Characterization of the nephrocystin/nephrocystin-4 complex and subcellular localization of nephrocystin-4 to primary cilia and centrosomes

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Nephrocystin and nephrocystin-4 are newly identified proteins involved in familial juvenile nephronophthisis, an autosomal recessive nephropathy characterized by cyst formation and renal fibrosis. Nephrocystin is an adaptor protein that is able to associate with signaling molecules involved in cell adhesion and actin cytoskeleton organization, such as p130Cas, Pyk2, tensin and filamins. Nephrocystin was recently shown to interact and to co-localize with the microtubule component β-tubulin to the primary cilia in renal epithelial cells, an organelle known to play a key role in the pathogenesis of cystic kidney diseases. In this study, we demonstrated that nephrocystin-4 also localizes to the primary cilia in polarized epithelial tubular cells, particularly at the basal bodies, and associates with microtubule component α-tubulin, suggesting a common role for the nephrocystin proteins in ciliary function. However, the co-localization of nephrocystin-4 with the microtubules is not restricted to the primary cilia, as nephrocystin-4 was also detected at the centrosomes of dividing cells and close to the cortical actin cytoskeleton in polarized cells. We also detected p130Cas and Pyk2 in the nephrocystin-4-containing complex, confirming the role of the nephrocystin proteins in cell–cell and cell–matrix adhesion signaling events. Finally, we refined the structural and functional regions involved in the interaction between nephrocystin and nephrocystin-4. These data suggest that nephrocystin and nephrocystin-4 belong to a multifunctional complex localized in actin- and microtubule-based structures involved in cell–cell and cell–matrix adhesion signaling as well as in cell division.

INTRODUCTION

Cystic kidney diseases are a clinically and genetically heterogeneous group of disorders that are all characterized by perturbed tubular architecture leading to the formation of cysts. These cysts form in any portion of the nephron in the dominant form of polycystic kidney disease (ADPKD), in the collecting duct in the recessive form (ARPKD), and mainly at the cortico-medullary junction in nephronophthisis. Recent studies have suggested that the primary cilium, a microtubule-containing organelle that protrudes from the apical cell surface, is involved in cyst formation. In a variety of mouse models of cystic kidney disease, the genes conferring disease susceptibility (orpk, cpk and inv) encode a cilia-associated protein (1–3). The genes responsible for ADPKD (PKD1 and PKD2) and ARPKD (PKHD1) are expressed in the primary cilia (4–6), as are the genes responsible for juvenile and infantile nephronophthisis (NPH1 and NPHP2) (7).

Familial juvenile nephronophthisis is an autosomal recessive tubulo-interstitial nephropathy responsible for chronic renal failure in children (8). The first sign of this disease is polyuria, followed by progressive deterioration of renal function during childhood or adolescence. The most prominent histological features are tubular atrophy, abnormal thickening of the tubular basement membrane, interstitial
fibrosis, and cyst formation at the cortico-medullary junction. Nephronophthisis can be associated with extrarenal manifestations such as retinitis pigmentosa (Senior–Løken syndrome) (9,10) and congenital ocular motor apraxia (Cogan syndrome) (11).

Two genes responsible for juvenile nephronophthisis have been identified: NPHP1 (12,13) and NPHP4 (14,15). NPHP1 encodes nephrocystin, a 732-amino acid protein that contains several protein–protein interaction modules: three coiled-coil domains in the N-terminal region, a Src homology 3 (SH3) domain and a ‘nephrocystin homology domain’ (NHD) comprising the highly conserved C-terminal two-thirds of the protein. The NHD is involved in both homodimerization and epithelial cell–cell junction targeting of nephrocystin (16). Nephrocystin interacts with proteins localized at focal adhesions, such as the adaptor protein p130Cas and the proline-rich tyrosine kinase 2 (Pyk2), as well with actin-binding proteins, tensin and filamins (16–18). The SH3 domain mediates the association with Pyk2 and p130Cas, whereas filamins interact with the NHD domain (16). Nephrocystin co-localizes to the cell–cell contact sites with p130Cas and E-cadherin in Madin-Darby canine kidney (MDCK) cells (17). In addition, nephrocystin was recently shown to interact and to co-localize with β-tubulin in the primary cilia of renal tubular epithelial cells (7). The second gene, NPHP4, encodes a 1426-amino acid cytosolic protein named nephrocystin-4 (14) or nephro- etinin (15). This protein does not contain any domains of known function, except for a proline-rich region between positions 458 and 514. We previously showed that nephrocystin, which is endogenously expressed in human embryonic kidney cells (HEK293), interacts with overexpressed nephrocystin-4 independently of the nephrocystin SH3 domain (14).

Two uncommon forms of nephronophthisis have been described on the basis of the age at onset of end-stage renal disease (ESRD). The infantile form (nephronophthisis type 2) is characterized by the early and widespread development of cysts, associated with interstitial fibrosis and tubular basement membrane disruption leading to ESRD in the first years of life (19). The phenotype of the adolescent form (nephronophthisis type 3) is similar to that of the juvenile form, although the renal disease was initially reported to occur later (20). The genes responsible for these two forms (NPHP2 and NPHP3, respectively) were identified recently. The NPHP2 gene encodes for invesin (7), a protein that is critical for the development of normal left–right asymmetry. This is particularly interesting, as the inv −/− mouse has situs inversus in addition to cysts in the kidneys (21). The lateralization process depends on the flow created by cilia located on the embryonic node cells (22). The NPHP3 product, nephrocystin-3, is a novel cytosolic molecule with several protein–protein interaction domains, such as a coiled-coil domain and a C-terminal tetratricopeptide repeat domain as well as a tubulin tyrosine ligase domain (23). Nephrocystin co-precipitates with both invesin and nephrocystin-3 (7,23) and colocalizes to the primary cilia of renal cells with invesin (7). These findings suggest that all these proteins are involved in the same signaling pathway and play a role in ciliary function.

The objectives of this study was to define the structural and functional regions involved in the interaction between nephrocystin and nephrocystin-4 and to determine the cellular localization of nephrocystin-4. Our main finding was that nephrocystin-4 is localized in microtubule-based structures, such as primary cilia, reinforcing the hypothesis that the nephronophthisis gene products play a co-operative role in cyst formation.

RESULTS

Nephrocystin and nephrocystin-4 interact in renal cell lines and in human kidney

To test the interaction between endogenous nephrocystin and nephrocystin-4, we first examined the expression of these proteins in various renal cell lines. Western blot analysis with a specific antibody directed against the human nephrocystin (AP-N1) detected an 80 kDa protein in human kidney extracts and in HK2 and HEK293 cell lines, but not in a renal tubular cell line from a patient carrying a homozygous deletion of the whole NPHP1 gene (NPHP1 −/− cells) (Fig. 1A). However, the full-length nephrocystin stably expressed in NPHP1−/− cells (NPHP1-TS) migrated at a higher molecular weight than the endogenous protein (Fig. 1A, left panel). The endogenous nephrocystin likely corresponds to a previously described shorter isoform (13), homologous to the canine and murine nephrocystin and lacking amino acids 258–312. RT–PCR predominantly amplifies this isoform in HEK293 and HK2 cells as compared to the full-length transcript which was only weakly detected (data not shown). This was confirmed by transfecting HEK293 cells with constructs expressing either the full-length nephrocystin (Flag-N1-fl) or the shorter isoform (Flag-N1-6A) (Fig. 1A, right panel).

A specific antibody against the C-terminal part of the nephrocystin-4 protein (AP-N4) detected a strong 175 kDa band, corresponding to the size of the full-length nephrocystin-4, in each extract including MDCK cells (Fig. 1B, left panel). An additional 150 kDa band was observed in HEK293 cells. This shorter variant may correspond to an isoform translated from the second in-frame methionine (M177) as it migrated to the same position as the nephrocystin-4 mutant lacking the first 176 amino acids (N4-ΔN176-myc) (Fig. 1B) and as it was not recognized by a specific antibody directed against the first 176 amino acids of the nephrocystin-4 protein (AP-N4A) (Fig. 1B, right panel). The AP-N4 antibody also detected a 175 kDa protein in adult mouse kidney and brain homogenates (data not shown).

Immunoprecipitation experiments with the anti-nephrocystin-4 antibody revealed that the endogenous nephrocystin and nephrocystin-4 interact with each other in human kidney and in all renal epithelial cell lines, except in NPHP1 −/− cells, confirming the specificity of the interaction (Fig. 1C). Experiments using the AP-N1 antibody showed that endogenous nephrocystin co-precipitates with the 175 kDa nephrocystin-4 isoform in human kidney and HK2 cells (data not shown), as well as in HEK293 cells, but fails to interact with the 150 kDa isoform of nephrocystin-4 expressed in HEK293 cells (Fig. 1C, right panel).
Figure 1. Expression and interaction of endogenous nephrocystin and nephrocystin-4 in various mammalian renal cell lines and human kidney. (A) Expression of nephrocystin. Human medullary extracts and lysates of NPHP1 −/−, NPHP1-TS (NPHP1 −/− cells re-expressing the full-length nephrocystin), HK2 and HEK293 cells (left panel), as well as lysates of HEK293 cells transfected with the full-length nephrocystin (Flag-N1-fl) or the short isoform (Flag-N1-6A) (right panel), were analyzed by western blot with the AP-N1 antibody. Arrow-heads in the right panel indicate Flag-tagged nephrocystin isoforms (Flag-N1-fl and Flag-N1-6A) expressed in HEK293 lysates. The size of the transfected full-length nephrocystin in NPHP1-TS or HEK293 cells (~83 kDa) is larger than the endogenous nephrocystin (~80 kDa), which corresponds to the short isoform (Flag-N1-6A). (B) Expression of nephrocystin-4. Various cell lysates and human medullary kidney homogenates were analyzed by immunoprecipitation using the AP-N4 antibody. In parallel, HEK293 cells were transfected with the N4-fl-myc and N4-ΔN176-myc expression vectors followed by anti-c-Myc immunoprecipitation (left panel). Immune complexes and cell lysates from transfected HEK293 were analyzed by immunoblotting with the AP-N4 antibody to reveal endogenous nephrocystin-4 and overexpressed c-Myc-tagged nephrocystin-4 proteins. The immunoprecipitates of endogenous and overexpressed nephrocystin-4 protein in HEK293 cell lysates (right panel) were subsequently immunoblotted with the AP-N4A antibody. Stars indicate the N4-fl-myc and N4-ΔN176-myc overexpressed proteins, which migrate to the same position as the long (~175 kDa) and the short (~150 kDa) nephrocystin-4 isoforms expressed in the HEK293 cell line, respectively. (C) Interaction between endogenous nephrocystin and nephrocystin-4. (Right panel) The AP-N1 antibody detected endogenous nephrocystin in immune complexes formed with the AP-N4 antibody in all cell lines, except in NPHP1 −/− cells. A faint band corresponding to nephrocystin was detected in medullary extracts; the intensity improved after longer exposure (data not shown). It should be remembered that the NPHP1-TS cells express the long isoform of nephrocystin. (Left panel) HEK293 cell lysates were submitted to immunoprecipitation using the AP-N4 or AP-N1 antibodies and nephrocystin-4 was revealed with the AP-N4 antibody. The short isoform of nephrocystin-4 (expressed only in HEK293 cells) does not interact with endogenous nephrocystin. The thin ~83 kDa band, immunoprecipitated and detected by the AP-N1 antibody, probably corresponds to the endogenous full-length nephrocystin (right panel).
The C-terminal residues of nephrocystin and the N-terminal residues of nephrocystin-4 are required for the interaction

We analyzed the interaction between nephrocystin and nephrocystin-4 by co-immunoprecipitating c-Myc-epitope-tagged nephrocystin-4 (N4-fl-myc) with FLAG-tagged nephrocystin variants, Flag-N1-fl and Flag-N1-6A, and a series of C-terminal truncated nephrocystin mutants (Fig. 2A). These co-immunoprecipitation experiments were performed in NPHP1−/− cells rather than in HEK293 cells to avoid dimerization between endogenous nephrocystin and overexpressed nephrocystin mutants (Fig. 2B). Flag-N1-6A and Flag-N1-Δ376-480, a mutant lacking the homodimerization region, co-precipitated with N4-fl-myc, whereas no interaction was detected with any of the C-terminal truncated mutants (Fig. 2B, lanes 2–4). These results indicate that the last 79 residues of nephrocystin are required for the interaction with nephrocystin-4. This interaction does not require nephrocystin homodimerization as Flag-N1-Δ376-480 still precipitated with nephrocystin-4. We also showed that nephrocystin-4 bound to glutathione S-transferase (GST) fusion proteins containing the C-terminal region of nephrocystin (GST-N1-C251 and GST-N1-C131) in vitro (Fig. 2C). These data indicate that a region containing the C-terminal 131 amino acids of nephrocystin is sufficient to mediate an interaction with nephrocystin-4.

Similar experiments were performed in HEK293 cells using truncated mutants of nephrocystin-4 (Fig. 3A) and Flag-N1-fl (Fig. 3B). All the C-terminal truncated nephrocystin-4 mutants (myc-N4-Q793X and myc-N4-E1124X) co-precipitated Flag-N1-fl and the endogenous nephrocystin (Fig. 3B, left panel). Conversely, the overexpressed N-terminal truncated mutants (N4-ΔN176-myc and N4-ΔN309-myc) coprecipitated far less Flag-N1-fl than the N4-fl-myc (Fig. 3B, right panel). Furthermore, the residual interaction of N4-ΔN176-myc with Flag-N1-fl was not any weaker when the proline-rich region (residues 458–514) was deleted (N4-ΔN176-ΔPro-myc) (Fig. 3B, right panel), indicating that this proline-rich region is not involved in the interaction between nephrocystin and nephrocystin-4. Moreover, the interaction between the endogenous nephrocystin and the N4-ΔN176-myc and N4-ΔN309-myc was dramatically reduced (Fig. 3C). These results indicate that the N-terminal part of nephrocystin-4 (residues 1–176) is critical for the interaction. In vitro binding assays showed that the GST-N1-C131 fusion protein associated with the C-terminal-truncated mutant (myc-N4-Q793X) but not with the N4-ΔN176-myc (Fig. 3D). Thus, the interaction involves at least the N-terminal 176 residues of nephrocystin-4 and the C-terminal 131 residues of nephrocystin.

Nephrocystin-4 interacts with nephrocystin partners

Nephrocystin associates with various proteins in living cells including p130Cas (17), Pyk2 (18) and β-tubulin (7). Here, we demonstrated that endogenous p130Cas and nephrocystin are present in the nephrocystin-4 complex in HEK293 cells transiently expressing N4-fl-myc (Fig. 4A). Furthermore, we found that the nephrocystin-4/p130Cas association is stronger when the full-length nephrocystin is overexpressed and that this association is mediated by the N-terminal region of nephrocystin-4 because p130Cas, like nephrocystin, was poorly precipitated with the N-terminal truncated mutant N4-ΔN176-myc (Fig. 4A). These data suggest that p130Cas associates with nephrocystin-4 via nephrocystin. Given the weak expression of endogenous Pyk2 in HEK293 and HK2 cells (data not shown), we analyzed the endogenous association of nephrocystin-4 and Pyk2 in human kidney and brain extracts as well as in NPHP1−/− and NPHP1-TS cells. Our results revealed that nephrocystin-4 co-precipitates with Pyk2 in various tissue and cell extracts, including NPHP1−/− cells, indicating that their interaction does not require nephrocystin (Fig. 4B). Finally, we showed that nephrocystin-4 and nephrocystin associate with α-tubulin in polarized and ciliated tubular MDCK cells (Fig. 4C).

Nephrocystin-4 is localized in multiple subcellular compartments in MDCK cells

We examined the subcellular distribution of endogenous nephrocystin-4 in polarized MDCK cells. We detected peripheral staining and partial co-localization with β-catenin at cell–cell contact sites (Fig. 5A and B). The membrane-associated staining was strongest in the basolateral areas but weak signals were also seen in the apical region (Fig. 5B).

The peripheral staining coincides with microtubule localization (Fig. 5C). Nephrocystin-4 was also detected in primary cilia where it partially co-localized with α-tubulin (Fig. 5D). As described for nephrocystin and inversin (7), nephrocystin-4 staining was intense at the base of the cilia (Fig. 5D, arrowhead) and punctuated along the axoneme (Fig. 5D, insets). Cells that had not yet developed primary cilia, nephrocystin-4 was localized at the basal bodies (Fig. 5D, arrow).

In subconfluent MDCK cells with an extended microtubule network, nephrocystin-4 was dispersed throughout the cytoplasm. Conversely, in confluent cells, nephrocystin-4 co-localized with the microtubule organizing center (MTOC), as indicated by the double labeling with AP-N4 and either anti-α-tubulin (Fig. 5E) or anti-γ-tubulin antibodies (data not shown). These data showed a dynamic localization of nephrocystin-4 that coincides partially with microtubule distribution.

DISCUSSION

Nephrocystin and nephrocystin-4 are two proteins involved in familial juvenile nephronophthisis, an autosomal recessive tubulo-interstitial nephropathy characterized by cyst formation and renal fibrosis (8). Nephrocystin is an adaptor protein that associates with cell signaling and actin-binding molecules involved in cytoskeleton organization and cell adhesion regulation (16–18). It was also recently shown to associate with the microtubule component β-tubulin and to co-localize with inversin at the primary cilia of renal tubular epithelial cells (7). Although we previously demonstrated that nephrocystin and overexpressed nephrocystin-4 associate in living cells, the function and the subcellular localization of nephrocystin-4 remained unknown. Here, we report the endogenous expression of nephrocystin-4 in renal epithelial...
Figure 2. Requirement for the C-terminal residues of nephrocystin for the interaction with nephrocystin-4. (A) FLAG-tagged nephrocystin variants used in this study. The black areas flanking the SH3 domain indicate the glutamic acid-rich regions. The hatched and striped boxes correspond to the coiled-coil and homodimerization domains of nephrocystin, respectively. (B) Co-immunoprecipitation of N4-fl-myc with FLAG-tagged nephrocystin variants in NPHP1−/− cells. (left panel) Lysates were immunoprecipitated with the anti-c-Myc antibody and immunoblotted with AP-N1 and AP-N4 antibodies. Levels of FLAG-tagged nephrocystin proteins and N4-FL-myc in cell lysates are shown in the right panel. Arrows in the right panel indicate Flag-tagged nephrocystin variants that migrate close to non-specific proteins in cell lysates recognized by the AP-N1 antibody. Flag-N1-FL, Flag-N1-6A and Flag-N1-Δ376-480 variants co-precipitate with the N4-fl-myc. (C) In vitro pull-down assay. Various GST–nephrocystin fusion proteins and GST alone were incubated with lysates from NPHP1−/− cells transiently overexpressing N4-fl-myc. GST-N1-Δ376-480 variants co-precipitate with the N4-fl-myc. Samples were analyzed by immunoblotting using the AP-N4 antibody. An aliquot of cell lysate was used to control the expression level of the transiently expressed nephrocystin-4. N4-FL-myc binds to GST-N1-C251 and GST-N1-C131, but not to GST-N1-N212 or GST alone.
Figure 3. Regions of nephrocystin-4 involved in the interaction with nephrocystin. Co-immunoprecipitation of c-Myc-tagged nephrocystin-4 mutants and FLAG-tagged nephrocystin (Flag-N1-fl) or endogenous nephrocystin in HEK293 cells. Cell lysates were subjected to immunoprecipitation using the anti-c-Myc antibody. The immunoprecipitates were then immunoblotted with the AP-N1, AP-N4 and anti-c-Myc antibodies to reveal co-precipitated nephrocystin and overexpressed c-Myc-tagged nephrocystin-4 mutants. (A) c-Myc-tagged nephrocystin-4 variants used in this experiment. Black boxes correspond to the proline-rich region of nephrocystin-4. (B) Interaction between Flag-N1-fl and various nephrocystin-4 truncated mutants. All C-terminal truncated mutants interact with nephrocystin (left panel), whereas the N-terminal truncated mutants precipitate far less nephrocystin (right panel). The arrowhead indicates endogenous nephrocystin. (C) Interaction between endogenous nephrocystin and N-terminal truncated nephrocystin-4 mutants. Deletion of the N-terminal part of nephrocystin-4 dramatically reduces the interaction with endogenous nephrocystin. The N4-fl-myc protein was able to precipitate both the 80 kDa nephrocystin and an 83 kDa protein, probably the endogenous full-length nephrocystin. (D) In vitro pull-down assay. GST-N1-C131, containing the 131 last C-terminal residues of nephrocystin, and GST alone were incubated with HEK293 lysates transiently expressing N4-fl-myc, N4-ΔN176-myc and myc-N4-Q793X. N4-fl-myc and myc-N4-Q793X proteins are indicated by stars. Bound proteins were revealed using the anti-c-Myc antibody. Although expressed at low levels in the cell lysates, N4-fl-myc and myc-N4-Q793X clearly bind to GST-C-131, whereas the N4ΔN176-myc protein does not.
cells and in human kidney extracts, its ability to form complexes with nephrocystin, Pyk2 and p130Cas, and its co-localization with the microtubule cytoskeleton component α-tubulin, especially to the primary cilia and the MTOC in epithelial MDCK cells. The fact that nephrocystin and nephrocystin-4 were present at several specific sites in multifunctional complexes indicates that they are involved in various cell processes.

Nephrocystin has been described as a scaffolding protein able to recruit signaling molecules into specialized protein complexes. Its presence in nephrocystin-4-containing immunoprecipitates indicates its involvement in various cellular processes.

**Figure 4.** Presence of p130Cas, Pyk2 and α-tubulin in nephrocystin-4-containing immunoprecipitates. (A) Lysates from HEK293 cells transfected with N4-fl-myc or co-transfected with Flag-N1-fl and N4-fl-myc or N4-ΔN176-myc were submitted to immunoprecipitation using the anti-c-Myc antibody and to western blotting analysis with a monoclonal p130Cas antibody and with AP-N4 and AP-N1 antibodies. p130Cas is present in the nephrocystin/nephrocystin-4-containing complex and is poorly precipitated by N4-ΔN176-myc. (B) NPHP1+/− and NPHP1-TS cell lysates, human medullary kidney and mouse brain extracts were incubated with the AP-N4 antibody and the anti-ATF4 antibody as a control (IP control) and then immunoblotted with a Pyk2-specific antiserum. Pyk2 is present in the nephrocystin-4-containing complex. (C) Lysates from MDCK cells previously maintained at confluence for 10 days to allow polarization and cilia formation were incubated with the AP-N4 antibody, and then immunoblotted with α-tubulin and AP-N1 antibodies, demonstrating the presence of α-tubulin in the nephrocystin-4/nephrocystin complex.
complexes (16,18). Our results clearly show that nephrocystin-4 is part of the nephrocystin focal adhesion signaling complex. Although nephrocystin contains a coil-coiled domain and an SH3 domain, it interacts with nephrocystin-4 through a region located in its extreme C-terminal part. This association could involve uncharacterized domains located within this defined region or it might also require an intermediary partner. Nephrocystin is no longer targeted to the cell–cell junction area when

Figure 5. Localization of endogenous nephrocystin-4 in MDCK cells. (A–D) MDCK cells were grown on filters and maintained at confluence for 5–9 days before fixation to allow epithelial polarization and cilia formation. Confocal sections were examined after paraformaldehyde fixation and co-staining with rabbit polyclonal anti-nephrocystin-4 (green) and either mouse monoclonal anti-β-catenin (red) (A and B) or monoclonal anti-α-tubulin antibody (red) (C and D). (A and C) Sections were taken 3.6 μm above the most basal plane (indicated by an arrow in z-sections presented in (B)]. Insets show 2-fold magnifications; (B) z-sections show the subcortical plasma membrane distribution of nephrocystin-4. (D) Sections in the apical plane reveal nephrocystin-4 in basal bodies (arrow) and primary cilia, mostly at their base as indicated by an arrow-head; insets represent a 2-fold magnification, revealing a punctuated distribution of nephrocystin-4 along primary cilia. (E) MDCK cells cultured on coverslips were fixed and co-stained with rabbit polyclonal anti-nephrocystin-4 (green) and mouse monoclonal anti-α-tubulin (red). DNA was labeled with propidium iodide (blue). Nephrocystin-4 co-localizes with α-tubulin to the MTOC. Scale bars, 10 μM.
the last 76 residues are deleted (16), suggesting that binding to nephrocystin-4 is required for nephrocystin subcellular localization. Furthermore, we show that the N-terminal region of nephrocystin-4 is crucial for the interaction with nephrocystin. The residual interaction between the nephrocystin and the overexpressed N-terminal truncated nephrocystin-4 mutants indicates the existence of further nephrocystin-binding regions within nephrocystin-4. However, neither the proline-rich region located between residues 450 and 500 nor the C-terminal part of nephrocystin-4 seemed to modulate the interaction with nephrocystin. Thus, this residual association may occur via several intermediate partners present within the nephrocystin/nephrocystin-4 complex.

We detected several isoforms of both nephrocystin and nephrocystin-4. A 677 amino acid splicing variant of nephrocystin previously isolated from a kidney cDNA library (13) appears to be the main isoform produced and functional in renal cell lines and human kidney, in agreement with its conservation among species (16,24). In contrast, a 150 kDa variant of nephrocystin-4, expected to lack the nephrocystin-binding region, is only produced in the embryonic kidney cell line HEK293, raising questions about its potential role during kidney development.

The fact that nephrocystin-4 interacts with p130Cas and Pyk2 strengthens the hypothesis that the nephrocystin protein complex plays a crucial role in the regulation of cell adhesion signaling events. However, although the interaction between nephrocystin-4 and p130Cas seems to be mediated by nephrocystin, the association between nephrocystin-4 and Pyk2 does not require its presence, as these proteins co-precipitated even in nephrocystin-deficient cells. The association of nephrocystin-4 with these proteins and its subcortical localization in polarized epithelial cells close to the cortical actin cytoskeleton suggest that nephrocystin-4, as well as nephrocystin, is involved in actin cytoskeleton organization at sites of epithelial cell–cell adhesion and in cell–matrix adhesion, which is essential for the establishment and maintenance of differentiated polarized epithelial cells (16,18). However, the fact that nephrocystin-4 interacts with α-tubulin and co-localizes with microtubule cytoskeleton networks in polarized epithelial cells and with the MTOC in dividing cells suggests that nephrocystin-4 plays a role in the various cell processes relying on connections between actin and microtubules (reviewed in 25).

Recent studies have shown that the genes underlying a growing number of cystic kidney diseases encode proteins that are localized to the primary cilium of renal epithelial cells, suggesting that cilial dysfunction results in cyst formation (26). Primary cilia may sense environmental cues, such as tubular flow, which may in turn trigger calcium currents mediating various downstream signaling pathways, which could thus be altered in cystic diseases (27). Nephrocystin and inversin co-localize to the primary cilium of renal tubular cells. Inversin is mutated in infantile nephronophthisis, which is characterized by the features of juvenile nephronophthisis and polycystic kidney disease (7). Nephrocystin-3, involved in the adolescent form of nephronophthisis, also associates with nephrocystin and localizes to ciliated groups of cells (23). We demonstrate, here, that nephrocystin-4 localizes to the basal bodies, forming the base of the cilium, and along the axoneme of the cilia. The distribution of nephrocystin-4 along the axoneme is punctuated, which is reminiscent of the distributions of nephrocystin and inversin (3,7). The presence of nephrocystin proteins in primary cilium of kidney epithelial cells suggests that they play a common role in cilia function. However, nephrocystin is not essential for ciliogenesis, as NPHP1+/−/− cells can form cilia (data not shown). Similar findings have been obtained in mice lacking inversin, which present epithelial cells with normal-sized cilia (28). Thus, nephrocystins appear to participate in cilia function rather than structure.

Primary cilia arise from centrosomes, are resorbed during mitosis and are reassembled during interphase. The analysis of nephrocystin-4 localization revealed a dynamic expression pattern from the cilia to the centrosome, overlapping microtubule distribution. A similar distribution has been observed for inversin (3,7). These data suggest that the nephrocystin complex might also be involved in the different phases of the cell cycle.

The role of nephrocystins in cilia function may also explain the extrarenal abnormalities observed in juvenile nephronophthisis, such as retinal dystrophy. The structure of the cilium that connects the outer segment of photoreceptor cells to the basal body is very similar to that of the primary cilium. When intraflagellar transport proteins (e.g. KIF3A and polaris) are mutated in mice, transport of phototransduction molecules to the junction of the inner and outer segment is impaired, resulting in retinitis pigmentosa and blindness due to degeneration of photoreceptor cells (29,30). The nephrocystin proteins may also participate in intraflagellar transport pathways in photoreceptor cells. It has recently been shown that the BBS8 gene, which is mutated in some patients with Bardet–Biedl syndrome and shares some phenotypic features with nephronophthisis including retinal dystrophy and renal anomalies, encodes a protein present exclusively in the basal bodies and the centrosomes of ciliated cells (31). Interestingly, BBS8 is expressed in the connecting cilium of the retina (31).

These data suggest that, through their association with actin- and microtubule-based structures, the nephrocystins integrate signals related to cell adhesion with those controlling cell cycle progression through the cilia sensor, thus maintaining the integrity of the renal epithelium.

**MATERIALS AND METHODS**

**Antibodies**

Anti-nephrocystin-4 antibodies (AP-N4A and AP-N4) were generated in rabbits following injection of recombinant Histagged human nephrocystin-4 fragments (amino acids 1–176 and 673–1032, respectively). Antisera were affinity-purified using a HiTrap-NHS activated column coupled with the corresponding recombinant nephrocystin-4 proteins (AgroBio). The affinity-purified antibodies were used at a 1:3000 dilution for western blot analysis and a 1:10 dilution for immunofluorescence studies.

The other antibodies used for immunoprecipitation, western blot analysis and immunofluorescence were: anti-nephrocystin antibody directed against amino acids 2–113 (AP-N1) (14), anti-p130Cas (for immunoprecipitation) and anti-c-Myc-epitope
(9E10) antibodies (Santa Cruz Biotechnology), anti-Pyk2 and anti-p130Cas (for western blot) antibodies and anti-ß-catenin (BD Transduction Laboratories), anti-α-tubulin antibody (Molecular Probes), anti-γ-tubulin antibody, anti-FLAG M2 beads and TRITC-conjugated phalloidin (Sigma), horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies (Amersham Pharmacia Biotech).

Construction of mammalian and bacterial expression plasmids

The expression vector for FLAG-tagged full-length nephrocystin (Flag-N1-fl) was constructed by inserting a PCR-amplified fragment containing the full-length human NPHP1 cDNA (GenBank accession number AJ001815) into the EcoRI and BamHI sites of a FLAG-tagged expression vector (pCMV-FLAG) (Stratagene). The full-length nephrocystin mutant constructs were obtained by site-directed mutagenesis: Flag-N1-6A, which corresponds to an isoform of nephrocystin lacking residues 258–312 (13), Flag-N1-212X, Flag-N1-481X and Flag-N1-653X, which lack the C-terminal 520, 251 and 79 residues, respectively, and Flag-N1-D376-480, deleted for the residues required for nephrocystin homodimerization (16). The full-length c-Myc-tagged nephrocystin-4 construct, N4-fl-myc, was obtained by inserting a 5’-RACE–PCR-amplified fragment (amino acids 1–309 of human NPHP4 cDNA) (GenBank accession number AY118228) in frame into the HindIII site of a c-Myc-tagged nephrocystin-4 construct (residues 310–1426) previously cloned into the pcDNA-Myc-His expression vector (Clonetech) (14). N4-fl-myc-based plasmids for the expression of N- and C-terminal c-Myc-tagged nephrocystin-4 deletion mutants were constructed by site-directed mutagenesis: myc-N4-Q793X (corresponding to a mutation found in one family with juvenile nephronophthisis) (14) and myc-N4-E1124X lack the C-terminal 633 and 302 residues, respectively; N4-ΔN176-myc and N4-ΔN309-myc lack the N-terminal 176 and 309 residues and start at the second and the third in-frame methionines (M177 and M310), respectively; myc-N4-ΔN176-ΔPro lacks the N-terminal 176 residues and the proline-rich region (residues 458–514). Partial NPHP1 cDNA sequences were amplified by PCR using the Flag-N1-fl construct as a template and subcloned into the pGEX4T-1 and pGEX5X-2 vectors (Amersham Pharmacia Biotech) for expression as GST-tagged fusion proteins in Escherichia coli: GST-C-251, GST-C-131, (nephrocystin residues 481–732 and 601–732, respectively) and GST-N-212 (residues 1–212 corresponding to the coiled-coil and SH3 domains of nephrocystin).

Generation of human renal tubular epithelial cell lines

Clean catch urine was obtained from a patient affected with nephronophthisis bearing a homozygous deletion of the NPHP1 gene (32). Cells were collected and cultured as described in Racusen et al. (33). Just before confluency, cells were transduced with a recombinant retrovirus expressing the SV40 large T antigen (34), yielding immortalized cells named NPHP1−/− cells. The NPHP1−/− cell line expressed epithelial-specific antigens (cytokeratin, type IV collagen) but no mesenchymal antigens (alpha-smooth muscle actin and vimentin). This cell line was positive for alkaline phosphatase and gamma-glutamyl transpeptidase activities, specific to the proximal renal tubule. To obtain a cell line that re-expressed nephrocystin, NPHP1−/− cells were transduced with a lentiviral vector containing the full-length NPHP1 cDNA sequence, which had been inserted into the BamHI site of pRMES-CMV (pRMES-CMV-NPHP1). The lentiviral vectors pSIV15 (encoding the Gag, Pol and Tat proteins), pG-Rev (encoding the VSV-G and Rev proteins) and pRMES-CMV were kindly provided by P. Moullier (Vector Core of the University Hospital of Nantes, France). The lentivirus was produced by transient calcium-phosphate cotransfection of HEK293 cells with 5 µg of pG-Rev, 15 µg of pSIV15 and 20 µg of pRMES-CMV-NPHP1 or 20 µg pRMES-CMV-GFP as a control. The supernatant was harvested, centrifuged to remove cellular debris and filtered. Viral supernatant (500 µl containing 10⁶ transduction units of infectious particles) was used to infect 2×10⁴ NPHP1−/− cells. Typically, >90% of the transduced cells expressed the NPHP1 transgene after two repetitive infections. The resulting cells were named NPHP1-TS.

Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 10% FCS. MDCK cells type II (gift from M. Caplan, Yale University, CT, USA) were grown in Eagle’s Minimum Essential Medium supplemented with 10% FCS. HK2 (human proximal tubular epithelial) (35), NPHP1−/− and NPHP1-TS cells were grown in the medium described in Racusen et al. (33) supplemented with 1% FCS. All cells were transfected with the different plasmids using FuGENE™6 (Roche Applied Science), except HEK293 cells, which were transfected by the calcium-phosphate method. Cells were cultured for 2 days prior to harvesting for co-immunoprecipitation studies.

Immunoprecipitation experiments

Human renal tissues were obtained from normal poles of kidneys removed for renal carcinoma. Tissues were crushed in liquid nitrogen and homogenized in lysis buffer (1% Triton, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl and 1 mM sodium orthovanadate) with complete protease inhibitor cocktail (Roche Applied Science). Cells were harvested and lysed for 10 min with lysis buffer. Insoluble debris was removed by centrifugation at 14 000g for 15 min. Co-immunoprecipitation was performed as previously described (14). Supernatants (~1 mg total proteins) were incubated with 2–10 µg of antibody for 1–3 h, followed by 40 µl of protein A–Sepharose for 1 h. Western blotting was performed with the indicated antibodies.

GST fusion protein pull-down assay

GST fusion proteins were expressed in the E. coli strain BL21 (Novagen) and purified by glutathione–agarose affinity chromatography according to the manufacturer’s instructions (Amersham Pharmacia Biotech). GST (10 µg) and the GST
fusion protein coupled to glutathione beads (Sigma) were incubated for 1 h at 4°C with cell lysates (∼500 µg total proteins). The resin was washed three times with lysis buffer and then analyzed by immunoblotting using the indicated antibodies. Lysates containing 50 µg of proteins were loaded onto gels as positive controls.

**Immunofluorescence**

MDCK cells were either grown on glass coverslips before confluence or plated at 10³ cells/well on 12 mm Transwell filters (Costar) and cultured for 5 days post-confluence. Cells were fixed in 4% paraformaldehyde in PBS for 15 min. The fixation reaction was quenched in 50 mM NH₄Cl dissolved in PBS and blocked successively with 10% donkey serum, 1% BSA, 0.5% Tween-20 in PBS, avidin blocking solution and then biotin blocking solution (Vectorshield, Vector Laboratories). Cells were co-stained for 1 h and then incubated with appropriate fluorescent secondary antibodies. To enhance anti-nephrocystin-4 signal, cells were incubated with biotin-labeled anti-rabbit antibodies (DakoCytomation) and then with Alexa 488-streptavidin (Molecular Probes). In some experiments, DNA was labeled with propidium iodide. Confocal images were taken using a ZEISS LSM 510 laser scanning microscope system with a X63.2 objective (Carl Zeiss North America, Thornwood, NY, USA). When cells were fixed and permeabilized with cold methanol, no anti-nephrocystin-4 immunoreactivity could be observed in mitotic spindles, preventing us from using this improved method to visualize the microtubule network.

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