Analysis of the cytoplasmic interaction between polycystin-1 and polycystin-2

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Autosomal dominant polycystic kidney disease (ADPKD) is a systemic hereditary disease characterized by renal and hepatic cysts. End-stage renal failure is characteristic of this disease, and ADPKD accounts for hemodialysis in 10% of patients in the United States (30). The consequences of mutations in Pkd1 and Pkd2 that cause ADPKD (27, 31) on the function of polycystin-1 (PC1) and PC2 are still not completely understood. However, some mutations may be linked to changes in intermolecular interactions of the components of the channel complex and consequent regulation of the channel formed by PC2.

PC2 is expressed in most adult and fetal tissues, including the kidney, heart, brain, ovaries, testis, and intestine (21, 22) and has been found in both the cilia and endoplasmic reticulum (ER) of renal tubular epithelial cells. The sequence and molecular architecture suggest that PC2 is a member of the transient receptor potential (TRP) family of channels (8, 22), consistent with its function as a nonselective cation channel that is highly permeable to Ca2+ (11, 16, 29). The role of Ca2+ in the function of the channel is not completely understood at the molecular level. However, Ca2+ regulates channel currents with a bell-shaped dependence, where currents can be measured in the presence of low concentrations and inhibited by high concentrations of Ca2+ (4). PC2 channel activity can also be modulated by intermolecular interactions with protein partners that include troponin I, α-actinin, and PC1 (17–19, 24). Alterations in the ability of PC2 to conduct Ca2+ have been seen in ADPKD, and some disease-associated point mutations result in complete loss of the ability of PC2 to function as a Ca2+ channel. This disruption of normal Ca2+ signaling by PC2 may be an important factor for development of ADPKD.

PC1 is a large protein that is expressed on the plasma membrane and primary cilia of renal cells, and it is also expressed in many other tissues including the heart, brain, muscle, and bone (10, 14). PC1 shares a region of high sequence similarity to PC2 (22), but it does not appear to be a cation channel and has less clearly understood functions. The N-terminal portion of PC1 encodes a large (~3,000 amino acid) extracellular fragment with multiple domains and predicted functions. The C-terminal cytoplasmic portion of PC1 is much smaller (~200 amino acids) and has been shown to translocate to the nucleus following regulated intramembrane proteolysis where it may modulate transcription (7). The PC1 C-terminal region also mediates an interaction with the PC2 C-terminal tail (13, 24, 28) and can activate channel activity of PC2 in bilayers (32).

The cytoplasmic C-terminal region of PC2 plays an important role in regulation of the channel, and truncation of this region results in the inability of the channel to conduct Ca2+. The molecular basis for this is not understood, but recent studies by us have begun to unravel the function of this region of PC2 by showing that there are two distinct domains in the PC2 cytoplasmic C-terminus (6). One of these domains is a Ca2+-binding EF-hand domain with predicted structural similarity to canonical EF-hand domains. The other is a coiled-coil domain (33) that may be important for channel formation. Here, we provide an investigation into the roles that these two PC2 C-terminal domains, the EF-hand domain (PC2-EF) and the coiled-coil domain (PC2-CC) have in the interaction of PC2 with PC1. These studies provide a framework to understand the functional role of the PC1 C-terminal tail in the regulation of PC2 channel activity.

MATERIALS AND METHODS

Protein expression and purification. Briefly (see supplemental text for detailed descriptions of all experimental procedures), N-terminally glutathione S-transferase (GST)-tagged PC1 C-terminal fragments (GST-PC1-C), PC1-4183-4270, and PC1-4202-4243 and N-terminally 6× polyhistidine (His)-tagged PC2 C-terminal fragments (His-PC2-C), PC2-704-968, PC2-720-817, PC2-720-829, PC2-798-927, and PC2-821-927 fusion constructs were expressed in Escherichia coli and purified using standard...
glutathione-Sepharose or nickel-affinity chromatography (all supplementary materials for this article are available on the journal web site). For mammalian expression, PC1 constructs PC1-4183-4270 and PC1-4183-4270-Q4215P were subcloned into mammalian expression vector pcDNA 3.1+. For the pull-down experiments, the PC1 proteins were bound to the glutathione-Sepharose beads. Protein concentration after elution was determined by a Bradford assay.

Pull-down assays of PC1 and PC2 protein fragments. GST-PC1-C constructs were immobilized on glutathione-Sepharose beads and washed twice. Purified His-PC2-C was incubated with the bound GST-PC1-C. Following incubation, the beads were pelleted, washed, and analyzed using SDS-PAGE. Each experiment was conducted at least three times, and His-PC2-C binding was confirmed by Western blotting. Control pull-downs conducted for all His-PC2 constructs using both GST alone and glutathione-Sepharose beads as the bait showed no His-PC2 binding above background.

Pull down of full-length PC2 from Madin-Darby canine kidney epithelial cells by PC1-4183-4270. PC1-4183-4270 and PC1-4183-4270-Q4215P were affinity purified as described above using glutathione-Sepharose 4B beads, separated by anion exchange, and bound to glutathione-Sepharose beads. A micromolar fraction was isolated from confluent Madin-Darby canine kidney epithelial (MDCK) cells using differential centrifugation procedures previously described (1). The MDCK microsomes were incubated with GST-PC1-C on glutathione-Sepharose beads overnight at 4°C. The beads were then pelleted, washed, analyzed using 4–20% SDS-PAGE, and visualized by Western blotting using a PC2 antibody (YCC2, provided by S. Somlo, Yale PKD Center) (5).

Surface plasmon resonance. PC1-4183-4270 and PC2-798-927 were purified as described above and eluted in 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 500 mM imidazole. Binding studies were performed at 25°C using a Biacore 1000 optical biosensor equipped with a sensor chip coated with an anti-GST antibody (Biacore BR-1002-23). GST-PC1-C or GST alone was captured on the anti-GST antibody surface, and PC2 binding was monitored using a twofold dilution series of 0.625, 1.25, 2.5, 5.0, and 10 μM PC2 injected in duplicate. Binding responses were double-referenced against blank injections of buffer over the GST-PC1 surface and the nonspecific binding to GST alone. Steady-state responses at the end of the association phase were used to determine Kd.

Cell culture, transfection, and imaging. MDCK cells were cotransfected with pcDNA3.1 + vector containing either wild-type or Q4215P PC1 constructs (PC1-4183-4270 or PC1-4183-4270-Q4215P) and pIRE2-DsRed2. Cells were loaded with 10 μM fura 2-AM (Molecular Probes-Invitrogen, Carlsbad, CA). Ratios of Ca2+ imaging was performed with a Zeiss Axiovert 100 microscope. Intracellular Ca2+ concentrations ([Ca2+]i) were derived from background-subtracted F360/F340 fluorescent ratios (R) after in situ calibration (12, 15) using [Ca2+]i = Kd × β × (R − Rmin)/(Rmax − R), where Kd is the dissociation constant of fura 2 for Ca2+ at 37°C (225 nM), Rmin and Rmax were experimentally determined, and β was the fluorescence ratio of the emission intensity excited by 380 nm for Ca2+-free compared with Ca2+-saturating imaging buffer. Nondetectable cells were excluded from the evaluation.

Statistical analysis. Released Ca2+ (peak and total) was calculated by subtracting baseline Ca2+ of each individual cell and plotted over time. Total Ca2+ release (area under the curve) was calculated using SigmaPlot and resting Ca2+ by averaging the baseline Ca2+ of each individual cell over a period of at least 30 s with Microsoft Excel. Statistical analysis was performed using one-way ANOVA (Student-Newman-Keuls method) for multiple group comparisons, and a P value of <0.05 was considered statistically significant.

RESULTS

PC2 interaction with PC1 is mediated by C-terminal coiled coils. The C-terminus of PC2 is critical for channel activity (16). We recently showed that there are two domains in this cytoplasmic region, an EF-hand domain and a coiled-coil domain (6) and that these domains are separated by an ~30-residue linker. We also demonstrated that the presumed function of these two domains is distinct, with biophysical analyses showing that the EF-hand domain binds Ca2+ with micromolar affinity and the coiled-coil domain oligomerizes (6). To investigate the role of these two domains for association with the PC1 C-terminal tail construct (PC1-4183-4270), we conducted pull-down experiments utilizing three constructs: PC2-704-968, PC2-821-927, and PC2-720-797. These constructs encoded the full cytoplasmic region of PC2, the newly identified coiled-coil domain, and the EF-hand domain, respectively (6) (Fig. 1). Our results showed that the C-terminal tails of PC1 and PC2 interact in this system, an observation that correlates well with results reported by others (24, 28, 32). More significantly, these results show that this interaction is mediated by the newly identified PC2 coiled-coil domain and not by the PC2 EF-hand domain (Fig. 2A).

PC2 linker is not sufficient to mediate interaction with PC1. We next investigated the role of the linker between the EF-hand and coiled-coil domains of PC2. The role of this linker region in PC2 channel activity is not fully described; however, posttranslational modification in this region (S812) alters PC2 channel activity and localization (4). To investigate whether the linker between the PC2 EF-hand and coiled-coil domains was sufficient for PC2 association with PC1, we made two sets of constructs. One set contained PC2 coiled-coil domain constructs with different N-terminal start residues, whose constructs began at R798 and G821 (Fig. 1). These constructs were likely to bind PC1-C due to the presence of the coiled-coil domain of PC2. We found that both constructs bind GST-PC1-C (Fig. 2A, lanes 2–7). The second set of constructs encoded the PC2-EF-hand domain, which does not bind to PC1, and portions of the PC2 linker. These constructs (PC2-720-817 and PC2-720-829) terminated either immediately following the phosphorylation site (S812) (4) or at the N terminus of the coiled-coil domain (6). These constructs were not expected to bind PC1-C because they lacked the coiled-coil domain of PC2. We found that both constructs bind GST-PC1-C (Fig. 2A, lanes 9 and 10 and 12–14). These results suggest that the interaction of PC2 with PC1 is mediated by the PC2 C-terminal coiled-coil domain and that the linker region between the EF-hand and coiled-coil domains is not sufficient to mediate the association by itself.

In similar studies, using shortened constructs of the PC2 coiled-coil domain that included variable lengths of the linker region, we observed a trend toward weaker binding for constructs that did not include the linker. We were, however, unable to obtain quantitative measurements of these interactions because of the inherent variability due to low overall binding. Nonetheless, the results suggest that the presence of the N-terminal linker to the PC2 coiled-coil domain allows a tighter association.
The PC1 coiled-coil domain is necessary and sufficient for association of PC1 and PC2 C-terminal tails (Fig. 2B, lanes 6 and 7).

Affinity of his-PC2-C for GST-PC1-C. The affinity of the C-terminal tails of PC1 and PC2 for one another has never been measured. In our pull-down analyses above, we showed that the EF-hand domain did not contribute to the binding of these two proteins. Therefore, we investigated the affinity of this interaction using the longer PC1-C construct and a PC2 construct that included the linker and coiled-coil domain (PC1-4183-4270 and PC2-798-927). To obtain an approximate value for $K_{0.5}$ between the C-terminal tails of PC1 and PC2, we conducted pull-down and surface plasmon resonance (SPR). Densitometric analysis of Coomassie-stained SDS-PAGE pull downs yielded a $K_{0.5}$ in the low-micromolar range (data not shown). To obtain a quantitative estimate of PC1-PC2 C-terminal tail interaction, we then conducted SPR (Fig. 3). In the SPR system, oligomerization of the analyte can result in avidity effects which preclude fitting the kinetics to a simple 1:1 binding model (23). These effects were present in our SPR analysis and indicate that the coiled-coil domain of PC2 is indeed multivalent for PC1, a result that suggests the presence of multiple PC2-PC1 interactions in the polycystin ion channel complex. For complicated systems such as this, a steady-state analysis can, however, provide a reasonable estimate of $K_{0.5}$. The steady-state analysis (Fig. 3, bottom) yields a $K_{0.5}$ of 2.9 ± 0.9 μM.

Helix-breaking disease mutant results in abrogation of interaction between PC1 and PC2. One of the point mutations in PC1 that has been documented to cause ADPKD in humans is Q4224P (3). The effect of this point mutation on the direct interaction of PC1 and PC2 has not been shown but may arise from the helix-breaking proline residue, preventing formation of an intact PC1 binding site for PC2. In the mouse, the equivalent mutation is Q4215P (see alignment in Fig. 1A). This mutation was incorporated into construct PC1-4183-4270 to investigate whether it does indeed abrogate the binding of PC1 and PC2 C-terminal tails. No interaction was detected with the mutated PC1. Densitometric analysis of the pull-down experiments showed virtually no association above background for PC1-4183-4270-Q4215P and PC2-704-968 (Fig. 4A).

Native full-length PC2 is pulled down by GST-PC1-C but not the disease mutant. To investigate whether full-length PC2 from native tissue was able to associate with the C-terminal tail of PC1 and whether the ADPKD mutation, Q4224P, abrogates this interaction, we conducted pull downs using native PC2 and beads coated with PC1-4183-4270 or PC1-4183-4270-Q4215P, the mouse ortholog of the disease mutant (Fig. 1A). Full-length PC2 is expressed in the ER, which is included in the cytoplasmic fraction of MDCK cells. The ER was isolated from MDCK cells as the microsomal fraction of the cytoplasm and used in pull-down experiments. We found that full-length PC2 is pulled down by PC1-4183-4270 but not by the construct containing the disease mutant (Fig. 4B). These results suggest that a major effect of the Q4224P ADPKD mutation is to interfere with the association of PC1 and PC2.

Role of C-terminal region of PC1 on intracellular PC2 Ca$^{2+}$ signaling. The functional role of the C-terminal tail of PC1 in maintaining normal Ca$^{2+}$ signaling within a cell has not been well described. We hypothesized that overexpression of the C-terminal portion of PC1 would alter Ca$^{2+}$ signaling by...
interfering with normal PC1-PC2 interaction and that overexpression of the disease mutant C-terminal portion of PC1 would not. To investigate this, we overexpressed the PC1-C constructs PC1-4183-4270 and PC1-C-4183-4270-Q4215P in MDCK cells (Supplementary Fig. S2). We found that expression of these constructs in MDCK cells did not alter the resting Ca\(^{2+}\) (Fig. 5A). Also, the ER Ca\(^{2+}\) load was not altered by expression of these constructs, as shown by the magnitude of the release of Ca\(^{2+}\) stored in the ER after addition of the inhibitor thapsigargin (data not shown); the released Ca\(^{2+}\) was similar in all groups monitored.

When cells were stimulated with a saturating concentration of agonist (15 \(\mu\)M ATP), the response was maximal for all conditions and it was not possible to separate out differences among the treatment groups (data not shown). However, when cells were stimulated with a lower concentration of agonist (3 \(\mu\)M ATP), we found that the peak Ca\(^{2+}\) level achieved was reduced when PC1-C was overexpressed (Fig. 5B), a situation where the normal interaction between full-length PC1 and PC2 would be lost; note that the duration of response was unchanged (Fig. 5C). Expression of the mutated version of PC1-C (PC1-C-4183-4270-Q4215P) did not alter the peak Ca\(^{2+}\) levels (Fig. 5B), as expected for a construct that does not interfere with the normal interaction between PC1 and PC2. These results show that the interaction between PC1-C and PC2-C can influence intracellular Ca\(^{2+}\) signals and that the modulation does not occur when the interaction between the two proteins is lost. The disease-related point mutation alters this functional interaction.

**DISCUSSION**

In this study, we show that the newly identified coiled-coil domain of PC2 (6) is critical for maintenance of the PC1-PC2 interaction and that disruption of the interaction of the C-terminal tails of PC1 and PC2 can alter normal cellular Ca\(^{2+}\) signaling. This result helps explain the pathological effect of extreme C-terminal truncations observed in PC2 in ADPKD patients (25); normal channel regulation by PC1 cannot be maintained in these patients. Other studies have shown that this interaction is important for PC2 channel regulation (32) but divergent expression patterns suggest that PC1 and PC2 are not always associated (9). Here, we provide the first quantitation of the affinity of this interaction and show that \(K\_o\) for C-terminal tail-mediated interaction between PC1 and PC2 is in the low-micromolar range. This is an interesting result in the context of the recent structural study showing crystallization of only the PC2 coiled-coil domain under crystallization condi-
tions that contained both PC1 and PC2 (33). Additionally, our observation that the linker may be important for binding provides a molecular basis for the finding that phosphorylation of S812 regulates PC2 channel activity (4).

Intracellular Ca\(^{2+}\) changes have been shown to control an increasingly large number of cellular events, and modulation of intracellular Ca\(^{2+}\) signaling is necessary to maintain cellular integrity. Recently, it has become clear that PC2 plays a role in maintaining Ca\(^{2+}\) signaling in a number of cell types. For example, there is synergy with both the inositol 1,4,5-trisphosphate receptor (20) and the ryanodine receptor (2), and there are profound changes in the intracellular Ca\(^{2+}\) transients when regulation by full-length PC2 is deleted (2) or overexpressed (16). Similarly, when pathogenic mutations are expressed intracellular signaling is altered (16). Here, we also show that mutation of a portion of PC1, the C-terminal region, disrupts its ability to bind to its cellular partner PC2 and that this change leads to alterations in Ca\(^{2+}\) signaling.

Multiple proteins have been suggested to interact with the C-terminal fragments of both PC1 and PC2. Many of these protein-binding partners can modulate channel activity (13, 24, 28, 32), and some of these interactions with PC2 have been suggested to take place in what is now described as the C-terminal coiled-coil domain (18, 19), overlapping with the region that we describe here as critical for PC1-PC2 associa-

![Fig. 4. Polycystin-1 Q4215P mutant does not bind PC2. A: pull downs show that introduction of the Q4215P mutation into PC1-4183-4270 interrupts association with PC2-798-927. Arrow indicates PC2-798-927. PC2, PC2-704-968 loaded; B, bound PC1 on beads; PD, pull down on beads following 3 wash; GST, GST marker; n = ≥3. B: GST-PC1-C pulls down full-length PC2 from Madin-Darby canine kidney epithelial cell (MDCK) microsomes; however, introduction of the disease-related mutation Q4215P into PC1 abrogates this pull down. PC1, PC1-4183-4270; “cells,” loaded MDCK microsomes; PD, pull down on beads; Sup, supernatant following pull down. Black lines indicate the intervening lanes have been spliced out. PC2 in the supernatant was in excess of PC1 on the beads to maximize any possible interaction. Blot is representative of >3 experiments.](http://ajprenal.physiology.org/)

![Fig. 5. Expression of PC1-C, but not PC1-C-Q4215P, alters intracellular Ca\(^{2+}\) signaling. MDCK cells were loaded with fura 2-AM, and changes in intracellular Ca\(^{2+}\) levels were monitored upon addition of extracellular ATP in the absence of extracellular Ca\(^{2+}\). In all cases, the values plotted represent the average of the response measured in 92-254 cells from experiments performed on at least 5 different days. Control, experiments where cells were mock transfected with empty vector. A: resting Ca\(^{2+}\) levels were unchanged by the expression of PC1-4183-4270 (wild-type) or PC1-4183-4270-Q4215P (Q-P mutant). B: peak Ca\(^{2+}\) release was attenuated after addition of 5 μM ATP in cells expressing PC1-C wild-type, but not the Q-P mutant, presumably because expression of PC1-C disrupts the normal interaction between full-length PC1 and PC2 in MDCK cells. C: duration of the response to 5 μM ATP was similar in cells expressing PC1-C wild-type and the Q-P mutant. *Statistically significant (P <0.05).](http://ajprenal.physiology.org/)
tion. It is not currently clear whether the multiple protein-binding partners of PC2 (13, 24, 28, 32) are exclusive of or cooperative with PC1, and how these proteins interact with PC2; however, the diversity in modulation of channel activity and the similarity in binding sites suggest that active protein-mediated regulation of PC2 Ca\(^{2+}\) signaling may occur in normal channel function.

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