where the expression of PKD2 is turned on (*Left*), but not in extracts from cells where the expression of PKD2 is turned off (*Right*). As a positive control the GST/Hax-1 fusion protein was precipitated with glutathione-agarose beads (GST).

Interaction Between Hax-1 and Cortactin Links PKD2 to the Actin Cytoskeleton. As already mentioned above, Hax-1 was first isolated in a search for HS1-associated proteins. HS1 is closely related to cortactin, an F-actin-associated protein, but whereas HS1 expression is restricted to cells of the hematopoietic cell lineage (20), cortactin has a much more widespread distribution (21). To establish a link between PKD2 and the actin cytoskeleton, we investigated whether Hax-1 and cortactin also interacted. COS-7 cells were transiently cotransfected with a plasmid coding for the GST/Hax-1 fusion protein and a plasmid coding for myc-tagged cortactin. Coimmunoprecipitation and GST-pull-down experiments with whole-cell lysates indicated that cortactin, like its paralog HS1, also interacts with Hax-1 *in vivo* (Fig. 6 *a* and *b*). Further double-immunofluorescence studies on the cells expressing PKD2 and the fusion protein between GST and Hax-1 indicated that Hax-1 and the endogenous cortactin colocalized in the cytoplasm as well as in lamellipodia (Fig. 6*c*).

Discussion

In the search for proteins interacting with loop 5 of the PKD2 protein we have identified Hax-1, a ubiquitous protein with a M_r of 35 kDa (19). The interaction between Hax-1 and PKD2 is very specific, because loop 5 of the PKD2-related protein PKD2L did not interact with Hax-1. Sequence alignments between PKD2, PKD2L, and several voltage-activated and store-operated calcium channels suggest that the segment between the membrane-spanning domains 5 and 6 of PKD2 and PKD2L forms a pore (10, 11). The major difference between PKD2 and PKD2L is a stretch of 8 aa in the last third of this region, thus suggesting that they are at least part of the interface that is necessary for interaction with Hax-1. Because this domain immediately follows a negatively charged (aspartic acid) residue, which is considered to be important for cation selectivity in Ca^{2+} channels (22), the interaction between PKD2 and Hax-1 may have important functional implications. Although it is not known exactly how the pore is formed, mutagenesis experiments indicate that in the case of human TRP3 loop 5 extends into the lumen of the endoplasmic reticulum at least temporarily (23).

Before our cloning of Hax-1 as a protein interacting with PKD2, it was already isolated in two other settings. Hax-1 (*syn.* SIG-111) was first identified as a partial cDNA in a differential screen for mRNAs induced upon exposure of macrophages to silica, the same article also describes an up-regulation of Hax-1 upon treatment of macrophages with the calcium ionophore A23187 (24). Subsequently to the description of Hax-1 as a silica-induced mRNA in

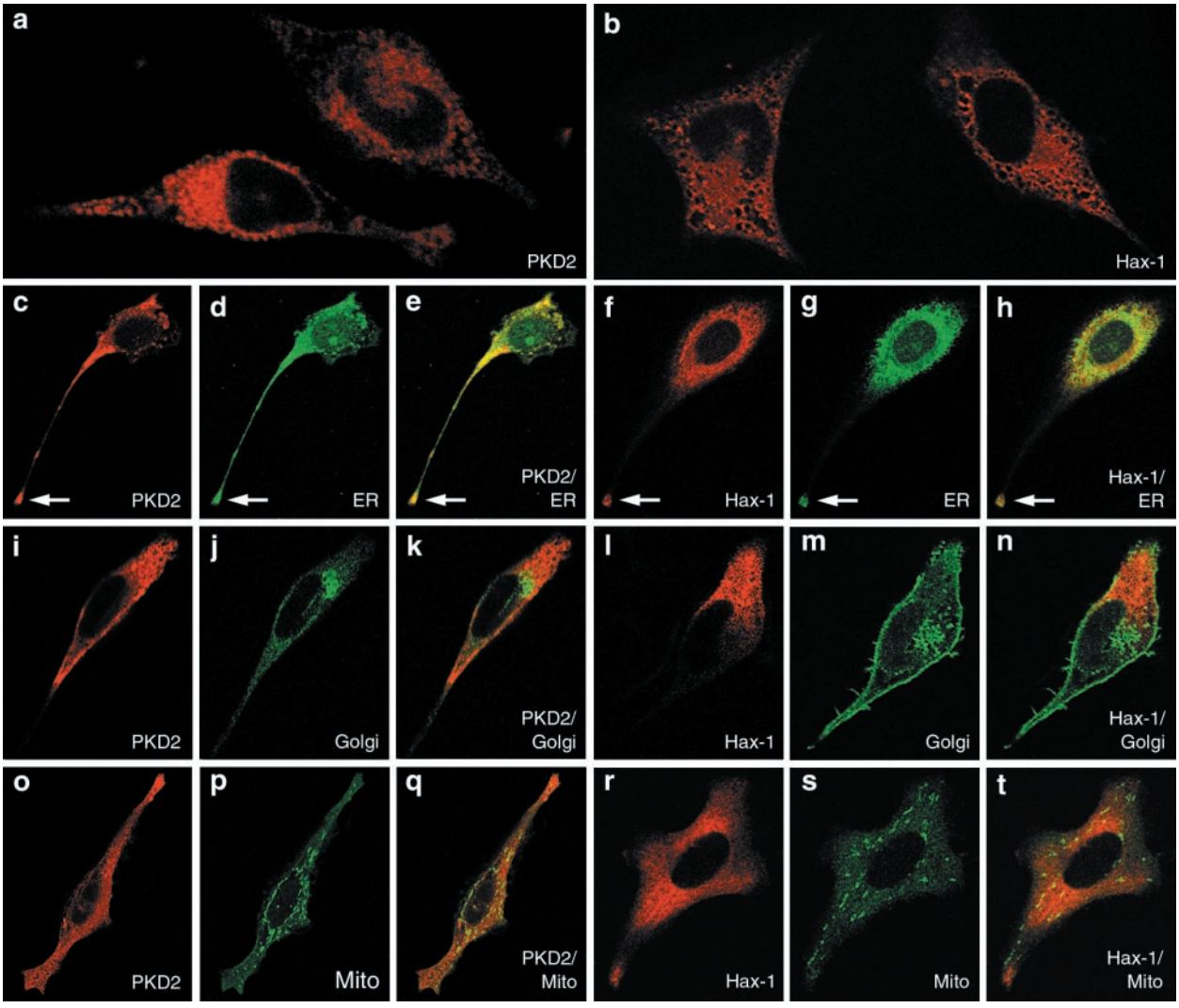

Fig. 4. PKD2 and Hax-1 are located in the endoplasmic reticulum. HeLa cells either inducibly expressing a HA epitope-tagged PKD2 protein or constitutively expressing a GST/Hax-1 fusion protein showed a reticular cytoplasmic distribution of both proteins when stained with the anti-HA epitope antibody (*a*) or with an anti-GST antibody (*b*). Double-staining with markers for the endoplasmic reticulum (anti-Sec61 β antibody, *c–h*), the Golgi apparatus (anti-Golgi 58K protein antibody, *i–k*; FITC-conjugated wheat germ agglutinin, *l–n*) and mitochondria (anti-Mn-superoxide dismutase antibody, *o–t*) demonstrates that PKD2 and Hax-1 are located in the endoplasmic reticulum (*c–h*), but not in the Golgi apparatus (*i–n*) nor in the mitochondria (*o–t*). The arrows in *c–h* point to the tips of long cell processes where PKD2 and Hax-1 were detected. Note that with the FITC-conjugated wheat germ agglutinin the outline of the cell also is stained because of the presence of *N*-glycosylated plasma membrane proteins (*m* and *n*).

macrophages, the Hax-1 cDNA was isolated in a two-hybrid screen for proteins interacting with HS1 (19). HS1, a protein specifically expressed in cells of the hematopoietic lineage (20), is thought to be involved in signal transduction in B cells. Upon antigen receptor crosslinking, a number of tyrosine kinases become activated and in turn phosphorylate cellular proteins including HS1. HS1 is closely related to the F-actin-binding protein cortactin, which in contrast to HS1 is expressed throughout the body (21). Despite their difference in tissue distribution both HS1 (25–27) and cortactin (28–30) are phosphorylated by nonreceptor tyrosine kinases. In this report we extend the earlier observation that Hax-1 interacts with HS1 by demonstrating an interaction between Hax-1 and cortactin. We show that cortactin coprecipitates with Hax-1; furthermore both proteins colocalize in the cytoplasm and at the cell periphery in lamellipodia, a similar distribution to that described before for cortactin (29, 31–34). The distribution of cortactin is tightly regulated, because the location in the cell cortex depends on an intact actin cytoskeleton (32), and the protein is translocated from the cell body into lamellipodia upon treatment of intact cells with phorbol ester (34) and platelet-derived growth factor (29, 34) or by the activation of the monomeric G-protein Rac1 and its downstream effector, the serine/threonine kinase PAK (34).

Our own data obtained from the analysis of a number of different cell lines show that the full-length PKD2 protein typically is located in the endoplasmic reticulum (ref. 35 and unpublished observations), and that only very rarely is it translocated into the cell periphery. Apparently a 34-aa region in its COOH terminus is responsible for the retention of PKD2 in the endoplasmic reticulum, because deletion of this motif results in the translocation of PKD2 toward the plasma membrane (35). Using double-staining for organelle-specific markers, we show that the intracellular pool of both PKD2 and Hax-1 is associated with the endoplasmic reticulum, whereas in a previous study evidence was provided that Hax-1 also associates with mitochondria (19). In their study, however, Suzuki *et al.* (19) used an antibody against Bcl-x to investigate the intracellular distribution of Hax-1. Because Bcl-x is not a stringent marker for mitochondria but also can associate with the endoplasmic reticulum, we

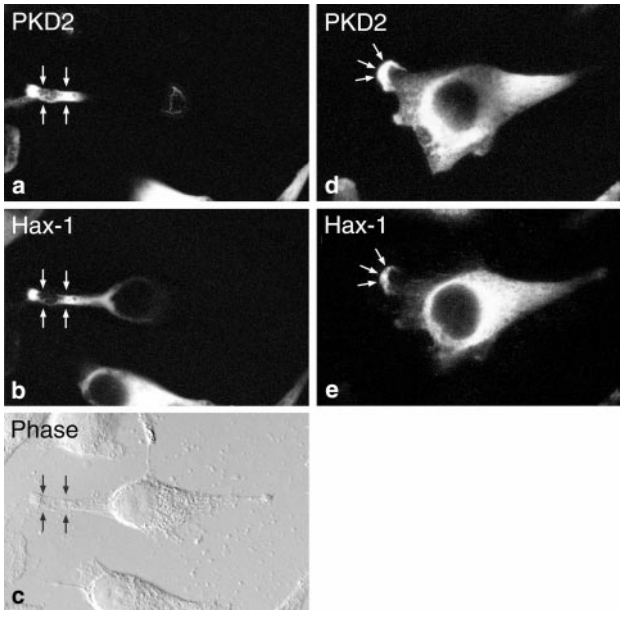

Fig. 5. Colocalization of PKD2 and Hax-1 in mammalian cells. Stably transfected HeLa cells inducibly expressing PKD2 and constitutively expressing a GST/Hax-1 fusion protein were double-stained with the polyclonal anti-PKD2 antibody (*a* and *d*) and a monoclonal anti-GST antibody (*b* and *e*). In most cells PKD2 and Hax-1 colocalize in the cytoplasm, but in those cells spreading out over the dish a striking redistribution into the tips of the cell processes (arrows) could be seen (*a*–*c*). A phase contrast view is included to better demonstrate the shape of the cell (*c*). In very few cells both proteins were sorted into lamellipodia (arrows in *d* and *e*). PKD2 and Hax-1 only partially colocalize in the body of the cell shown in *d* and *e*, suggesting a dynamic interaction between PKD2 and Hax-1.

suggest to reinterpret the data shown in the previous publication (19). Immunohistochemical investigation of the distribution of the rat Pkd2 protein complements the characterization of PKD2 in cell lines. Whereas in the kidney and salivary gland Pkd2 assumes a basolateral distribution, a reticular cytoplasmic staining pattern is detected in the adrenal gland and the ovary (36). It therefore is very likely that an additional signal exists, which determines the location of PKD2 inside the cell.

We propose two models to illustrate how PKD2 is integrated into a protein complex involved in cell-matrix interactions. In the first model integrins as integral plasma membrane proteins mediate binding to the extracellular matrix; they signal through nonreceptor tyrosine kinases to cortactin. Cortactin connects to Hax-1, which in turn binds to PKD2. Because loop 5 is the putative pore-forming region of PKD2 and related proteins (10, 11), Hax-1 may not only be an adaptor between cortactin and PKD2, but in addition a key element to regulate the function of PKD2. The association of Hax-1 with loop 5 of PKD2 could either induce or block local ion fluxes originating from the endoplasmic reticulum. This first model is supported by the finding that in all cell lines investigated so far the full-length PKD2 protein has been detected in the endoplasmic reticulum. Furthermore there is biochemical evidence that also in the kidney PKD2 is associated with the endoplasmic reticulum (35).

In the second model PKD2 itself is located in the plasma membrane and through its interaction with PKD1 (6, 7) is associated with the extracellular matrix. Signaling would occur by the interaction between PKD2 and Hax-1, which through its binding to cortactin would mediate the association with the actin cytoskeleton. There are also data in support of this model. Using biotinylation of intact cells we have demonstrated that a mutant PKD2 protein can be inserted into the plasma membrane (35), suggesting that there are certain conditions that permit the transit of PKD2 from the endoplasmic reticulum to the plasma membrane. Both PKD1 (37) and PKD2 (38) may regulate monomeric G-proteins, and these in turn are necessary for the translocation of cortactin to the cell periphery (34). In addition

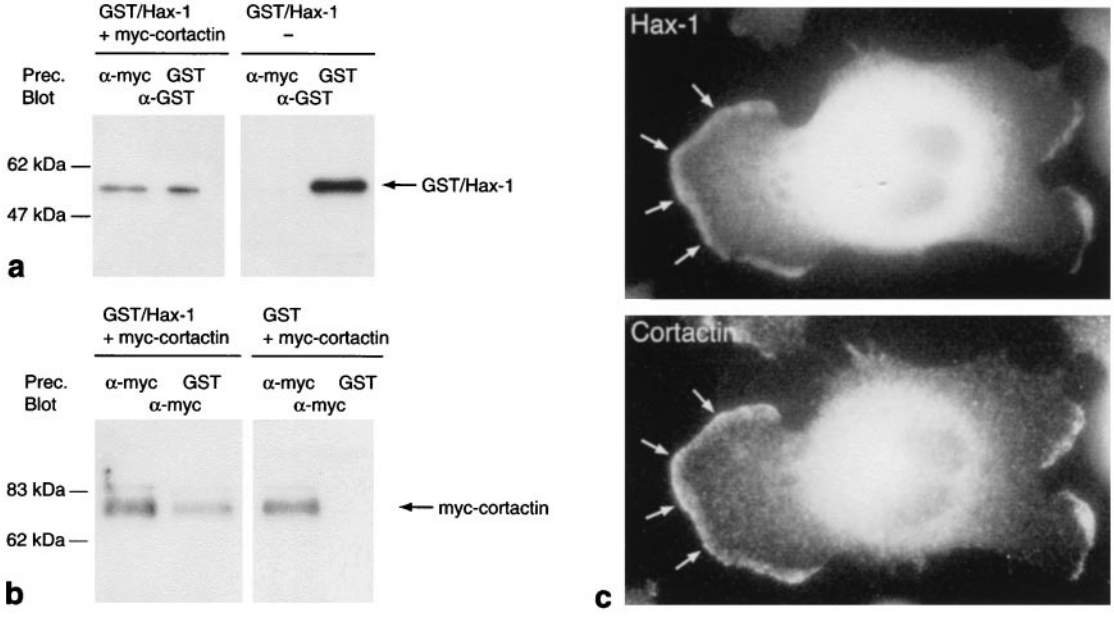

Fig. 6. Hax-1 is associated with cortactin. (*a* and *b*) COS-7 cells were transiently transfected with various combinations of expression plasmids coding for myc-tagged cortactin, a GST/Hax-1 fusion protein and GST only. Protein extracts from transfected cells were used for immunoprecipitation with an anti-myc-epitope antibody and for GST pull-down assays (Prec.). The precipitates then were analyzed by Western blot with an anti-GST (*a*) and anti-myc-epitope antibody (*b*) (Blot). In either case cortactin and Hax-1 are coprecipitated, demonstrating an association between the two proteins. (*c*) Stably transfected HeLa cells constitutively expressing a GST/Hax-1 fusion protein were double-stained with a mouse mAb against GST (*Upper*) and a rabbit polyclonal antibody against cortactin (*Lower*). It can be easily seen that the two proteins colocalize in lamellipodia (arrows).

the PKD1 protein, which probably exists in a complex with PKD2, has been shown to coprecipitate with actin cytoskeleton-associated proteins (39, 40).

A central role for cell contacts in cyst formation also is suggested by the fact that the inactivation of the genes coding for tensin (41) and Rho GDIα (42) as well as the overexpression of a constitutively active β-catenin mutant (43) result in the development of polycystic kidneys. Furthermore there is evidence that PKD1 stabilizes β-catenin (44). In this context it should be pointed out that cortactin also has been localized to invadopodia, specialized sites of extracellular matrix breakdown (45), and that overexpression of cortactin leads to increased cell motility (28, 33). During cyst expansion the epithelial cells have to degrade the surrounding extracellular matrix and this process may be started by a dysregulation of cortactin caused by mutations in the PKD2 protein.

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