Alpha-actinin associates with polycystin-2 and regulates its channel activity

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INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a common, potentially lethal genetic disease. It is characterized by massive cystic enlargement in renal tubules, a consequence of aberrant cell proliferation, apoptosis, under-differentiation, mispolarization of ion and fluid transport and extracellular matrix adhesion abnormalities (1–4). ADPKD is a monogenic disease caused by mutations in the PKD1 or PKD2 genes, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively (5,6). PC1 is a large integral glycoprotein with 11 transmembrane domains, a short intracellular C-terminus containing a putative coiled-coil structure and a large extracellular N-terminal region containing multiple motifs (7). PC1 is expressed in various tissues and acts as a cell surface receptor (8,9). PC1 interacts with multiple proteins including PC2 (10), extracellular matrix proteins (collagen, fibronectin and laminin) (11), components of micro- and intermediate filaments (α-actinin, cytokeratin, vimentin and desmin) (12–14) and focal (cell–matrix) adhesion and cell–cell adherens junction proteins (12,13).

Polycystin-2 (PC2) is the product of the PKD2 gene, which is mutated in 10–15% patients of autosomal dominant polycystic kidney disease (ADPKD). PC2 is an integral transmembrane protein and acts as a calcium-permeable cation channel. The functional modulation of this channel by other protein partners remains largely unknown. In the present study, using a yeast two-hybrid approach, we discovered that both intracellular N- and C-termini of PC2 associate with α-actinins, actin-binding and actin-bundling proteins important in cytoskeleton organization, cell adhesion, proliferation and migration. The PC2-α-actinin association was confirmed by in vitro glutathione S-transferase pull-down and dot blot overlay assays. In addition, the in vivo interaction between endogenous PC2 and α-actinins was demonstrated by co-immunoprecipitation in human embryonic kidney 293 and Madin-Darby canine kidney (MDCK) cells, rat kidney and heart tissues and human syncytiotrophoblast (hST) apical membrane vesicles. Immunofluorescence experiments showed that PC2 and α-actinin were partially co-localized in epithelial MDCK and inner medullary collecting duct cells, NIH 3T3 fibroblasts and hST vesicles. We studied the functional modulation of PC2 by α-actinin in a lipid bilayer electrophysiology system using in vitro translated PC2 and found that α-actinin substantially stimulated the channel activity of reconstituted PC2. A similar stimulatory effect of α-actinin on PC2 was also observed when hST vesicles were reconstituted in lipid bilayer. Thus, physical and functional interactions between PC2 and α-actinin may play an important role in abnormal cell adhesion, proliferation and migration observed in ADPKD.
PKD2 is a 968 amino acid membrane protein with six putative transmembrane domains and intracellular N- and C-termini (6) and functions as a Ca\(^{2+}\)-permeable non-selective cation channel (15,16). Apart from its heterodimerization with PC1, PC2 can form a homodimer (17). PC2 also co-assembles with a number of partners including transient receptor potential channel TRPC1 (18), Golgi- and endoplasmic reticulum-associated protein PIGEA-14 (19), mammalian diaphanous protein mDia1 (20), actin filament-associated proteins CD2AP (21), Hax-1 (22), tropomyosin-1 (23) and troponin I (24). PC2 is present in a variety of tissues including kidney, testis, intestine, cardiac and skeletal and smooth muscles (6), but not all organs display known phenotypes associated with PKD2 pathogenic mutations. Mice with PKD2 targeted mutations display renal failure and cardiac defects (25).

Alpha-actinins, which are actin-binding and -bundling proteins and belong to the spectrin superfamily, are abundant in both muscle and non-muscle cells (26). Multiple homologous isoforms of α-actinin are found in human and encoded by at least four distinct genes. In non-muscle cells, α-actinin-1 and -4 crosslink between actin filaments to form filament bundles such as stress fibers and mediate Ca\(^{2+}\)-dependent membrane attachment at cell–cell and cell–matrix adherens junctions (27). In muscle cells, α-actinin-2 and -3 are major structural components of sarcomeric Z-lines of striated muscle and analogous dense bodies of smooth muscle where they anchor actin filaments in a constitutive manner. In fact, α-actinin-2 is expressed in all muscle fibers whereas α-actinin-3 is limited to a subset of fast-twitch skeletal fibers (28). Two identical α-actinin molecules can form a rod-shaped head-to-tail homodimer whereas α-actinin-2 and -3 together can form a heterodimer (29).

Structurally, α-actinins have a molecular mass of 94–103 kDa, composed of three distinct domains: an N-terminal actin-binding domain, a central rod-shaped domain with four internal spectrin-like repeats and a C-terminal calmodulin-like domain containing a pair of Ca\(^{2+}\)-binding EF-hands (30). Binding of Ca\(^{2+}\) to those isoforms possessing functional EF-hands leads to the release of an actin monomer from their actin-binding domain. Therefore, there are two distinct groups of α-actinins based on the sensitivity to Ca\(^{2+}\): muscle α-actinins with non-functional (Ca\(^{2+}\)-insensitive) EF-hands and non-muscle α-actinins with functional (Ca\(^{2+}\)-sensitive) EF-hands (26). In the last decade, numerous studies demonstrate that α-actinin not only associates with actin filaments but also interacts with several cytoskeletal and membrane-associated proteins such as integrins, catenins, L-selectin, zyxin, the PDZ-LIM family of proteins, N-methyl-D-aspartate (NMDA) receptors and other ion channels (31–37). In addition, it is proposed that α-actinin acts as an effective tumor suppressor (27,38,39).

In the present study, we identified by a yeast two-hybrid approach that both muscle α-actinin-2 and non-muscle α-actinin-1 are interacting partners of PC2. These specific interactions were further demonstrated under various in vitro and in vivo conditions. Electrophysiological analysis using reconstituted PC2 in a lipid bilayer system showed that α-actinin substantially increases PC2 channel activity.

RESULTS

Interaction of PC2 with α-actinins revealed by yeast two-hybrid system

The yeast two-hybrid system was used to screen proteins that associate with the PC2 intracellular N-terminus (PC2N, amino acids M1-K215). A bait construct, pGBKTT7-PC2N, was used to screen a human heart cDNA library (Clontech, Palo Alto, CA, USA). One plasmid isolated from the library represented the C-terminal two-thirds of human muscle-type α-actinin-2 (GenBank accession no. XM_002047) (Fig. 1). No β-galactosidase activity was detected by colony lift assay in the absence of PC2N or when other unrelated proteins replaced PC2N, as negative controls (data not shown).

The identified α-actinin-2 cDNA is a 3 kb fragment starting at nucleotide C1187 (amino acid L341) and encodes the majority of the central domain (including three spectrin-like repeats) and C-terminus (Fig. 1B). Given that mammalian α-actinin has four isoforms with high sequence similarity, we further sought to explore whether non-muscle α-actinin-1 can also bind PC2. Indeed, α-actinin-1 associated with PC2N albeit not as strong as the α-actinin-2–PC2N interaction (Fig. 1A).

We performed a similar yeast two-hybrid assay to determine whether the PC2 C-terminus (PC2C, amino acids 682–968) also interacts with α-actinins. Interestingly, although the entire cytoplasmic PC2C exhibited no interaction with α-actinin-2 or -1 (data not shown), shorter segments within PC2C displayed firm interaction with both α-actinins (Fig. 1A), suggesting the possibility that a domain within the first part (amino acids 682–820) of PC2C inhibits the PC2C–α-actinin interaction. We narrowed down to a small segment of 58 amino acids (PC2CC, amino acids 821–878) responsible for association with α-actinins. Of note, PC2CC overlaps with the domain that interacts with tropomyosin-1 (23). In contrast, although the spectrin-like domain II of α-actinin-2 seemed to be required for association with PC2CC and the fragment amino acids 821–968 (called PC2CA), we found that the domain IV alone was responsible for mediating association with PC2N (Fig. 1B). Our results indicate that the PC2–α-actinin interaction is through at least two domains of contact.

In vitro binding of PC2 with α-actinins

In vitro biochemical methods were applied to further characterize the interaction between PC2 and α-actinins. We first employed a gluthatione S-transferase (GST) fusion protein affinity binding method. For this purpose, polypeptides PC2N, PC2C, PC2CA, PC2CB (amino acids 821–927), PC2CC and PC2CD (amino acids 872–927) were fused in frame with a GST epitope and expressed in bacterial strain BL21 in the presence of 1 mM IPTG. All fusion proteins were successfully expressed in Escherichia coli with variable yields (Fig. 2A). Cell extracts containing respective fusion protein were incubated with purified non-muscle or muscle α-actinin. Using monoclonal α-actinin antibodies, we showed that GST–PC2N, –PC2C, –PC2CA, –PC2CB and –PC2CC but not GST–PC2CD or controls (GST alone and buffer without fusion protein lysates), co-precipitated with both types of α-actinin (Fig. 2B and C). These results
further demonstrated that both the PC2N and the small C-terminal segment amino acids 821–878 mediate association with α-actins. In this assay, GST–PC2C clearly showed binding to α-actins. Taking into account the fact that the molecular weight of GST–PC2CA is about 2/3 that of GST–PC2C, the protein yield of GST–PC2C is much higher than that of GST–PC2CA (Fig. 2A). However, larger amounts of α-actinin were pulled down by GST–PC2CA than by GST–PC2C, indicating stronger association between GST–PC2CA and α-actins, in agreement with the suggestion from our yeast two-hybrid assays that the fragment amino acids 682–820 in PC2C exhibits an inhibitory effect on the PC2C–α-actinin interaction.

We also performed dot blot overlay experiments to examine the PC2–α-actinin interaction. Purified α-actinin proteins were spotted onto a nitrocellulose membrane, followed by incubation with either human embryonic kidney (HEK) 293 or Madin-Darby canine kidney (MDCK) II cell lysates in the presence of Ca\(^{2+}\) (1 mM) or in its absence (1 mM EGTA). The membrane was then washed extensively and probed with PC2 antibody 1A11 (23,24). Immunoreactive spots were observed in both cell lysates for the two types of α-actinin, but not bovine serum albumin (BSA) or binding buffer alone (Fig. 3), indicative of specific binding between α-actinins and PC2. Similar PC2-binding signal intensities were displayed on membranes with or without Ca\(^{2+}\), indicating a Ca\(^{2+}\)-independent association between the two proteins. On the other hand, PC2 seemed to exhibit a stronger binding to muscle α-actinin than to non-muscle α-actinin, which is consistent with our yeast two-hybrid data (Fig. 1).
Co-immunoprecipitation of endogenous PC2 and α-actinins

We first tested the specificity of the two anti-PC2 antibodies used in this study, the custom-made mouse monoclonal 1A11 (Santa Cruz), by immunoprecipitation (IP) using MDCK cells. As shown in Fig. 4A and B, both 1A11 and G20 recognized a single band of the expected size. In addition, both signals were completely blocked when the respective blocking peptides were incubated with the antibodies before western blotting, indicative of the specificity of both antibodies. We then utilized mouse 1A11 for further IP studies because it yielded stronger signals than goat G20.

We also validated the goat anti-PC2 antibody G20 by immunofluorescence (IF). In MDCK cells stably expressing GFP-tagged PC2, staining with G20 basically coincided with the GFP signal (Fig. 4C). Moreover, the G20-stained signal was abolished when G20 was pre-incubated with its antigen peptide (Fig. 4D). Thus, G20 specifically recognized the PC2 signal. Of note, when MDCK cells became ciliated, staining by G20 was also observed in primary cilia, demonstrating the presence of PC2 in MDCK cilia (Fig. 4E).

To determine whether PC2 interacts with α-actinin in vivo, we performed co-IP experiments using HEK293 and MDCK cells and rat kidney and heart tissues. Using 1A11, anti-α-actinin BM75.2 (mainly recognizing the non-muscle type α-actinin-1) and anti-α-actinin EA53 (mainly recognizing the muscle type α-actinin-2) for precipitation, we detected associated proteins via immunoblotting. PC2 was detected in the immunoprecipitates from rat heart tissue using EA53 but not in the control immunoprecipitates using non-immune serum (Fig. 5A). Reciprocal co-IP using 1A11 also precipitated muscle α-actinin (Fig. 5B). These data firmly demonstrate that PC2 associates in vivo with muscle α-actinin. Under the same conditions, PC2 was also detected in the immunoprecipitates of HEK and MDCK cells and rat kidney tissue using BM75.2 (Fig. 5C, E and G). A reciprocal signal corresponding to non-muscle α-actinin from these cells or tissue lysates was observed in the immunoprecipitates of the same cell/tissue lysates using 1A11 (Fig. 5D, F and H). Taken together, these results demonstrate that endogenous PC2 and α-actinins form a complex in vivo in cells and tissues.

Because α-actinin belongs to the spectrin superfamily, we tested whether PC2 also interacts with spectrin. As shown in Fig. 5I and J, spectrin signal was not detectable in the 1A11-precipitated lysates of rat heart but was clearly present in total lysates. When the spectrin antibody was used to precipitate both tissue lysates, PC2 was also undetectable (data not shown), suggesting that spectrin is not in the complex containing PC2 and α-actinin.

Subcellular co-localization of PC2 and α-actinin

Using indirect double IF, we found that endogenous non-muscle α-actinin was dispersed along stress fibers and in the cytoplasm of mouse NIH 3T3 fibroblasts (Fig. 6A1), in agreement with the previous reports (27,40). Our current (Fig. 6A2) and previously published (23) data as well as reports from other groups (15,41) demonstrated that over-expressed PC2 exhibited endoplasmic reticulum localization in 3T3, LLC-PK1 and Chinese hamster ovary cells. Over-expressed PC2 and endogenous α-actinin displayed partial co-localization in endoplasmic reticular and perinuclear compartments (Fig. 6A3).

To determine the localization of endogenous PC2 and α-actinin, we performed IF assays utilizing epithelial MDCK and mouse inner medullary collecting duct (IMCD) 3 cells.
Figure 4. Characterization of polycystin-2 antibodies G20 and 1A11. (A) Left panel, total protein from MDCK cells was precipitated with goat G20, non-immune goat IgG (G20), mouse 1A11 or non-immune mouse IgG (1A11) and detected with 1A11. Right panel, the same membrane was stripped off and re-probed with 1A11 after incubation with the fusion protein GST–PC2C as the blocking peptide. (B) Precipitated samples in panel A were detected with G20 (left panel) or with G20 following pre-incubation with its antigen peptide (right panel). (C and D) MDCK cells stably expressing GFP–PC2 were stained with G20 (red) or visualized for GFP (green, no staining), in the absence (C) and in the presence (D) of the blocking peptide. (E) Ciliated MDCK cells (5 days post-confluency) were co-stained for acetylated α-tubulin and for PC2 (with G20).
We found that non-muscle α-actinin exhibited clear cell surface/periphery localization, notably at cell–cell contacts (Fig. 6B and C). Faint staining was also observed along stress fibers and in the perinuclear region (Fig. 6B1). Similar subcellular localization of α-actinin has previously been shown in other cell lines such as A431, breast cancer MCF7, oral floor cancer IMC2 and bladder KU7 (27,32). On the other hand, consistent with recent reports (42–44), we found that subconfluent MDCK cells express endogenous PC2 in intracellular compartments as well as on the plasma membrane and at the cell–cell contacts (Fig. 6B2). The subcellular localization of PC2 in subconfluent IMCD cells is

Figure 5. Association between endogenous PC2 and α-actinins in HEK293 and MDCK cells and rat kidney and heart tissues. (A) Total protein from rat heart was precipitated with either muscle α-actinin antibody EA53 or non-immune mouse IgG (−EA53) and detected with antibody 1A11. (B) Reciprocal to A, total protein from rat heart was precipitated with 1A11 antibody or non-immune mouse IgG and probed with EA53. Total cell lysates from HEK293 (C) and MDCK cells (E) and total protein from rat kidney (G) were precipitated with non-muscle α-actinin antibody BM75.2 or non-immune mouse IgG (−). The precipitates were detected with 1A11. In reciprocal co-IP experiments, cell lysates from HEK293 (D) and MDCK cells (F) and total protein from rat kidney (H) were precipitated with 1A11 or non-immune mouse IgG (−). The precipitates were detected with BM75.2. Total protein from rat kidney and heart (I and J) was precipitated with 1A11 or non-immune mouse IgG (−1A11). These precipitates and the total lysates from rat kidney and heart were detected by the spectrin antibody.
comparable to that in MDCK cells, with relatively lower abundance in IMCD intracellular compartments (Fig. 6B2 and C2). The fluorescence patterns of α-actinin and PC2 were substantially overlapped both on the plasma membrane and at cell–cell junctions (Fig. 6B3 and C3), indicating that both proteins are partially co-localized in these two cell lines. As α-actinin is an important member of the adherens junction protein complex, the co-localization of PC2 and α-actinin in renal epithelial cell junctions suggests that PC2 may play a role in cell junction signaling/communication.

We utilized cell surface biotinylation to confirm the presence of PC2 in the plasma membrane of renal epithelial cells. PC2 was detectable in IMCD but not MDCK cells using 1A11 in western blot analysis. The cell surface biotinylation assay revealed that PC2 is present on both the plasma membrane and intracellular organelles of IMCD and MDCK cells.

Figure 6. Subcellular co-localization of PC2 and α-actinin in NIH 3T3, MDCK and IMCD cells and hST vesicles. (A) Localization of endogenous non-muscle α-actinin and transiently transfected PC2 in 3T3 cells, revealed using IF with antibodies BM75.2 and G20, respectively. (B and C) Localization of endogenous non-muscle α-actinin and PC2 in subconfluent MDCK (B) and IMCD (C) cells. Lower panels are expanded pictures of cells from within the square boxes shown in B3 and C3 upper panels. Triangles and arrows in B3 and C3 lower panels indicate the plasma membrane and cell–cell junction localizations, respectively. (D) Cell surface biotinylation in MDCK and IMCD cells. Cells were first labeled with Sulfo-NHS-biotin and precipitated with avidin beads (upper panel, Surface). The remaining flow-through samples were then precipitated with 1A11 and protein G beads and detected by 1A11 in western blotting. Total lysates were also loaded as shown. IMCD cell extracts (Lysate, Surface and Flow-thru without enrichment) were also detected for Na⁺/K⁺ ATPase α1 subunit, as a plasma membrane marker (positive control), and calreticulin, as an endoplasmic reticulum marker (negative control) (lower panels). (E) Interaction and distribution of endogenous non-muscle α-actinin and PC2 determined in hST apical membrane vesicles in the absence and presence of cytochalasin D (CD) (10 μg/ml for 24 h) treatment. For co-IP, vesicles were lysed with CellLytic-M buffer, precipitated with BM75.2 and detected by 1A11. Total lysates were loaded on the left two lanes to indicate the PC2 protein levels. For IF, vesicles were co-stained with goat anti-PC2 G20 (green) and mouse anti-α-actinin BM75.2 (red). Representative merged images (green plus red) with and without cytochalasin D treatment were shown. Horizontal bars = 20 μm.
Figure 6. Continued.
When compared with MDCK cells, IMCD cells have a relatively higher proportion and amount of PC2 proteins on the cell surface consistent with our IF data (Fig. 6B and C).

**Functional modulation of PC2 channel by α-actinin**

Alpha-actinin has been shown to regulate various types of ion channels including calcium and potassium channels and NMDA receptors (37,45,46). We thus examined whether α-actinin modulates the channel function of reconstituted PC2 using a lipid bilayer single-channel electrophysiology system described previously (16). We found that addition of non-muscle α-actinin to the cis chamber (cytoplasm) elicited a substantial increase in single-channel activity (Fig. 7A and B). In average, the mean single-channel currents increased 15-fold by α-actinin. The currents averaged 1.7 ± 0.5 pA from seven control measurements (in the absence of α-actinin) and 27.0 ± 5.4 pA from six paired measurements (in the presence of α-actinin, P < 0.01), i.e. from the same...
membranes for control measurements (Fig. 7C). While open-
nings of different single-channel conductance states were eli-
cited by α-actinin, large-amplitude states seemed to be
elicited more (Fig. 8A). However, α-actinin did not signifi-
cantly alter the current–voltage relationship of the main
(largest) conductance state of PC2 (Fig. 8B).

To further assess the regulatory role of α-actinin on PC2
channels, we used human syncytiotrophoblast (hST) apical
membrane vesicles, in which we previously showed that
PC2 is the most abundant cation channel present (16,47,48).
We recently demonstrated that both PC2 and α-actinin are
present in hST apical membrane vesicles. Here, our co-IP
experiment showed that both proteins are present in the
same complex (Fig. 6E upper panel). We see that, in the
presence of the actin filament-disrupting agent cytochalasin D
(10 μg/ml for 24 h), the PC2 protein level was not affected
but the strength of this interaction was decreased. Consis-
tently, co-localization of both proteins was partially disrupted
by cytochalasin D (Fig. 6E lower panels). These data
suggest that filamentous actin networks modulate the PC2–
α-actinin interaction. Of note, similar modulation effect was
observed in the interaction between PC2 and troponin I (24).

After hST vesicles were reconstituted in the lipid bilayer
system, addition of α-actinin to the cis chamber substantially
increased hST PC2 cation channel activity (Fig. 9A and B), in
agreement with the results obtained using in vitro translated
PC2. We also tested the effect of amiloride and indeed
observed that 100 μM amiloride applied to the trans side of
the chamber substantially inhibited (by 91%) the channel
activity (Fig. 9C), consistent with our previous report (16).

Figure 7. Effect of α-actinin on in vitro translated PC2 channel activity in a lipid bilayer system. (A) Representative tracings of in vitro translated, reconstituted
PC2 at +40 mV before and after addition of 250 nM (final concentration) of non-muscle α-actinin to the cis chamber. Cis chamber, 150 mM K⁺; trans chamber,
15 mM K⁺ (Materials and Methods). Tracings at sites (a–c) are shown below with an expanded scale. (B) Representative tracings recorded at +40 and +60 mV
before and after α-actinin addition. (C) Averaged PC2 single-channel currents recorded at +40 mV before (N = 7) and after (N = 6, paired) addition of
α-actinin.
Our present study demonstrates that PC2 physically and functionally interacts with α-actins, important components of the actin cytoskeleton. To document this interaction, we used a variety of approaches, including a yeast two-hybrid system, in vitro biochemical assays, IF and co-IP in cultured cells and tissues. Using the lipid bilayer reconstitution system, we further documented functional interaction where the channel activity of both in vitro translated and endogenous hST PC2 was substantially increased in the presence of α-actinin.

Actin filaments are of crucial importance to cell morphology, adhesion, proliferation and migration. Alpha-actinin, a widely distributed actin-bundling protein, is a prominent component for cell–cell and cell–matrix adhesion: it associates with integrin receptors at cell–matrix focal contacts and with the cadherin/catenin complex at cell–cell adhesion belts (31,32). Clustering and engagement of integrin receptors or the cadherin/catenin complex lead to the formation of complete adhesion junctions, where integrins or cadherins are linked to intracellular cytoskeletal complexes and bundles of actin filaments (49). In addition to α-actinin, these protein assemblies also contain paxillin, vinculin, filamin and talin and may serve as frameworks for association with signaling proteins that regulate signal transduction pathways and lead to integrin- or cadherin-induced changes in cell behavior (50). Interestingly, PC1, a PC2 partner, associates with numerous adhesion cluster proteins including vinculin, talin, paxillin, focal adhesion kinase, α2β1-integrin, E-cadherin, βγ-catenin and α-actinin. Given the associations between PC1 and PC2 (10), PC1 and α-actinin (12), and PC2 and α-actinin (this report), it seems that the formation of cell–matrix and cell–cell adhesion junctions requires polycystins as well as structural and signaling anchoring junction proteins.
Figure 9. Regulation of hST PC2 channel function by α-actinin. (A) Representative single-channel tracing (left panels) at +40 mV of reconstituted hST apical membranes in asymmetrical KCl solutions (Materials and Methods). The corresponding all-point histograms are shown on the right panels. Upper panels, in the absence of α-actinin; lower panels, in the presence of non-muscle α-actinin in the cis chamber. (B) Upper left panel, representative tracings recording at +40 mV before and after the addition of α-actinin (as indicated). Lower left panel, averaged tracing from six experiments. Right panel, channel open probability (NPo) averaged from the six experiments before and after the application of α-actinin. (C) Cation channel activity in the presence of trans amiloride (100 μM) averaged from six membrane patches, under the same ionic conditions as in (A) and (B) and at +40 mV. This is compared with channel activity in the absence of amiloride averaged from eight patches of membrane (P < 0.0002).
PC1 brings together extracellular matrix, cell membrane and actin cytoskeleton in conjunction with integrins and cadherins, whereas PC2 links the actin cytoskeleton to the cell membrane and may regulate local ions such as Ca\(^{2+}\), which is important for the regulation of protein–protein interactions within the polycystin–junction protein complex. Thus, further characterization of this complex may ultimately provide a deeper insight into the molecular mechanism of ADPKD.

Besides, α-actinin has also been reported to bind specific phospholipids (51–53). This association decreases α-actinin bundling activity through competitively blocking the interaction between its actin-binding domain and the actin filament. This association is also proposed to affect the subcellular localization of α-actinin through controlling the amount of α-actinin available in the cytoplasm for bundling and organization of the actin cytoskeleton. It remains to be determined whether phospholipids can regulate the interaction between PC2 and α-actinin.

Recent reports have demonstrated that α-actinin can regulate the activity of a number of channels. The muscle actinin isoform, α-actinin-2, for example directly binds voltage-gated K\(^+\) channel Kv1.5 and modulates its channel gating and current density (37). Alpha-actinin-2 was also found to bind the NR1 and NR2B subunits of the NMDA-type glutamate receptor and regulates its channel function (34,54). Over-expressed α-actinin-2 was found to compete with calmodulin for binding the NMDA receptor (34). More recently, it was revealed that the muscle type α-actinin regulates L-type Ca\(^{2+}\) channel function (45). Our present data demonstrate that α-actinin connects PC2 to the actin cytoskeleton for both structural and regulatory purposes. This suggests that PC2 channel function can also be modulated through dynamic alteration of the actin networks. Indeed, we recently found that cytochalasin D, monomeric actin and actin filament-severing protein gelsolin modulate PC2 channel function in human placenta. Thus, cytoskeletal networks exert a dynamic interaction with PC2 protein, i.e. directly and indirectly modulate its channel function, in addition to help its anchorage to the membrane. Given the involvement of α-actinin and actin filaments in cell morphogenesis and growth, the functional and physical interaction between PC2 and α-actinin may be important for the link between cyst formation and altered cell adhesion, proliferation and migration.

Alpha-actinin is present in various types of renal cells including epithelial and blood vessel cells. Evidence of the relevance of α-actinin to renal diseases includes the findings that experimental nephritic syndrome is induced by α-actinin up-regulation (55) and that mutations in α-actinin-4 cause familial focal segmental glomerulosclerosis (56). It was also reported that α-actinin exhibits tumor suppressor activity. For instance, mouse NIH 3T3 fibroblasts over-expressing non-muscle α-actinin displayed a significant reduction in cell motility, whereas 3T3 cells in which the expression of α-actinin was reduced to 25–60% of the control level had increased cell motility (38). Such α-actinin-deficient cells form tumors upon injection into nude mice. Moreover, α-actinin-4 regulates the actin cytoskeleton, increases cell motility and its inactivation abolishes the metastatic potential of human cancer (27). Stable clones isolated from highly malignant neuroblastoma stem cells [BE(2)-C] after transfection with α-actinin-4 exhibits decreased anchorage-independent growth, loss of tumorigenicity in nude mice and decreased expression of N-myc proto-oncogene (39).

Recent studies suggest plausible causal link of both dominant and recessive PKD to structural and functional defects in microtubule-dependent primary cilia of renal epithelial cells (57,58). In fact, major cystoproteins, including PC1, PC2, polaris (orpk), cystin (cpxk) and inversin (inv), are present in renal primary cilia or the basal part of cilia (or centrosome) (57,59) and demonstrated functional importance. For example, a complex containing PC1 and PC2 acts as a mechanosensor in renal primary cilia for fluid flow and defects in cilia mechanosensation due to dysfunctional polycystins may result in cyst formation (57). Targeted disruption of KIF3A, a subunit of the microtubule-associated motor protein kinesin II, results in cyst development (60). Interestingly, PC2, polaris, inversin, kinesin II and dynein are involved in defining directional flow at the cilia-containing node and left–right asymmetry (61). Human inversin is responsible for nephronophthisis type 2 and linked to PKD phenotypes, ciliary function and left–right asymmetry (62). These findings indicate that microtubules should play an important role in the link between cilia structure/function and cystoproteins.

In addition to PC2s cilium targeting, its distribution on the endoplasmic reticulum and plasma membranes is still controversial (15,19,42–44), which seems to be cell type and developmentally dependent, possibly due to different interacting partners or signaling molecules and the various stages of the cell cycle. Most recently, it was reported that PC2 binds phosphofurin acidic cluster sorting proteins PACS-1 and PACS-2 in a CK2 phosphorylation-dependent manner (63) and that this binding is required for the routing of PC2 between the endoplasmic reticulum, Golgi and plasma membranes. An increasing body of evidence supports that PC2 plays different cellular functions such as flow sensing, cell division and cell morphology via its different subcellular localizations (e.g. plasma membrane, endoplasmic reticulum, mitotic spindle and cilium) where it interacts with different partner proteins, in particular, cytoskeleton components. Other cystoproteins, such as PC1, polaris, cystin and inversin, also associate with a variety of cytoskeletal proteins, which may be important for their multiple cellular functions. Future studies on physical and functional interactions of cystoproteins with cytoskeleton networks will be beneficial to understanding the molecular mechanisms of cystic diseases.

MATERIALS AND METHODS

Yeast two-hybrid system

A yeast two-hybrid screen using the intracellular N-terminus of human PC2 as bait was performed using human heart library (Clontech) in the yeast strain AH109 containing Ade2, His3 and LacZ reporter genes under the control of the GAL4 upstream activating sequences as described recently (23). Recovered primary positive clones and various cDNA constructs of PC2 (PC2N, PC2C, PC2CA, PC2CB, PC2CC and PC2CD) and α-actinin (Fig. 1) were transformed into
the yeast host strain Y187 containing LacZ reporter gene for specific pair interaction confirmation.

**Cell culture and transfection**

Mouse fibroblast NIH 3T3, HEK 293, MDCK and IMCD cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells of fewer than 25 passages were cultured to full confluence before collection. Transient transfection of PKD2 was performed on 3T3 cells cultured to 90% confluence using Lipofectamine 2000 (Invitrogen, Toronto, Canada) according to the manufacturer’s recommendation.

**Plasmids and antibodies**

Alpha-actinin-2 cDNA was amplified from human heart cDNA library and α-actinin-1 cDNA was a generous gift of Dr S. Hirohashi (National Cancer Center Research Institute, Japan). Mouse monoclonal anti-PC2 antibody IA11 was raised against GST-tagged PC2C (23,24) and purified for co-IP (1 : 50 dilution) and dot/western blots (1 : 1500). Goat polyclonal anti-PC2 antibody G20 (Santa Cruz Inc., Santa Cruz, CA, USA) was used for IP (1 : 50), western blot (1 : 500) and IF (1 : 50). Mouse monoclonal antibodies BM75.2 and EA53 (Sigma-Aldrich Canada) were used against non-muscle and muscle types of α-actinin, respectively. Rabbit polyclonal antibodies against GST (a kind gift of L. Fliegel), spectrin (Sigma-Aldrich Canada, Cat no. S1515), Na+K+ATPase α1 subunit (Upstate, Charlottesville, VA, USA) and calreticulin (Santa Cruz) were also used for western blotting. The following secondary antibodies were used: rabbit anti-goat IgG fluorescein isothiocyanate (FITC) and donkey anti-mouse IgG-rhodamine (Chemicon International, Temecula, CA, USA) for IF (1 : 70); goat antimouse, -rabbit and -goat IgG-horseradish peroxidases (Chemicon International) for dot and western blots.

**GST pull-down**

The cDNA fragments encoding PC2N, PC2C, PC2CA, PC2C, PC2CC and PC2CD were subcloned into vector pGEX5X (Pharmacia, Piscataway, NJ, USA). Expression and purification of GST or GST fusion proteins were performed as described (23,24). Pre-cleared bacterial protein extracts (250 μl) containing GST-tagged PC2C, PC2C, PC2CA, PC2CB, PC2CC and PC2CD or GST alone were incubated with 2 μg of purified non-muscle α-actinin from chicken gizzard (Sigma-Aldrich Canada) or muscle α-actinin from rabbit skeletal muscle (Cytoskeleton Inc., Denver, CO, USA) in the binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl2). The mixture was incubated at room temperature (RT) for 1 h with gentle shaking, followed by another hour of incubation after addition of 10 μl glutathione–agarose beads (Sigma-Aldrich Canada). The beads were then washed several times with 140 mM NaCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.5 and the remaining proteins were eluted using 10 mM glutathione, 50 mM Tris, pH 8.0. The protein samples were resolved by SDS–PAGE (10%) and transferred onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, USA). The filters were then blocked with 3% skim milk powder, immunoblotted with α-actinin antibodies BM75.2 or EA53 and visualized with enhanced chemiluminescence (Amersham, Baie d’Urfe, Canada).

**Dot blot overlay**

Purified chicken gizzard non-muscle α-actinin, rabbit skeletal muscle α-actinin and bovine albumin (1 μg each) (Sigma-Aldrich Canada) were spotted on dry nitrocellulose membrane strips. Membranes were then allowed to air dry for 10 min and saturated with phosphate-buffered saline (PBS) containing 3% BSA for 1 h at RT. The strips were subsequently incubated at 4°C overnight with native HEK293 or MDCK cell lysates in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl2 or 1 mM EGTA, 1% BSA, washed with PBS twice (10 min each) and immunoblotted with PC2 antibody IA11. Purified GST–PC2C fusion protein was used as positive control.

**Co-immunoprecipitation**

Reciprocal co-IP was performed using fresh lysates of native HEK293, MDCK cells (2 × 106 cells), rat adult kidney and heart tissues (10 mg total protein). Cell monolayers in 100 mm dishes were washed twice with PBS and solubilized in ice-cold CellLytic-M lysis buffer and protease inhibitor cocktails (Sigma-Aldrich Canada). Adult Sprague-Dawley rat kidney and heart tissues were flash-frozen in liquid nitrogen, macerated to fine powder and solubilized in lysis buffer. Supernatant was collected following two consecutive centrifugations at 16 000 g for 20 min and 100 000 g for 1 h to remove cell debris. Equal amounts of total protein from final supernatants were pre-cleared for 1 h with protein G or A-sepharose (Sigma-Aldrich Canada) and then incubated with antibody IA11 for another hour on ice. After the addition of 150 μl of 50% protein G or A-sepharose, the mixture was incubated for another hour with gentle shaking. The immune complexes absorbed to protein G or A-sepharose were washed four times with the lysis buffer (1.2 ml each). The precipitated proteins were analyzed by western blotting (6–8% SDS–PAGE) using antibody BM75.2, EA53 or antibody against spectrin. Co-IP experiments were conducted reciprocally.

**Immunofluorescence**

Cells were grown on coverslips, fixed with methanol/acetone (1 : 1, from −20°C) for 2 min at RT and washed five times with PBS. Cells were then blocked in PBS with 5% skim milk powder for 1 h and incubated with G20 and BM75.2 primary antibodies at RT for 1 h, followed by another hour of incubation with secondary antibodies. Cells were finally washed with PBS containing 0.1% Tween 20. Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) was used to protect IF signals from fading. Fluorescent images were captured on a Zeiss 510 confocal laser scanning microscope with argon 488 nm and helium–neon 543 nm lasers or a motorized Olympus IX81 microscope installed with a CCD cooling RT SE6 monochrome camera (Diagnostic Instruments, Sterling Heights, MI, USA). Final composite
The authors are grateful to Dr S. Hirohashi for providing samples (in the Vectashield mounting medium) at 37°C on the slide. We minimized these problems by incubating samples (in the Vectashield mounting medium) at 37°C for 10 min and pre-treating slides with phospholipids to better stick vesicles prior to photograph.

Cell surface protein biotinylation

MDCK and IMCD cells and cells were grown to 90–95% confluence in 10 cm Petri dishes. Cell surface membranes were labeled with Sulfo-NHS-biotin (Pierce, Rockford, IL, USA) following instructions provided by the manufacturer with modifications. Briefly, cells were rinsed twice with PBS and then Sulfo-NHS-biotin (0.5 mg/ml) was added and incubated at RT with gentle agitation for 30 min. The reaction was terminated by addition of NH₄Cl (1 m) to a final concentration of 10 mM. After washing cells with PBS three times, ice-cold Sigma Celllytic™-M reagent supplemented with protease inhibitor cocktail (Sigma-Aldrich Canada) was added to lyse the cells. After pre-clearing the lysates biotinylated proteins were pulled down by monomeric avidin beads while remaining, flow-through proteins were enriched by the anti-PC2 antibody 1A11. The positive control Na⁺/K⁺-ATPase α₁ and negative control calreticulin were conducted in the same way but the flow-through samples were not enriched. Samples were separated on 8% SDS–PAGE and detected using one of these antibodies.

Protein preparation and lipid bilayer electrophysiology

Commercial chicken gizzard α-actinin (Sigma) was used as modulator of PC2 channel. In vitro translated PC2 and PC2-containing hST apical membrane vesicles were prepared and reconstituted in a lipid bilayer system as previously (16,47,48) to assess the channel activity. Briefly, lipid bilayers were formed with a mixture of 1-palmitoyl-2-oleoyl phosphatidylinositol (Avanti Polar Lipids, Birmingham, AL, USA) in a 7:3 ratio. The lipid bilayer was bathed with a solution containing MOPS–KOH, 10 mM, and MES–KOH, 10 mM, pH 7.40, and 10–15 μM Ca²⁺, with 150 and 15 mM K⁺, in the cis and trans compartments, respectively. Electrical signals were obtained with a PC501A patch-clamp amplifier (Warner Instruments, Hamden, CT, USA) with a 10 GΩ, feedback resistor and pClamp 5.5.1 (Axon Instruments, Union City, CA, USA). Signals were low-pass filtered at 700 Hz (~3 dB) with an eight pole, Bessel type filter (Frequency Devices, Haverhill, MA, USA). Signals were stored and analyzed as previously reported (16).

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Conflict of Interest statement. None declared.

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