Polycystin-2 traffics to cilia independently of polycystin-1 by using an N-terminal RVxP motif

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Summary

Primary cilia play a key role in the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD). The affected proteins, polycystin-1 (PC1) and polycystin-2 (PC2), interact with each other and are expressed in cilia. We found that COOH-terminal truncated PC2 (PC2-L703X), lacking the PC1 interaction region, still traffics to cilia. We examined PC2 expression in several tissues and cells lacking PC1 and found that PC2 is expressed in cilia independently of PC1. We used N-terminal deletion constructs to narrow the domain necessary for cilia trafficking to the first 15 amino acids of PC2 and identified a conserved motif, R_6VxP, that is required for cilial localization. The N-terminal 15 amino acids are also sufficient to localize heterologous proteins in cilia. PC2 has endogenous cilia trafficking information and is present in cilia of cells lining cysts that result from mutations in *PKD1*.

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Introduction

The primary cilium has taken center stage in a broad class of human diseases with pleiotropic manifestations that often include cyst formation and fibrosis in the kidney and liver (Pazour, 2004; Zhang et al., 2004). Of these diseases, autosomal dominant polycystic kidney disease (ADPKD) is the most common and invariably manifests with cysts in the kidney. The respective *PKD1* and *PKD2* gene products associated with ADPKD, polycystin-1 (PC1) and polycystin-2 (PC2), are integral membrane proteins that localize in the plasma membrane overlying the cilial axoneme of kidney tubule epithelial cells (Pazour et al., 2002; Yoder et al., 2002a). The single cilium on the luminal (apical) surface of these cells functions as a sensory organelle (Pan et al., 2005) and PC1 and PC2 form part of the signal transduction pathway in this structure. The precise nature of the extracellular signal is uncertain but a role for mechanical fluid shear stress has been proposed (Praetorius and Spring, 2001; Nauli et al., 2003). PC1 has structural features of a cell surface receptor and undergoes proteolytic cleavage that is required for its function (Qian et al., 2002; Chauvet et al., 2004). PC2 is a cation channel belonging to the TRP channel family (Mochizuki et al., 1996; Gonzalez-Perrett et al., 2001; Koulen et al., 2002). PC1 physically associates with PC2 (Qian et al., 1997; Tsiokas et al., 1997; Hanaoka et al., 2000) and Ca^{2+} is involved in the intracellular signaling steps from this complex (Nauli et al., 2003; McGrath et al., 2003).

Although inherited as a dominant trait, cyst formation in ADPKD occurs after somatic second step mutations (Qian et al., 1996; Wu et al., 1998a). Cyst cells lack functional forms of either PC1 or PC2, depending on the genotype of the affected family. Several lines of evidence support the functional inter-relationship of PC1 and PC2 in the kidney, liver and pancreas. The spectrum of clinical features of human ADPKD is the same for *PKD1* and *PKD2* except that the former is more severe at any given age (Hateboer et al., 1999). $Pkd1^{-/-}$ and $Pkd2^{-/-}$ mice as well as the respective heterozygous mice have very similar kidney, liver and pancreas phenotypes (Lu et al., 1997; Lu et al., 2001; Wu et al., 2000; Wu et al., 2002; Kim et al., 2000). Mutations in the orthologous genes in *C. elegans*, *lov-1* and *pkd-2*, result in male mating defects that are identical to each other (Barr et al., 2001). By contrast, compound heterozygous $Pkd1^{+/-}$:$Pkd2^{+/-}$ mice differ from singly heterozygous animals by a mild extra-additive effect in cyst formation (Wu et al., 2002), raising the possibility of a partial epistatic relationship between these genes. Outside of the kidney, liver and pancreas, there is more striking evidence of functional divergence for PC1 and PC2. $Pkd2^{-/-}$ mice develop defects of left-right axis formation with associated heart defects reflective of heterotaxy syndrome (Pennekamp et al., 2002; Wu et al., 2000). This results from failure to express functional PC2 in nodal cilia (McGrath et al., 2003) and is not seen in $Pkd1^{-/-}$ mice (S.S., X.T. and S.S., unpublished observations), separating PC2 from PC1 in the nodal cilial pathway.

Under non-ciliated conditions, PC2 expression is confined to the endoplasmic reticulum (Cai et al., 1999) where it can release Ca^{2+} from intracellular stores (Koulen et al., 2002).

Truncation of the cytosolic COOH terminus allows trafficking of the peptide to the plasma membrane where it acquires resistance to endoglycosidase H (Endo H) and can be biotinylated in living cells (Cai et al., 1999; Cai et al., 2004). Heterologously expressed, epitope-tagged PC2 traffics into cilia once they form in post-confluent monolayers but the full-length epitope-tagged protein still cannot be detected on the remainder of the plasma membrane (Cai et al., 2004). Although a cell surface location of endogenous PC2 has been reported using a variety of native protein antisera (Luo et al., 2003; Scheffers et al., 2004; Li et al., 2005), none of these studies showed expression of epitope tagged PC2 in the plasma membrane. This has led to some controversy regarding PC2 expression in the plasma membrane outside cilia (reviewed by Witzgall, 2005). The domain responsible for restricting PC2 to the ER and cilia is a stretch of acidic residues containing a casein kinase 2 phosphorylation site at S812 (Cai et al., 1999; Cai et al., 2004; Kottgen et al., 2005). Loss of phosphorylation at S812 does not abrogate cilial localization of PC2 (Cai et al., 2004). An interdependence with PC1 for PC2 trafficking to the cell surface was inferred from the presence of a novel channel conductance in cells over-expressing both proteins, although the identity of that conductance with the PC2 channel was never demonstrated (Hanaoka et al., 2000). More recently, native PC2 was deemed to be absent from cilia of cultured epithelial cells lacking functional PC1 (Nauli et al., 2003).

A role for protein trafficking in the pathogenesis of polycystic disease has been suggested by the discovery of two genes for polycystic liver disease whose products function in ER maturation and quality control of integral membrane and secreted proteins (Li et al., 2003; Drenth et al., 2003; Davila et al., 2004). Mutations in the genes encoding the β subunit of the glucosidase II enzyme (Li et al., 2003; Drenth et al., 2003) or the SEC63 component of the protein translocon (Davila et al., 2004) lead to bile duct cysts histopathologically indistinguishable from those seen in ADPKD. Given the primacy of cilia in the pathogenesis of cyst formation, we have hypothesized that a second step mutation in either of these genes in mature bile duct cells results in failure to properly mature integral membrane or secreted proteins including those necessary in the cilial polycystic disease pathway(s) (Davila et al., 2004). A corollary to this hypothesis is that a subset of amino acid substitution mutations in proteins such as PC1, PC2 or polyductin (the autosomal recessive polycystic disease protein) are pathogenic as a result of aberrant trafficking to cilia.

There is now general agreement supporting the functional role of PC1 and PC2 in cilia. Many of the trafficking studies for both proteins were performed before the roles of cilia were widely appreciated. We sought to examine the mechanism for cilia trafficking of PC2 with the view that this is central to pathogenesis of polycystic diseases. Specifically we set out to test the hypothesis that PC2 has endogenous cilial trafficking information within its primary sequence and that this trafficking is independent of PC1. We based these hypotheses on our observation that COOH-terminal truncated forms of PC2 that do not interact with PC1 still traffic to cilia. We found that the motif, R_6VXP, at the N-terminus of PC2 is necessary for cilial location of the protein. We also found that the first 15 amino acids of PC2 are sufficient to localize heterologous proteins to cilia that do not otherwise traffic there. Finally, we examined PC2 expression in cells lacking PC1 and found that both native and epitope-tagged over-expressed full-length PC2 are expressed normally in cilia. The implications of these studies are that PC2 is present in cilia of cells lining cysts of patients with mutations in *PKD1*. Therapeutic strategies that make use of channel agonists or antagonists targeted directly at PC2 should prove effective in altering the course of polycystic kidney disease in patients with mutations in *PKD1*.

Results

PC2 trafficking to cilia is independent of PC1

Full-length PC2 is retained in the ER in cultured epithelial cells in the non-ciliated state. Once cells form cilia, in addition to its ER location, full-length epitope-tagged PC2 traffics to the plasma membrane overlying primary cilia in both LLC-PK1 and MDCK epithelial cells. PC2 is not detected on the remainder of the cell surface plasma membrane (Cai et al., 1999; Koulen et al., 2002; Cai et al., 2004). Truncation of the COOH terminus of PC2 upstream of amino acid E787 results in trafficking of the residual protein to the general cell surface even before cilia form (Cai et al., 1999; Koulen et al., 2002). For the current study, we determined that addition of a COOH-terminal EGFP fusion to full-length PC2 does not alter cilial trafficking properties of the full-length protein (Fig. 1A). The truncation mutant, PC2-L703X, lacking the bulk of the COOH terminus including the putative PC1 interaction domain (Qian et al., 1997; Tsiokas et al., 1997) also shows robust expression in cilia in both LLC-PK1 and MDCK cells (Fig. 1B). Previous studies had suggested that co-assembly with PC1 is required for surface expression of PC2 (Hanaoka et al., 2000) and that PC2 is not expressed in cilia in the absence of PC1 (Nauli et al., 2003). The specific trafficking of full-length PC2 to cilia and the ability of the truncated PC2-L703X form to also achieve this localization led us to investigate whether full-length PC2 can traffic to cilia independently of PC1.

To test whether PC1 is necessary for localization of PC2 to cilia, we examined PC2 trafficking in the absence of PC1 in several experimental systems. We found that native PC2 is expressed in nodal cilia of *Pkd1*$^{-/-}$ mice, indicating that in nodal cilia, PC2 traffics to cilia independently of PC1 (Fig. 2A). Similarly, in cells lining kidney cysts formed by inactivation of *Pkd1* (S.S., X.T. and S.S., unpublished observations), cilia express PC2 (Fig. 2B) in a pattern indistinguishable from that seen in non-cystic tubule cells. PC2 is also expressed in cilia of *Pkd1* null cell lines (Fig. 2C) constructed from our mice (Z.Y., X.T. and S.S, unpublished observations) (Chauvet et al., 2004). Finally, we expressed full-length PC2 as a COOH-terminal EGFP fusion protein in *Pkd1*$^{-/-}$ cells and found EGFP epifluorescence in the cilia (Fig. 2D). The data indicate that PC2 localizes in cilia in the absence of PC1.

An N-terminal domain necessary for cilial location of PC2

We sought to define the domains of PC2 that direct its specific trafficking properties. For this purpose, we produced a series of deletion and fusion constructs of human PC2 (Fig. S1 in supplementary material). Deletion of amino acids 5-72 resulted in loss of cilial location of the COOH-terminal truncated peptide Δ(5-72)PC2-L703X (Fig. 3A). By contrast, deletion of amino acids 72-130 or 130-220 had no effect on trafficking to

cilia of the respective Δ(72-130)PC2-L703X and Δ(130-220)PC2-L703X peptides (Fig. 3B,C). We excluded the possibility that absence from the cilia resulted from differences in the expression of the mutant peptides. Robust expression of the proteins deleted of amino acids 5-72 was seen in cell body using confocal microscopy (Fig. 3A, right) and the proteins showed the expected expression and migration in SDS-PAGE of total cell lysates detected by immunoblotting anti-HA epitope antibodies (Fig. 4C).

Since the PC2-L703X backbone lacks the COOH terminus of PC2, we considered the possibility that the intact COOH terminus of PC2 may override the role of the N-terminus in localizing full-length PC2 to cilia. In contrast to full-length PC2 (Fig. 1), the Δ(5-72)PC2 construct deleted for amino acids 5-72 but containing the intact COOH terminus failed to traffic into cilia (Fig. 3D). The finding that (Δ5-72)PC2 protein does not traffic into cilia, indicates that N-terminal sequences are essential for cilial localization and that the capacity to interact with PC1 via the COOH terminus is not sufficient to deliver PC2 to cilia.

Subcellular trafficking of N-terminal domain mutants of PC2

We examined the differences in the intracellular trafficking of PC2-L703X and Δ(5-72)PC2-L703X. The proteins both showed a perinuclear and cytosolic expression pattern (Fig. 4A,B) and co-localization with calnexin (data not shown) indicative of location in the ER. PC2-L703X also showed partial co-localization with the Golgi marker GM130 (Fig. 4A) (Nakamura et al., 1995). The Δ(5-72)PC2-L703X form did not co-localize with this Golgi marker (Fig. 4B), indicating that deletion of the N-terminal domain of PC2 results in failure to traffic into the Golgi.

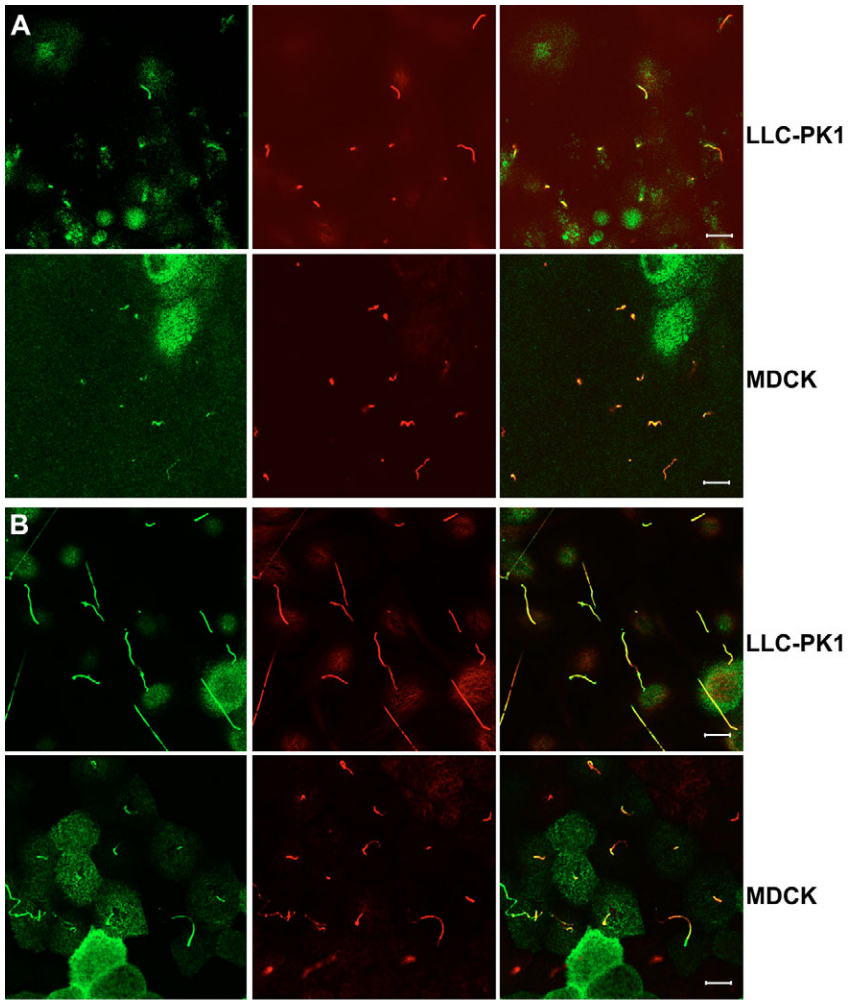

Fig. 1. Trafficking of full-length and truncated PC2 to cilia. (A) Expression of full-length PC2 with a COOH-terminal EGFP tag in the cilia of LLC-PK1 (top panel) and MDCK (bottom panel) cells. (B) Expression of PC2-L703X with a COOH-terminal EGFP in the cilia of LLC-PK1 (top panel) and MDCK (bottom panel) cells. Cells expressing PC2-L703X form longer cilia more readily than cells expressing only native PC2 or the full-length PC2 construct. (Left panels) EGFP epifluorescence (green) shows expression of PC2 , (middle panels) acetylated α-tubulin (red) marks the cilia; (right panels) merged images. Bars, 10 μm.

We used glycosylation analysis in ciliated cells to further examine trafficking of these proteins (Fig. 4C,D). Normally, the fraction of PC2-L703X that is expressed on the cell surface acquires resistance to endoglycosidase H (Endo H) as it passes through the middle Golgi on its way to the plasma membrane. The Endo H-resistant species appears as a slower migrating band in immunoblots (Fig. 4C,D) (Cai et al., 1999; Cai et al., 2004). It is important to emphasize that forms of PC2 with the COOH terminus deleted that traffic to cilia (e.g. PC2-L70X, Δ(72-130)PC2-L703X and Δ(130-220)PC2-L703X) also traffic to the general plasma membrane outside the cilia and do so even in the absence of cilia formation (see below) (Cai et al., 1999; Cai et al., 2004)]. By contrast, PC2 with an intact COOH terminus is only expressed on the cell surface once cells form cilia, and then is only found in the plasma membrane overlying the cilia and not expressed in the remainder of the cell surface. Therefore, there are far greater amounts of the truncated forms of PC2 on the cell surface than of the intact protein, even in ciliated cells (see below). Since passage through the middle Golgi is required to develop Endo H resistance, mutant forms of PC2 that reach the cell surface are expected to acquire Endo H resistance. The Δ(5-72)PC2-L703X form of PC2 is expected to remain completely sensitive to Endo H digestion since it does not traffic into the Golgi or reach the cell surface. We found that PC2-L703X, Δ(72-130)PC2-L703X and Δ(130-220)PC2-L703X acquire Endo H resistance (Fig. 4D). By contrast, the N-terminal deleted Δ(5-72)PC2-L703X protein did not acquire Endo H resistance (Fig. 4D). This is consistent with the observed failure of Δ(5-72)PC2-L703X to reach the Golgi compartment (Fig. 4A,B) and the cilia (Fig. 3A,D) and supports the conclusion that deletion of amino acids 5-72 of PC2 results in retention of the protein in pre-Golgi compartments.

We used surface biotinylation in living cells to assess cell

surface expression of PC2 in ciliated cells. We had previously found absence of detectable surface-biotinylated PC2 in non-ciliated cells (Cai et al., 1999; Cai et al., 2004). We now sought to determine if surface-biotinylated PC2 is detectable in ciliated cells. Full-length PC2 with an epitope tag traffics to cilia (Fig. 1) (Cai et al., 2004), yet we were unable to detect biotinylated full-length PC2 on either the apical or basolateral surface of living, polarized, ciliated epithelial cells (Fig. 4E, left panel). Full-length PC2 also does not have detectable Endo H resistant forms (Cai et al., 1999; Koulen et al., 2002; Cai et al., 2004). We conclude that cell surface biotinylation and Endo H resistance do not have sufficient sensitivity to detect glycoproteins such as PC2 whose surface expression is restricted only to cilia. The most probable interpretation of our data is that surface expression of full-length PC2 is restricted to the plasma membrane compartment overlying cilia in ciliated cells and that PC2 does not otherwise traffic to the general plasma membrane in epithelial cells.

Removal of the COOH terminus allows PC2-L703X protein to traffic to the general plasma membrane as well as the cilia. As a result, PC2-L703X is biotinylated on both the apical and basolateral surfaces in polarized, ciliated cells (Fig. 4E, middle panel) (Cai et al., 1999; Cai et al., 2004). Only the slower migrating, Endo H-resistant form of PC2-L703X is biotinylated, confirming the interpretation that the Endo H-resistant form of PC2-L703X represents the fraction of the protein on the cell surface (Fig. 4D,E). Despite absence of the COOH terminus, no detectable biotinylated form of Δ(5-72)PC2-L703X is seen in ciliated polarized cells (Fig. 4D, right panel). In contrast to full-length PC2, the absence of detectable surface biotinylation and Endo H resistance in Δ(5-72)PC2-L703X is coupled with absence of detectable immunofluorescent signal in cilia. Taken together, this shows that Δ(5-72)PC2-L703X is not expressed on the plasma membrane at all, even when cells form cilia. We conclude that amino acids 5-72 are necessary for trafficking of PC2 to the plasma membrane and cilial compartments.

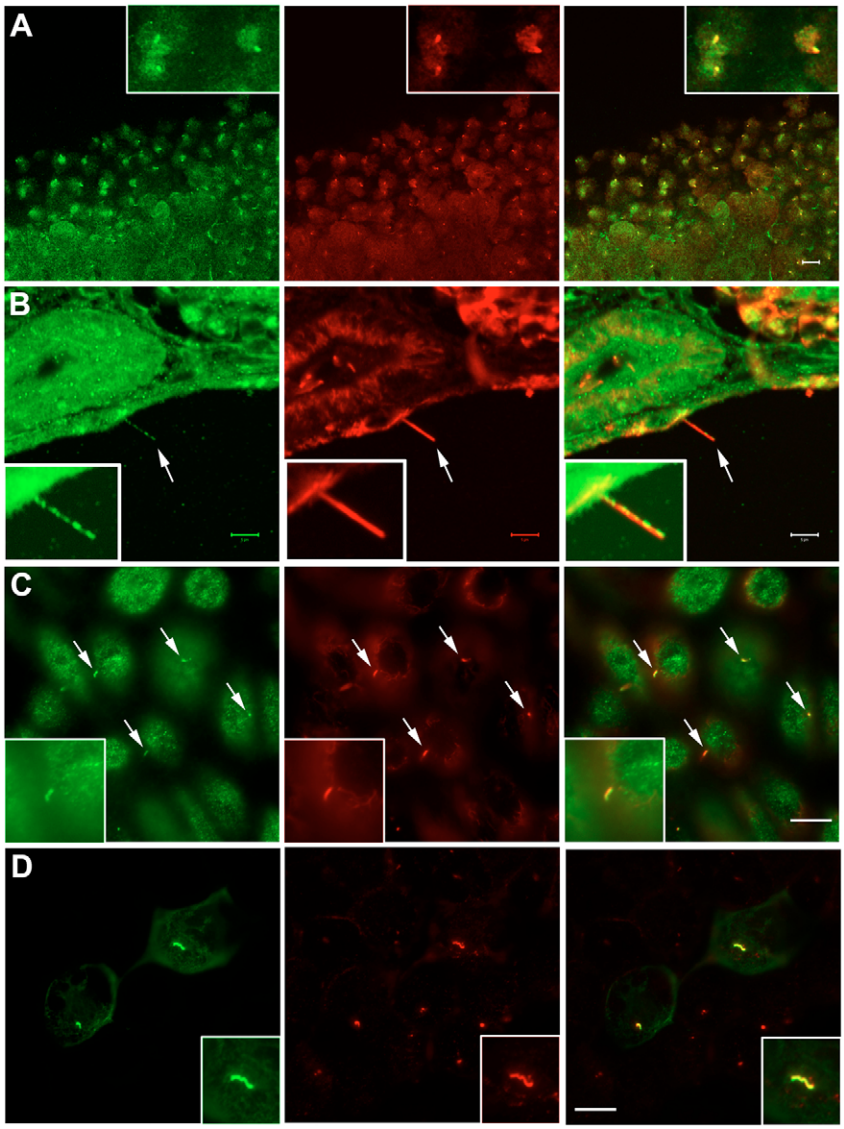

Fig. 2. PC2 traffics to cilia independently of PC1. (A) PC2 is expressed in cilia at the embryonic node in *Pkd1*$^{-/-}$ mice. (B) PC2 is expressed in cilia (arrow) of cells lining kidney cysts formed in *Pkd1* mutant mice. (C) PC2 is expressed in the cilia (arrows) in cultured mouse kidney cells lacking PC1. (D) PC2-GFP fusion protein expressed in cells lacking PC1 traffics into cilia as evidenced by EFFP epifluorescence in cilia. Adjacent cells not expressing the transfected protein do not show EGFP epifluorescence. (A-C) Anti-PC2 (YCC2), green; (D) EGFP epifluorescence, green; (A-D) anti-acetylated α-tubulin, red; right panels, merged images. Bars, A, C, D, 10 μm; B, 5 μm. Insets show selected cilia in each field.

The PC2 homolog, PKD2L1, is not expressed in cilia in epithelial cells

We evaluated whether cilial location is a general property of the polycystin class of TRP channels (TRPP). PC2 and PKD2L1 have ~60% identity and 80% similarity over the region between the first and sixth membrane spans (the TRP channel region) and have 40% identity and 60% similarity in the first 200 amino acids of their cytosolic COOH-termini. PC2 and PKD2L1 have no similarity in the N-terminus and diverge significantly in the remainder of their respective COOH-termini. We reasoned that these proteins are similar in structure and both function as Ca^{2+} channels. We sought to examine the specificity of the N-terminal trafficking domain of PC2 by comparing it with the trafficking of PKD2L1.

We completed the cloning of the 5′ end of our partial *PKD2L1* sequence (Wu et al., 1998b) and identified a novel splicing pattern from exon 1 to exon 2. This splice occurred at a site 22 base pairs upstream of the exon 2 splice acceptor site reported by Nomura et al. (Nomura et al., 1998). Our novel variant extended exon 2 in the 5′ direction and changed the predicted in-frame translation start site in exon 1 (Fig. S2 in supplementary material). As a result, the first 78 amino acids

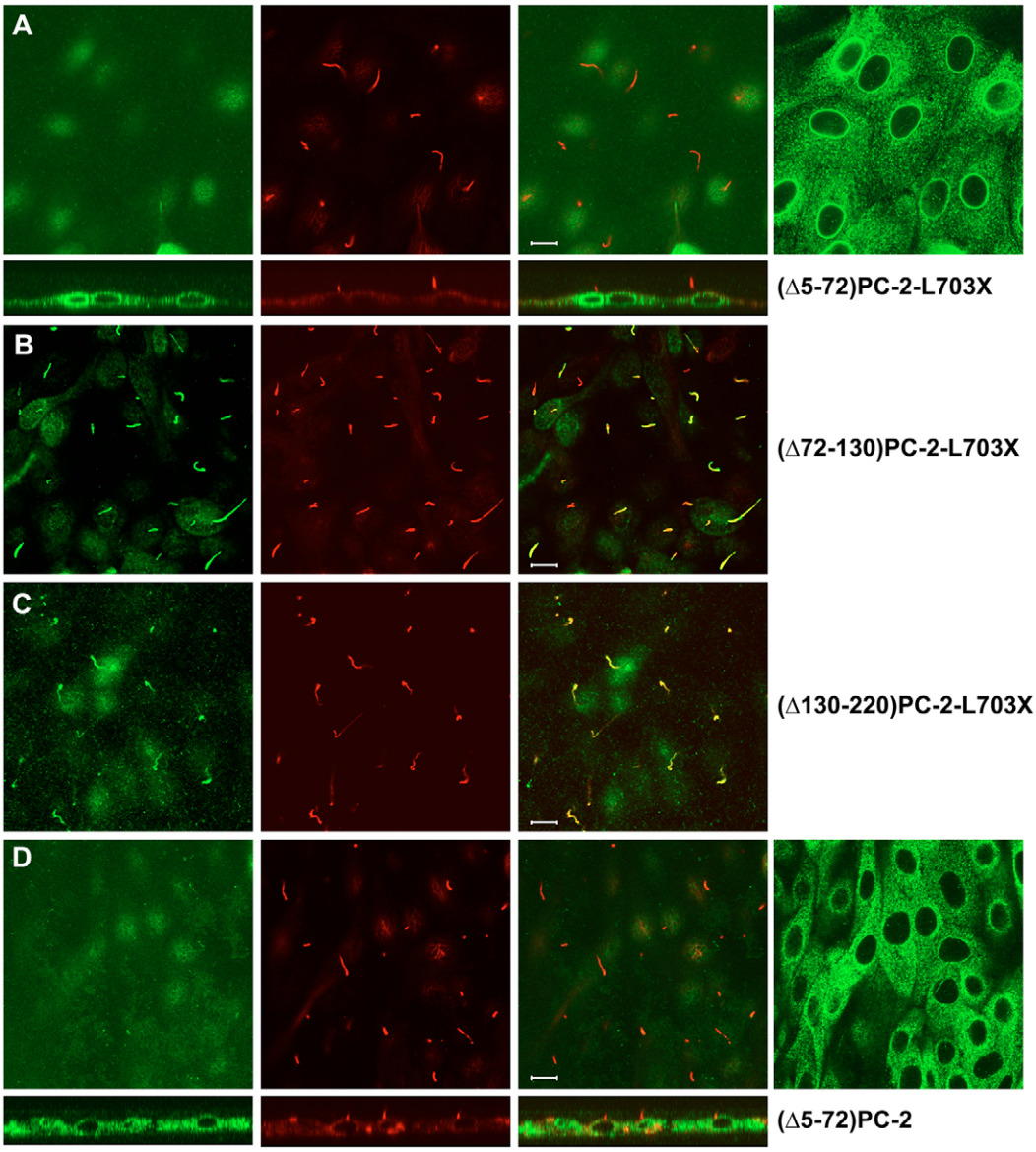

Fig. 3. Amino acids 5-72 of PC2 are necessary for its trafficking to cilia. Deletion of amino acids 5-72 in either (A) the PC2-L703X truncated peptide [(Δ5-72)PC2-L703X] or (D) the full-length PC2 [(Δ5-72)PC2] with an intact COOH terminus results in PC2 absence in the cilia. Robust expression of the respective proteins at the level of the cell body is shown in the right-most panel as well as in the *xz*-plane reconstructions in these confocal images. (B,C) Deletion of amino acids (B) 72-130 [(Δ72-130)PC2-L703X] or (C) 130-220 [(Δ130-220)PC2-L703X] does not abrogate trafficking of the respective peptides into cilia. Anti-HA epitope (green) indirect immunofluorescence indicates expression of PC2 constructs and acetylated α-tubulin (red) marks the cilia; right panels, merged images. The cell lines were made in LLC-PK1. Bars, 10 μm.

of the previously reported PKD2L1 sequence (GenBank accession no. NM016112) are replaced by a novel 31 amino acid sequence (GenBank accession no. DQ084244); the remainder of the protein is unchanged. Using direct sequencing of RT-PCR products from multiple tissues formed with closely spaced PCR primers flanking the exon 1-2 splice site, we did not find evidence for the previously reported splicing pattern between exons 1 and 2 (Fig. S2 in supplementary material). Unless otherwise noted, we used PKD2L1 expression constructs with the newly defined N-terminal sequence in our studies.

PKD2L1 with either EGFP or triple-HA COOH-terminal epitope tags was only detected in the cytoplasm and perinuclear areas of LLC-PK1 cells stably expressing the proteins. PKD2L1 does not traffic to cilia in polarized, ciliated LLC-PK1 cells (Fig. 5B,C). We also engineered PKD2L1 with the N-terminal sequence described by Nomura et al. (Nomura et al., 1998) and found that this form of PKD2L1-EGFP also does not traffic to cilia (Fig. S3 in supplementary material). PKD2L1 does not acquire Endo H resistance, consistent with the finding that it does not traffic to the cell surface in epithelial cells (Fig. 5C, left panel). Cell surface biotinylation in living LLC-PK1 and CHO-K1 cells similarly produced no evidence of PKD2L1 on the cell surface in either cell type (Fig. 5C, middle panel). Finally, we constructed a truncation of PKD2L1, PKD2L1-L524X (Fig. S1 in supplementary material), removing the predicted cytosolic COOH terminus to produce a protein analogous to PC2-L703X. PKD2L1-L524X was also not biotinylated on the cell surface (Fig. 5C, right panel) and did not have a cell surface pattern of immunofluorescent cell staining (data not shown). Altogether, these data indicate that PKD2L1 does not contain plasma membrane or cilia targeting information in its primary sequence and does not normally traffic to the plasma membrane.

We evaluated the channel function of PKD2L1 in LLC-PK1 cells. Since this protein was not expressed on the cell surface, we assayed its ability to release Ca^{2+} from intracellular stores

in the absence of extracellular Ca^{2+} in a manner similar to that of PC2 (Koulen et al., 2002) (Fig. S4 in supplementary material). PKD2L1 was able to transiently increase cytosolic Ca^{2+} in response to AVP stimulation in a manner similar to PC2, suggesting PKD2L1 can function as a Ca^{2+} channel in this assay system (Koulen et al., 2002; Chen et al., 1999; Rundle et al., 2004).

The N-terminal domain of PC2 is sufficient to direct trafficking to cilia

We next tested whether the N-terminal 72 amino acids of PC2 are sufficient to direct proteins to cilia that do not normally go there. We replaced the initiation codon of PKD2L1 with codons 1-72 of PC2 to make the chimeric protein (1-72-PC2)-PKD2L1 that contained the first 72 residues of PC2 and the entire PKD2L1 sequence (Fig. S1 in supplementary material). This chimeric protein trafficked into cilia of epithelial cells (Fig. 6A). To further confirm that the N-terminus of PKD2L1 did not contain a latent cilia targeting motif, we produced a chimeric protein in which we replaced amino acids 1-328 of PC2-L703X with the corresponding region (amino acids 1-230) of PKD2L1 [PKD2L1-Δ(1-328)PC2-L703X; Fig. S1 in supplementary material]. The chimeric region contained the entire cytosolic N-terminus, the first transmembrane span and part of the first extracellular loop of PKD2L1, replacing the comparable region of PC2-L703X. This chimeric protein failed to traffic to cilia (Fig. 6B). When amino acids 1-72 of PC2 were added

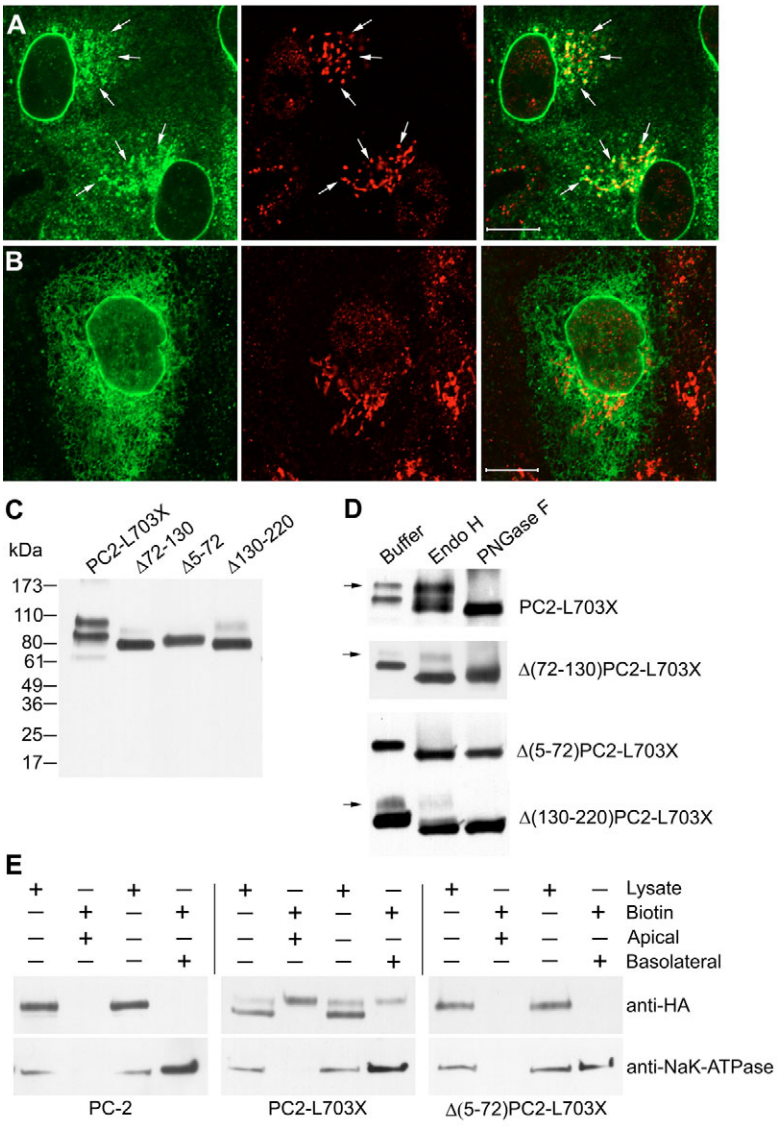

Fig. 4. Aberrant trafficking of N-terminal mutant forms of PC2. (A) PC2-L703X (anti-HA, green) shows partial co-localization (arrows) with the Golgi marker GM130 (red) in LLC-PK1 cells. (B) Δ(5-72)PC2-L703X (anti-HA, green) shows a typical ER pattern of expression but does not co-localize with GM130 (red), indicating that the protein does not traffic to the Golgi. Bars, 10 μm. (C) Immunoblot using anti-HA on lysates of LLC-PK1 cell lines stably expressing PC2-L703X or its respective N-terminal deleted forms (Δ72-130)PC2-L703X, (Δ5-72)PC2-L703X and (Δ130-220)PC2-L703X. Each cell line shows similar expression levels of the respective proteins, indicating comparable stability of the various deleted forms (20 μg of protein from total cell lysate in each lane). (D) Immunoblots using anti-HA of lysates incubated either with enzyme buffer alone or Endo H or PNGase F. PC2-L703X, (Δ72-130)PC2-L703X and (Δ130-220)PC2-L703X have slower migrating species (arrows) that show resistance to Endo H as indicated by the persistence of the bands after Endo H digestion. The Endo H resistance indicates that these peptides traffic past the middle Golgi. By contrast, (Δ5-72)PC2-L703X does not have an Endo H-resistant component, suggesting that it does not traffic past the middle Golgi. The latter finding is consistent with absence of co-localization with the Golgi marker GM130 (B). All proteins are completely sensitive PNGase F indicating the migration of the deglycosylated peptide backbone. (E) Expression of PC2, PC2-L703X and (Δ5-72)PC2-L703X on the cell surface, evaluated by selective apical and basolateral biotinylation in living, ciliated LLC-PK1 cells. Live cells forming confluent monolayers on semipermeable supports were biotinylated from either the apical or basolateral surface, followed by pull-down with streptavidin-conjugated agarose beads. Lanes on the immunoblots were alternately loaded with total protein from the starting material after cell lysis but before streptavidin pull down (lysate) or with eluted protein after streptavidin pull down from cells biotinylated from either the apical or basolateral surfaces, respectively. Immunoblotting with anti-HA was used to detect PC2 related peptides. Na^+,K^+-ATPase, which is expressed exclusively on the basolateral surface, was used to show the selectivity of the biotinylation reaction. No biotinylated PC2 was detectable in either membrane compartment (left panel), consistent with the conclusion that PC2 is only expressed in the cilial plasma membrane in amounts not detectable by biotinylation (see text for details). PC2-L703X is biotinylated on the both basolateral and apical surfaces (middle panel). It is noteworthy that only the slower migrating band corresponding to the Endo H resistant form of PC2-L703X is biotinylated, confirming the expression of the Endo H resistant form on the plasma membrane. Δ(5-72)PC2-L703X is not biotinylated on the cell surface in either membrane compartment (right panel). Coupled with the immunofluorescence data showing lack of expression in cilia, the failure to co-localize with Golgi markers and the absence of Endo H resistance, we conclude that Δ(5-72)PC2-L703X is not expressed in the plasma membrane.

back to the N-terminus of PKD2L1-Δ(1-328)PC2-L703X (Fig. S1 in supplementary material), the doubly chimeric protein localized to cilia (Fig. 6C).

We developed a heterologous trafficking assay using the human transferrin receptor (hTFR) to test whether this N-terminal domain of PC2 was effective in targeting other type II membrane proteins to cilia. hTFR has a 67 amino acid N-terminal cytoplasmic tail containing a Y_{20}xRF internalization motif (Jing et al., 1990; Collawn et al., 1990) followed by a single membrane span and a 671 amino acid extracellular COOH-terminal domain. We placed a triple HA-epitope at the extracellular COOH terminus of hTFR and confirmed that the full-length protein does not traffic into the cilia (Fig. 7A). We next replaced amino acids 1-61 in the cytosolic N-teminus of hTFR, with amino acids 1-56 from the N-terminus of PKD2L1. This chimeric protein was confined to intracellular membrane compartments and failed to traffic to cilia (Fig. 7B). When we replaced the first 61 amino acids of hTFR with the first 72 amino acids of PC2, the chimeric protein trafficked selectively to the apical membrane and showed robust localization in the cilial membrane of these cells (Fig. 7C). A doubly chimeric protein in which the first 56 amino acids of PKD2L1 preceded the first 72 amino acids of PC2 fused with hTFR did not traffic to cilia (data not shown). This suggests that accessibility of the PC2 trafficking domain at the N-terminus is important to its function. These data strongly suggested that the first 72 residues of the N-terminus of PC2 contains a domain sufficient for directing targeting to cilia.

An RVxP motif is required for trafficking of PC2 to cilia

We next sought to better define this domain and identify the responsible motif. L703X-HA deleted of amino acids 31-72 [Δ(31-72)PC2-L703X] still trafficked to cilia, whereas deletion of amino acids 5-31 [Δ(5-31)PC2-L703X] abrogated cilial trafficking (Fig. 8A,B). We checked the trafficking to cilia of hTFR chimeras containing the first 31 and first 15 amino acids of PC2 and found both localized to cilia (Fig. 8C,D). We confirmed the apical and cilial location of the fusion of the first 15 amino acids of PC2 with hTFR by labeling the outside HA epitope in living, non-permeabilized cells (Fig. 8E). The first 15 amino acids of PC2 define a minimal domain sufficient for apical membrane and cilial location.

We compared sequences of the first 31 amino acids of PC2 from several species and found that the highest degree of conservation occurred within the first 15 amino acids (Fig. 9A). A potentially conserved motif S-x-R-V-x-P occurred in vertebrate forms of PC2 as well as sea urchin (Fig. 9A). This motif is not conserved in PKD2L1. We introduced the following point mutations into the PC2-L703X backbone: S4A, R6G, V7A and P9A within this putative motif and P12A, R17G and P18A in non-conserved residues (Fig. 9A). Mutations at residues S4, P12, R17 and P18 had no effect on cilia localization of PC2-L703X (Fig. 9B and data not shown). Mutations R6G, V7A and P9A each resulted in loss of cilia localization of PC2-L703X (Fig. 9C-E). These data suggest that the residues in the motif RVxP are necessary for location of PC2 in cilia.

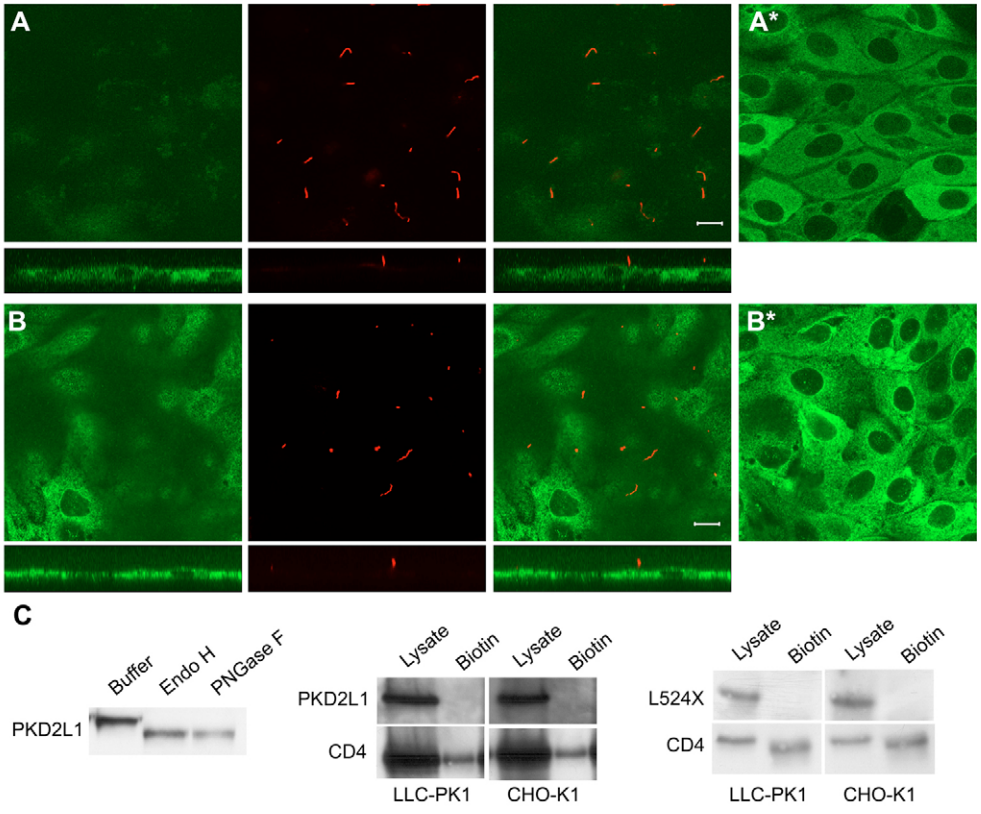

Fig. 5. The trafficking properties of PKD2L1. (A) Confocal images above the apical surface of the cell and in the *xz* plane show cilia devoid of PKD2L1 with either COOH-terminal EGFP (A) or triple-HA (B) epitope tags. EGFP epifluorescence (A) and double indirect anti-HA immunofluorescence (B) are shown in green; anti-acetylated α-tubulin, red. (A*,B*) Confocal images at the level of the nucleus showing that all cells express pPKD2L1. Bars, 10 μm. (C) PKD2L1 does not acquire detectable Endo H resistance (left panel) and is not detectably biotinylated on the cell surface in either LLC-PK1 of CHO-K1 cells (middle panel). Truncation of the predicted COOH terminus of PKD2L1 does not result in trafficking to the cell surface as indicated by the absence of a biotinylated species (right panel). 'Lysate' is the total cellular protein from the starting material before streptavidin pull down; 'biotin' is the eluted material after streptavidin pull down. PKD2L1 was detected by anti-HA in immunoblots. CD4 was co-transfected with PKD2L1 as a positive control for live cell surface biotinylation and streptavidin immunoprecipitation and was detected with anti-CD4 monoclonal antibody 1F6.

Discussion

Cilia play a central role in a spectrum of human diseases that include autosomal dominant and recessive polycystic kidney disease, nephronophthisis and Bardet-Biedl syndrome (Pazour, 2004; Pan et al., 2005). The proteins encoded by genes mutated in these diseases localize to the cilium complex that includes the basal body, the cilial axoneme and the overlying plasma membrane. From an alternative perspective, induced mutations in genes encoding several intraflagellar transport (IFT) proteins not known to be involved in human disease nonetheless result in polycystic phenotypes in model organisms (Pazour et al., 2000; Yoder et al., 2002b; Lin et al., 2003; Sun et al., 2004). It follows that polycystic kidney disease can result from defective ciliogenesis or from loss of discrete cilial proteins in otherwise apparently structurally normal cilia. Human ADPKD falls into the latter category (Thomson et al., 2003). Understanding how the ADPKD proteins get to cilia is essential to understanding both the function of the respective disease gene products and the role of cilia in the disease.

Cilial targeting of PC2

The cilium is a privileged compartment with restricted access from the contiguous cytosolic and membrane compartments (Rosenbaum and Witman, 2002). Vesicles containing integral membrane proteins destined for cilia travel to the area at the base of the cilium (Bouck, 1971) where there is a structural delimitation of the cilial and extra-cilial domains (Satir et al., 1976), perhaps corresponding to the transition zone (O'Toole et al., 2003). It has been proposed that there are cilial targeting signals for directing membrane and axonemal proteins to the cilial compartment (Rosenbaum and Witman, 2002). A protein may traffic to cilia either by having such a signal in its primary sequence or by forming a pre-assembly complex in the cytoplasm with another protein(s) that has such a signal. The compartmental restriction between the cilium and the remainder of the cell (Nir et al., 1984) is mediated via a 'flagellar pore complex' composed of transition fibers extending from the distal portion of the basal body to the plasma membrane (Deane et al., 2001). It is generally accepted that vesicles do not traffic into cilia (Kozminski et al., 1993). Instead, cilia integral membrane proteins such as the polycystins are synthesized in the ER and trafficked through the Golgi into post-Golgi vesicles that dock near the cilial pore complex between the basal body and cilium (Bouck, 1971; Rosenbaum and Witman, 2002). A plausible alternative mechanism would require that vesicles fuse with the apical membrane compartment adjacent to the cilium, and from there enter the cilial compartment via an intramembranous process (Witzgall, 2005).

Distinct compartmentalization of the cilial and apical plasma membranes is illustrated by the distribution of PC2, which is concentrated in the plasma membrane overlying the cilial axoneme and is not detected on the remainder of the cell surface (present study) (Cai et al., 1999; Cai et al., 2004), and of gp135 (podocalyxin), which marks the apical membrane but is excluded from cilia (Meder et al., 2005). The restricted distribution of PC2 requires a balance of two processes – one that is permissive for entry into the cilial compartment and the other that is either restrictive of exit from, or exclusive of entry into, the contiguous apical plasma membrane compartment. The presence of wild-type PC2 only in the cilial plasma membrane suggests that both permissive and restrictive (or exclusive) processes are active. They are probably mediated by distinct domains. The distribution of the PC2-L703X truncation to both the cilial and non-cilial

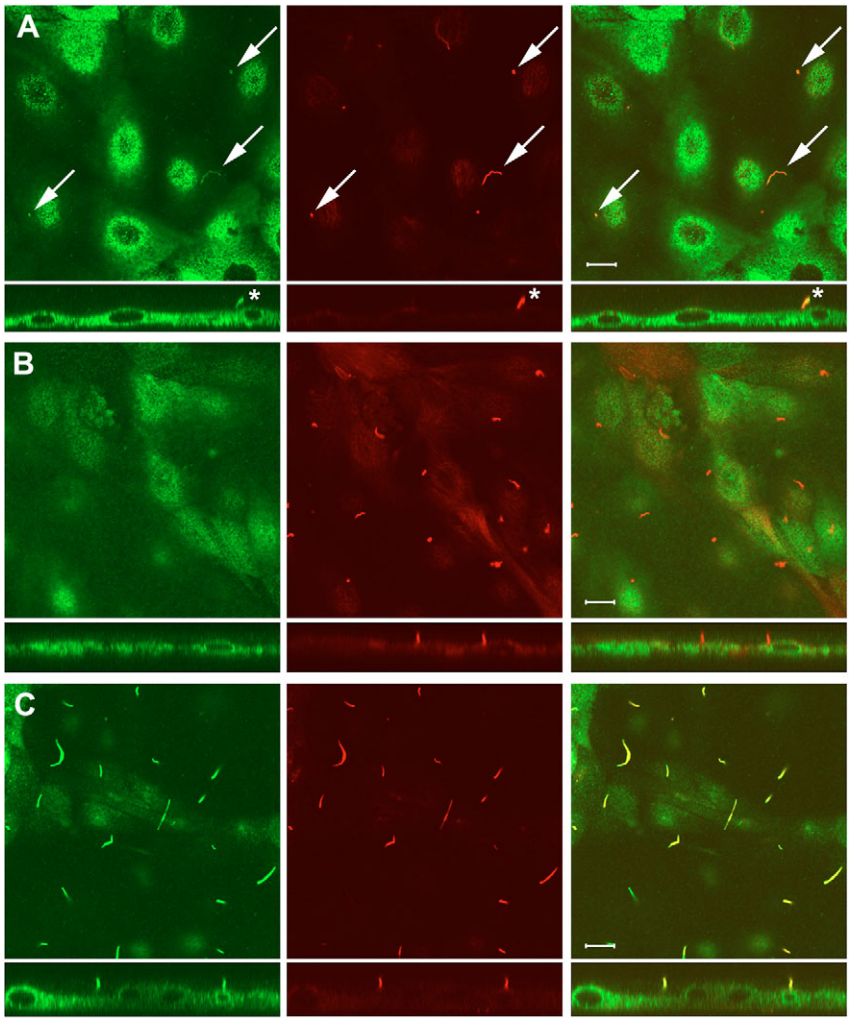

Fig. 6. The N-terminal domain of PC2 can direct cilial localization of heterologous non-cilial proteins. (A) Addition of the first 72 amino acids of PC2 to PKD2L1 results in trafficking of the chimeric protein, (1-72-PC2)-PKD2L1, into cilia (arrows). (B) Replacing the N-terminal region of PC2-L703X with the N-terminal region of PKD2L1 results in failure of the chimeric protein, PKD2L1-Δ(1-328)PC2-L703X, to traffic to cilia. (C) A doubly chimeric protein in which the first 72 amino acids of PC2 are fused to the PKD2L1-Δ(1-328)PC2-L703X chimera localizes to cilia. Anti-HA, green; anti-acetylated α-tubulin, red; right panels, merged images. Bars, 10 μm.

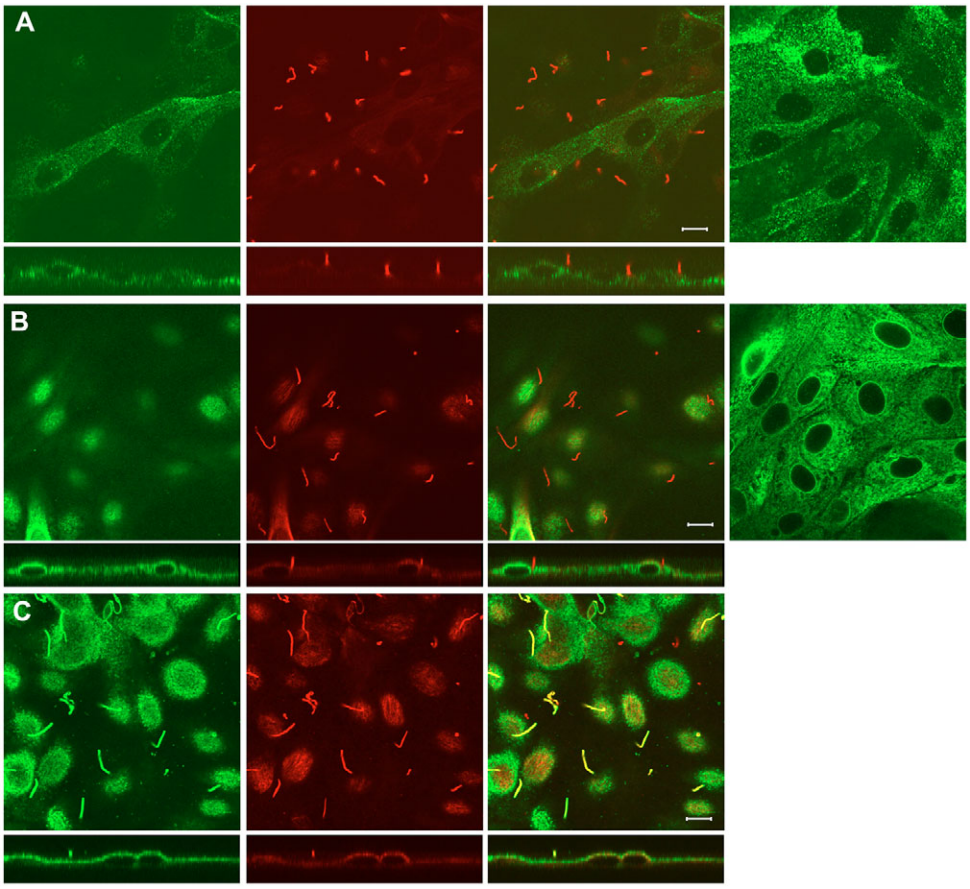

Fig. 7. A heterologous cilia trafficking assay using transferrin receptor. LLC-PK1 cells stably over-expressing (A) wild-type transferrin receptor (hTFR), (B) (1-56)PKD2L1-hTFR and (C) (1-72)PC2-hTFR. All heterologous proteins have a triple HA epitope at their COOH-termini and are immunolabeled by anti-HA (green). Cilial axonemes are marked by anti-acetylated α-tubulin (red). (A) The wild-type transferrin receptor (hTFR) does not traffic into cilia. (B) Replacement of the cytosolic N-terminus of hTFR with the first 56 amino acids of PKD2L1 does not result in trafficking of the chimeric protein to cilia. The right-most panels in A and B are confocal sections through the cell body at the level of the nucleus to show the robust expression of the heterologous protein in all cells in the field. (C) Replacement of the cytosolic N-terminus of hTFR with the first 72 amino acids of PC2 results in expression of the chimeric protein in cilia, indicating this domain contains cilia-targeting information. Bars, 10 μm.

plasma membrane suggests that PC2-L703X retains the signals permissive of entry to the cilial membrane but has lost signals restricting it only to that compartment.

We found a signal permitting access to the cilial plasma membrane that resides within the primary sequence of the N-terminus of PC2 in an RVxP motif that is conserved among vertebrate forms of the protein. Previous to this, four flagellar/cilial targeting domains had been reported (Nasser and Landfear, 2004; Ersfeld and Gull, 2001; Godsel and Engman, 1999; Tull et al., 2004). All are in species of the protozoans *Leishmania* and *Trypanosoma* and only one of these is in a flagellar membrane protein (Nasser and Landfear, 2004). The ISO1 glucose transporter in *Leishmania enriettii* contains the necessary targeting information in at least two segments of its cytosolic N-terminus (Nasser and Landfear, 2004). The other three reported domains are in flagellar axonemal proteins. In *Trypanosoma brucei*, the paraflagellar rod protein A (PFRA) and an actin-related protein (TrypARP) share a COOH-terminal histidine-leucine-alanine (HLA) motif that is necessary for targeting to the flagellum (Ersfeld and Gull, 2001). The flagellar Ca^{2+}-binding protein (FCaBP) of *Trypanosoma cruzi* as well as the SMP-1 protein of *Leishmania major* require N-terminal myristoylation and palmitoylation on respective glycine and cysteine residues to achieve flagellar location (Godsel and Engman, 1999; Tull et al., 2004). The motif in FCaBP is the only one previously shown to be both necessary and sufficient for flagellar location (Godsel and Engman, 1999).

The RVXP motif is probably part of a protein interaction domain. It is not likely to be a lipid interaction domain since it does not have an N-terminal G or a C typical of the myristoylation and palmitoylation sites, respectively, as observed for axonemal proteins in unicellular organisms (Godsel and Engman, 1999; Tull et al., 2004). The highly selective trafficking to the apical membrane and cilia of the (1-15)PC2-hTFR chimeric protein suggests that this motif directs trafficking of post-Golgi vesicles to either the cilial pore complex or the apical membrane. The trafficking of the polytopic PC2-L703X is more complex since it also detectable on the basolateral membrane as well. Once cells polarize and form cilia, PC2-L703X appears to preferentially accumulate in the apical membrane compartment (Fig. 4C, middle panel, and our unpublished observations). The only N-terminal binding partner for PC2 described to date is α-actinin (Li et al., 2005). It is associated with the actin rather than microtubule cytoskeleton, is not known to be localized in cilia and has been proposed to function in modulating the channel activity of PC2 (Li et al., 2005). It is an unlikely candidate for a role in trafficking of PC2 into cilia. PC2 has been found to interact with intraflagellar transport proteins (IFT57) and pericentrin, a protein that is necessary for proper formation of cilia (Jurczyk et al., 2004). The association with pericentrin occurs at the level of the centriole/basal body (Jurczyk et al., 2004) while the association with a putative trafficking partner would be expected to occur earlier in the trafficking pathway, perhaps at the level of the trans-Golgi network. Nonetheless, the class of IFT proteins as well as pericentrin are excellent candidate partners for directing PC2 to cilia. Several other binding

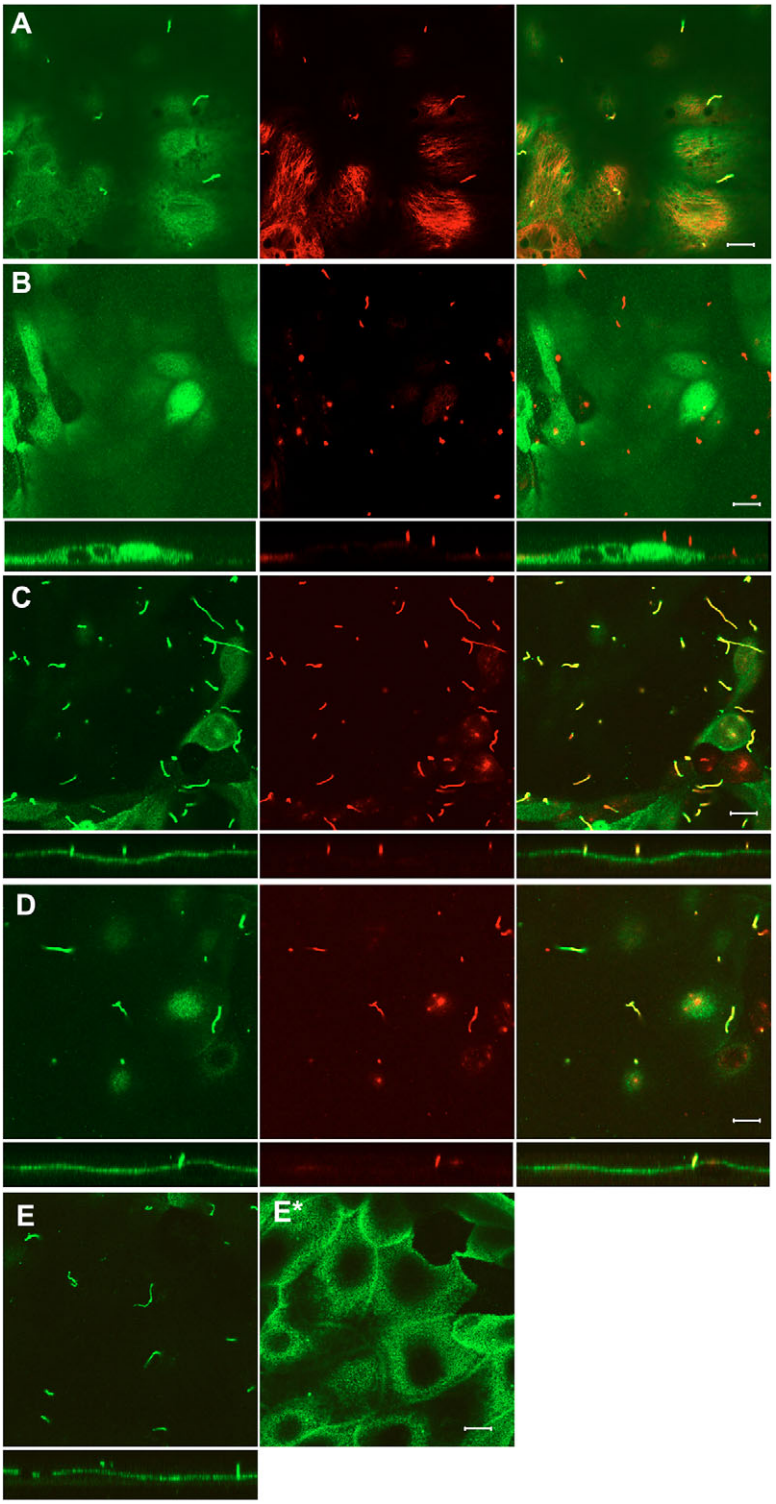

Fig. 8. Refinement of the cilia trafficking domain of PC2. (A) Deletion of amino acids 31-72 from PC2-L703X does not alter trafficking to cilia, whereas (B) deletion of amino acids 5-31 results in complete absence of Δ(5-31)PC2-L703X from cilia. Replacement of the cytosolic N-terminus of hTFR with either (C) the first 31 amino acids or (D) the first 15 amino acids of PC2 is sufficient to traffic the chimeric reporter molecule to cilia. (E) Non-permeabilized living cells have epitopes available to anti-HA on cilia and (E*) at the level of the apical plasma membrane. Anti-HA was applied on both the apical and basolateral aspects but there was no detectable immunoreactivity on the basolateral surfaces, indicating that the chimeric protein only trafficked to cilia and the apical membrane. Anti-acetylated α-tubulin gave no signal in non-permeabilized, living cells (not shown) confirming that the observed signal is indeed extracellular. Anti-HA, green; anti-acetylated α-tubulin, red. Bars, 10 μm.

partners for PC2 have been described including actin cytoskeleton interacting protein CD2AP (Lehtonen et al., 2000), PIGEA-14 which modulates PC2 distribution within the Golgi (Hidaka et al., 2004) and PACS-1 and PACS-2 which are involved in trafficking of PC2 between ER and Golgi and plasma membrane compartments (Kottgen et al., 2005). These latter three partners bind in the COOH terminus of PC2 and are unlikely to play a direct role in the mechanism of trafficking by the RVxP motif.

PC2 trafficking to cilia is independent of PC1

The most common and severe form of ADPKD results from mutations in *PKD1*. Genetic studies suggest that *PKD1* and *PKD2* function in the same genetic pathway and biochemical studies support the hypothesis that PC1 and PC2 work together in regulating key functions in polarized, lumen forming epithelial tissues (Barr et al., 2001; Hanaoka et al., 2000; Wu et al., 2002). The cation channel activity of PC2 is crucial to the function of the polycystin signaling complex, as evidenced by the pathogenic amino acid substitution mutation, D511V, that results in loss of channel activity (Reynolds et al., 1999; Koulen et al., 2002) without affecting interaction with PC1 or to cilia localization (our unpublished observations). Our bilayer experimental data supports the view that PC2, like other TRP channels, can form a functional channel complex without PC1 (Koulen et al., 2002; Gonzalez-Perrett et al., 2001). In cilia, PC1 is thought to exert control of the PC2 channel that ultimately renders PC2 ineffective in ADPKD patients who have *PKD1* mutations resulting in loss of PC1 (Nauli et al., 2003). One level at which PC1 exerts this control has been hypothesized to be in the proper trafficking of PC2 (Hanaoka et al., 2000; Nauli et al., 2003).

Previous studies had suggested that co-assembly with PC1 is required for surface expression of PC2 (Hanaoka et al., 2000) and that PC2 is not expressed in cilia in the absence of PC1 (Nauli et al., 2003). Our current studies indicate that PC1 is not required for trafficking of PC2 to cilia. Full-length, heterologously expressed PC2 can leave the ER and reach the cilia without PC1. Furthermore, PC2 is expressed in cilia in kidney cysts that result from null mutations in *PKD1*. Given the evidence of functional interdependence for the two polycystins, loss of PC1 probably results in loss of crucial regulatory signals, rendering PC2 ineffective, despite expression of the latter in cilia. It is also possible that PC1 participates more actively in the channel function, perhaps forming part of a heteromeric channel with PC2. However, data

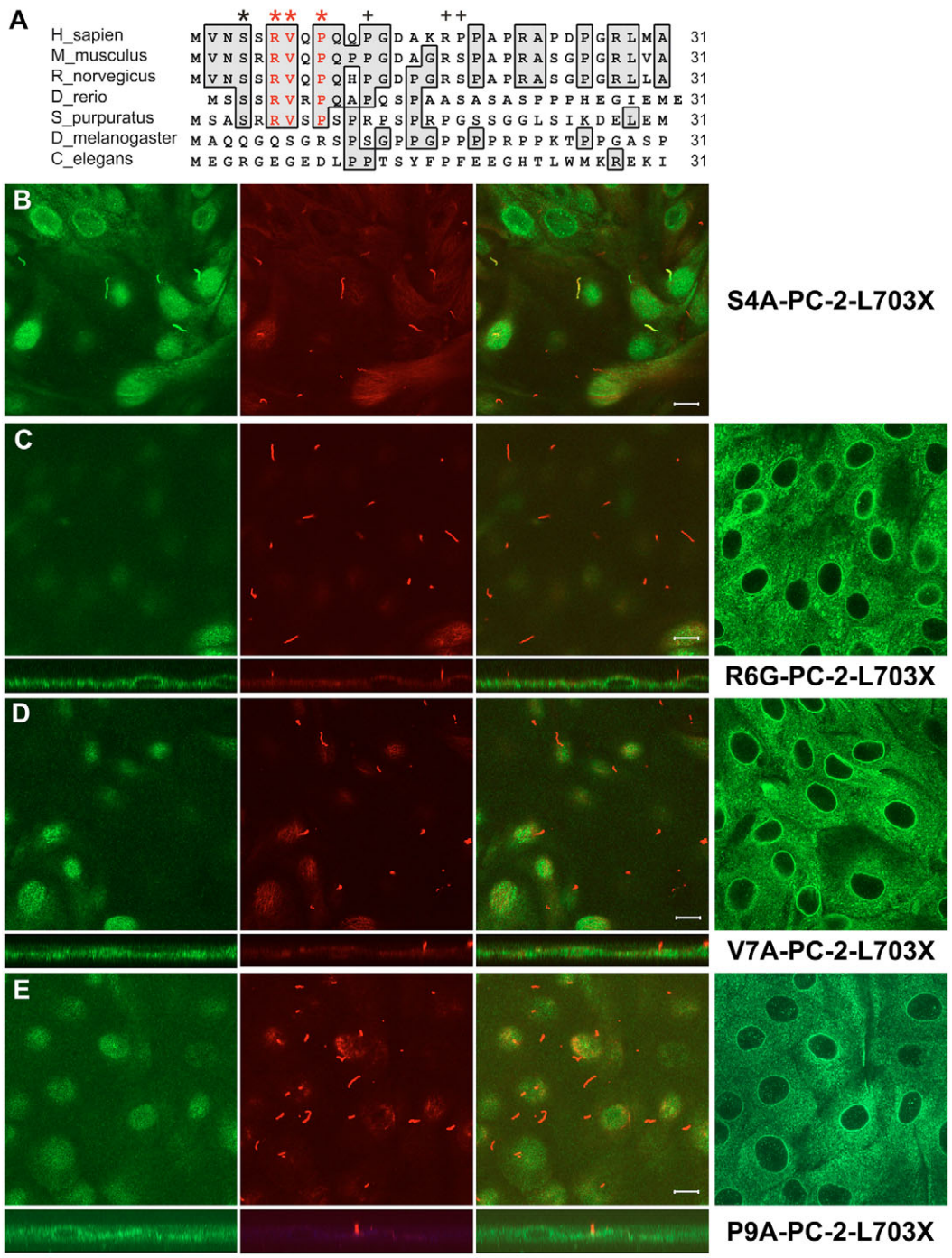

Fig. 9. RVxP is necessary for cilial targeting of PC2. (A) Alignment of the first 31 amino acids of PC2 from several species. *, location of mutations in the most conserved residues whose cilial trafficking properties are shown in B-E. +, other mutation sites tested that had no effect on cilial localization. Identical residues are highlighted and boxed; residues in red abrogated cilial location of PC2-L703X. (B) Amino acid substitution S4Adoes not affect trafficking of PC2-L703X to cilia. (C-E) Substitutions R6G (C) V7A (D) and P9A (E) result in loss of cilial localization of PC2-L703X. Green, anti-HA; red, anti-acetylated α-tubulin, third row, merged images; far right panels in C-E are confocal sections at the level of the nucleus showing robust expression of the respective mutant proteins in all cells. Bars, 10 μm.

cited above do support the hypothesis that PC2 has channel function independently of PC1. Whereas it is nominally possible that the role of PC1 is to turn off a constitutively active PC2 channel, this seems very unlikely given that the phenotype from loss of PC1 is indistinguishable from that from loss of PC2. Therefore, the most likely formulation is that activation of the PC1 receptor protein by relevant stimuli results in activation of the PC2 cation channel, which in turn propagates a signal that uses Ca^{2+} as a second messenger. It follows that selective PC2 agonists may be able to reconstitute polycystin signaling in patients with *PKD1* mutations by bypassing the receptor defect and acting directly on the effector channel protein. We propose that selective PC2 channel agonists may be effective therapeutic agents for the most common and severe forms of ADPKD.

Materials and Methods

cDNA expression constructs

Schematics of the expression constructs used in this study are presented in Fig. S1 in supplementary material. All constructs were sequence verified. PC2 and PC2-L703X expression constructs in pCDNA3.1 (Invitrogen, Carlsbad, CA, USA) have been previously reported (Cai et al., 1999). N-terminal deletions took advantage of the presence of a unique *Sac*II site in codon 72 of *PKD2*. We used site-directed mutagenesis to introduce a *Sac*II site codon 5 to form Δ(5-72) deletion construct and in codon 130 to produce the Δ(72-130) deletion constructs. For the Δ(130-220) deletion, we first made a large deletion from codons 72-220 by introducing a *Sac*II

site at codon 220, followed by in-frame insertion of codons 72-130, using the *Sac*II sites generated above. We used RT-PCR amplification of a segment from the vector arm *Xba*I site upstream of codon 1 to codon 31 that included an engineered *Sac*II site to replace codons 1-72 and form the Δ32-72 deletion construct. The Δ5-31 deletion was produced by inserting an RT-PCR product with codons 32-72 flanked by *Sac*II sites into the Δ5-72 deleted peptide. Full-length COOH-terminal EGFP-tagged PC2 was generated by cloning *PKD2* in-frame into the pEGFP-N2 vector (Clontech, Mountain View, CA, USA).

We completed cloning of the full-length PKD2L1 into pCDNA3.1/Zeo by RT-PCR from human brain cDNA using primers designed from published sequences (Wu et al., 1998b; Nomura et al., 1998) and ligating the complete N-terminus using the *Bam*HI site in PKD1L1. A PKD2L1-EGFP fusion protein was constructed by inserting the complete PKD2L1 sequence in-frame into the pEGFP-N2 vector. Since RT-PCR from multiple tissues yielded an alternative form of the 5′ end of PKD1L1 (see Results), we generated a sequence to match that published by Nomura et al. (Nomura et al., 1998) using site-directed mutagenesis.

Full-length human transferrin receptor (hTFR) cDNA was kindly provided by P. De Camilli (Yale University, New Haven, CT). We used PCR strategies to attach a 3XHA epitope tag to the COOH terminus and clone it into pCDNA3.1. We introduced a *Sac*II site at codon 61 of hTFR by site directed mutagenesis to allow replacement of the first 61 amino acids by various N-terminal segments of PKD2 and PKD2L1.

We took advantage of the unique *Bam*HI site in the *PKD2* and *PKD2L1* cDNAs to swap the respective N-terminal domains of each.

Point mutations were constructed using either the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) or conventional PCR.

Cell culture and transfection

LLC-PK1 (CRL1392, ATCC) or MDCK (CRL6253, ATCC) cells were maintained in DMEM:F12 (1:1), 5% FBS, and penicillin/streptomycin at 37°C in a 95% O_2/5% CO_2 incubator. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) using 1 μg of plasmid DNA. Cells were selected 24 hours after transfection with 400 μg/ml G418 (Invitrogen, Carlsbad, CA, USA) for 3 weeks. Positive colonies were verified by immunoblot and immunofluorescence analyses. Cells were grown on 0.4 μm pore size cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) for up to 21 days post confluence for analysis of cilia.

Immunofluorescent cell staining and antibodies

For immunofluorescent labeling, cells were washed and fixed in freshly prepared 3.5% paraformaldehyde for 30 minutes followed by permeabilization with 0.1% Triton X-100. The cells were washed three times with PBS and then incubated with primary antibodies followed by subsequent incubation with the goat anti-mouse or anti-rabbit Alexa Fluor 488 or 494-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). Primary antibodies: monoclonal acetylated α-tubulin (6-11B-1, Sigma, St. Louis, MO, USA); polyclonal anti-HA epitope (71-5500, Zymed Laboratories, San Francisco, CA, USA); rabbit polyclonal YCC2 (COOH terminus) and YCB9 (N-terminus) anti-PC2 (Cai et al., 1999), mouse monoclonal anti-Na^+,K^+-ATPase (clone 6H; kind gift from Michael Caplan, Yale University, New Haven, CT), and mouse monoclonal anti-GM130 (kind gift from Graham Warren, Yale University, New Haven, CT). Images were acquired using a Zeiss LSM 510 confocal microscope.

Immunoblot analysis

Cell extracts were prepared by incubation in lysis buffer (10 mM Tris pH 7.0, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin) for 30 minutes at 4°C. Lysate was centrifuged 8000 ***g*** for 15 minutes and dissolved in Laemmli SDS buffer followed by incubation at 65°C for 5 minutes. Proteins were separated by SDS-PAGE (4-20%) and electrophoretically transferred to PVDF membranes overnight at 20 mV in Tris-glycine-methanol. After blocking for 1 hour with 5% dried milk, membranes are washed three times with TBS-Tween (0.05%), incubated for 1 hour with the primary antibody, washed, incubated for 1 hour with horseradish peroxidase-coupled secondary antibody, washed extensively and processed for chemiluminescence by ECL (Amersham, Arlington Heights, IL, USA).

Glycosylation and cell surface biotinylation analysis

Cell lysate and membrane fraction protein were treated with peptide:*N*-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (New England Biolabs, Beverly, MA, USA) and analyzed by SDS-PAGE followed by immunoblotting as described previously (Cai et al., 1999; Koulen et al., 2002). Cell surface proteins were biotinylated as described by Gottardi et al. (Gottardi et al., 1995). Solutions were pre-chilled and all incubations performed at 4°C. Cells, grown as described above, were washed once in serum-free DMEM and twice with PBS-CM (PBS supplemented with 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$) followed by two incubations of 25 minutes each with biotin solution [10 mM triethanolamine pH 9.0, 150 mM NaCl, 2 mM $CaCl_2$, and 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA)]. The biotin reaction was quenched with PBS-CM and 100 mM glycine for 20 minutes. Cells were rinsed twice with PBS-CM and incubated in 1 ml lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl and 1% Triton X-100) for 60 minutes. Lysates were centrifuged at 14,000 ***g*** for 10 minutes, and 800 μl of the cleared supernatant was immunoprecipitated with 30 μl of agarose-conjugated streptavidin overnight at 4°C. The beads were washed three times with lysis buffer, once with high salt buffer (lysis buffer supplemented with 500 mM NaCl and 0.1% Triton X-100) and once with no-salt buffer (10 mM Tris, pH 7.4). Beads were re-suspended in protein sample buffer and subjected to SDS-PAGE and immunoblotting.

Live cell immunofluorescent labeling

Solutions were pre-chilled and all incubations performed at 4°C. Cells grown on cell culture inserts were washed twice with surface buffer: PBS supplemented with 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$ and incubated with anti-HA antibody on both apical and basolateral surfaces, followed by three washes and incubation with goat anti-mouse Alexa Fluor 488-conjugated antibody in surface buffer. Cells were washed and fixed for immunofluorescence as described above.

Cytosolic Ca^{2+} measurements

Cells were plated on glass coverslips and used within 16-48 hours. The coverslips were rinsed in HBSS (19.7 mM Hepes pH 7.4, 130 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1 mM $MgCl_2$, and 1.25 mM $CaCl_2$) and incubated with fluo-4AM (10 μM in HBSS) for 30 minutes at 37°C. The coverslips were rinsed in HBSS, mounted in a perfusion chamber, and analyzed with a Zeiss Aviovert S100 confocal microscope. Fluo-4 was excited at 488 nm and emission was time-lapse recorded at 522 nm. Fluorescence amplitudes (F) were normalized to baseline (F_0) and reported as F/F_0. The $t_{\frac{1}{2}}$ value corresponds to the time required for the fluorescence to decrease to one-half its peak amplitude.

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