Polycystin-2 Interacts with Troponin I, an Angiogenesis Inhibitor†

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ABSTRACT: Polycystin-2 (PC2), encoded by the *PKD2* gene, is mutated in 10−15% of autosomal dominant polycystic kidney disease (ADPKD) patients. PC2 is a Ca^{2+}-permeable nonselective cation channel and is present in kidney and many other organs. Likewise, *PKD2*-mutated patients and mice exhibit extrarenal abnormalities. In comparison with cysts in the kidney, liver, and pancreas, abnormalities in the heart, brain, and vascular vessels are less understood. In particular, roles of PC2 in muscle and endothelia remain largely unknown. In the present study, using a yeast two-hybrid screening, we discovered that the PC2 carboxyl terminal domain (D682-V968) interacts with the cardiac troponin I, an important regulatory component of the actin microfilament in cardiac muscle cells. This interaction was demonstrated by GST pull-down and microtiter binding assays. Dose-dependent binding between PC2 and troponin I followed a Michaelis−Menten relationship, indicating a 1:1 binding stoichiometry. The interacting domains were located to the R872−H927 segment of PC2 and the M1−V107 and K106−L158 segments of troponin I. Co-immunoprecipitation experiments demonstrated that the cardiac and two skeletal isoforms of troponin I were all associated with PC2, when coexpressed in mouse fibroblast NIH 3T3 cells and *Xenopus* oocytes. Furthermore, reciprocal co-immunoprecipitation verified the interaction between the native polycystin-2 and troponin I in human adult heart tissues. This study thus provides new evidence for a direct attachment of PC2 to the actin microfilament network, in addition to the recently identified association between PC2 and trypomyosin-1. Troponin I functions as an inhibitory subunit of the troponin complex for calcium-dependent regulation of muscle contraction and as an inhibitor of angiogenesis seen in ADPKD. It is possible that altered interaction due to pathogenic polycystin-1 or -2 mutations can account for angiogenesis in ADPKD and may be corrected to some extent by exogenous troponin I.

Autosomal dominant polycystic kidney disease (ADPKD)[1] is one of the most frequent genetic diseases, affecting approximately 1:1000 of population (*1*). Affected individuals can develop kidney cysts, and ∼50% manifest end-stage renal failure by age 60 (*1*). ADPKD is a systemic, rather than organ-specific, disorder. Extrarenal manifestations include cysts in the liver, pancreas, ovaries, and spleen, cerebral and intracranial aneurysms, mitral valve prolapse, and aorta dissections (*1*, *2*). Mutations in *PKD1* and *PKD2*, which have been mapped on chromosomes 16p13.3 and 4q21−23, respectively (*3*, *4*), are responsible for most cases (∼95%) of ADPKD. The *PKD1*-encoded protein polycystin-1 (PC1) is a large cell-membrane glycoprotein of ∼462 kDa with 11 membrane spans, a short intracellular carboxyl terminus and a large extracellular amino terminus containing multiple domains (*3*). The *PKD2*-encoded protein polycystin-2 (PC2) is a membrane protein of ∼110 kDa containing six transmembrane domains and intracellular N- and C-termini (*4*). PC2 shares significant sequence homology and membrane topology with α-subunits of voltage-activated cation channels and transient receptor potential channels (TRPC) (*5*, *6*) and, indeed, has been shown to be a Ca^{2+}-permeable nonselective cation channel (*7*−*10*). PC2 is present in a variety of tissues. In addition to its marked presence in kidney proximal and distal tubules and collecting ducts, it is also present in epithelia (e.g., pancreas, liver, lung, intestine, and reproductive organs), endothelia (e.g., brain, placenta, and vascular vessels), myocardial and endocardial cells, smooth muscle cells, and skeletal muscle cells (*11*). Mice with targeted *PKD2* mutations display renal, pancreatic and hepatic failure, and cardiac defects (*12*, *13*). In general, the physiological roles for its presence in muscle and endothelia are not well understood.

It is still elusive why ADPKD manifestations may vary from one tissue to another. Among different possibilities, interaction of PC2 with tissue-specific partners may be of interest for investigation. PC2 associates with PC1 through their C-termini (*10*, *14*−*16*), forms a homodimer (*15*), and oligomerizes with TRPC1 channel (*17*). PC2 also interacts with CD2AP (*18*) and Hax-1 (*19*), indicating an indirect attachment to the cytoskeletal network. We recently demonstrated that PC2 is directly associated with the cytoskeleton

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[1] Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; *PKD1*, polycystic kidney disease-1 gene; PC1, polycystin-1; *PKD2*, polycystic kidney disease-2 gene; PC2, polycystin-2; *PKDL*, polycystic kidney disease-like gene; PCL, polycystin-like; TnI, troponin I; GST, glutathione-S-transferase; co-IP, co-immunoprecipitation.

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via tropomyosin-1 (TM-1), an important regulatory component of the actin microfilament (*20*).

Troponin I (TnI) is a constituent protein of the troponin complex which is located on the thin filament of striated muscles that provides a Ca^{2+}-sensitive switch for muscle contraction (*21*, *22*). Multiple isoforms of TnI are found in birds and mammals and encoded by at least three distinct genes: slow-twitch skeletal (*TnI1*, or *ssTnI*), fast-twitch skeletal (*TnI2*, or *fsTnI*), and cardiac isoform (*TnI3*, or *cTnI*) (*23*−*26*). Skeletal and cardiac TnIs consist of 182−210 amino acids. They share substantial sequence homology and are polar proteins with excess of positively charged residues and *p*I value of around 9.9 (*21*). It was recently demonstrated that TnI plays various functional roles, such as acting as angiogenesis inhibitor (*27*) and Ca^{2+} channel modulator (*28*), in addition to being a constituent part of the troponin complex.

In the present study, we used a yeast two-hybrid approach and identified the interaction between the putative intracellular C-terminus of PC2 and TnI3. We further studied this interaction under various in vitro and in vivo conditions and discussed potential roles of TnI in angiogenesis and cystogenesis of ADPKD.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Analysis. The human *PKD2* cDNA encoding the PC2 C-terminal domain (amino acids D682-V968, named PC2C) was amplified from a kidney cDNA library (Clontech, Palo Alto, CA) and subcloned into the GAL4 DNA binding domain of vector pGBKT7. PC2C served as bait to screen a human heart cDNA library constructed with vector pACT2, which contains the GAL4 activation domain (Clontech). Transformants in yeast strain AH109 were grown on synthetic dropout (SD) medium lacking leucine, tryptophan, adenine, and histidine. Obtained colonies were replica-plated and consequently assayed. Putative interacting library plasmids were isolated from the yeast cells and co-introduced in yeast strain Y187 with pGBKT7−PC2C, pGBKT7−PC1C (PC1C refers to the C-terminus G4088-T4302 of polycystin-1), or negative controls, including pGBKT7, pGBKT7-LamC (human lamin C), and pGBKT7-p53 (murine p53). The transformants were then assayed to confirm the interaction and to eliminate potential artifacts. A liquid culture assay was used to quantify β-galactosidase activity using *o*-nitrophenyl β-D-galactopyranoside (ONPG, Sigma-Aldrich Canada) as a substrate.

Cloning of the Full-Length TnI Genes. We isolated three isoforms of *TnI* cDNA from a human heart cDNA library (Clontech) through a standard PCR approach. Three pairs of specific primers were used to amplify the *TnI* cDNA fragments spanning the entire open reading frames: *TnI1* forward 5′ ACCGGAATTcATGCCGGAAG TCGAGAGAAAAC and reverse 5′ ACGCCTCGAGCTATTGTGAGGTCGGAGCATTG; *TnI2* forward 5′ aCcgGAATTcATGGGAGATGAGGAGAAGCGGA and reverse 5′ ACGCCTCGAGCTAGGACTCGGACTCAAACATC; *TnI3* forward 5′ ACCGGAATCCGGCCTGAGTCTCAGCATGG and reverse 5′ ACCGCTCGAGGGCAGGAAGGCTCAGCTCTCA. E*co*R I and X*ho* I sites (as underlined) were engineered into the primers to facilitate subsequent cloning. The authenticity of all PCR-derived constructs was confirmed by sequencing.

GST Pull-Down. The cDNA fragment encoding PC2C was cloned into the B*am*H I site in pGEX-5X-3 (Pharmacia, Piscataway, NJ). The full-length cDNA of *TnI3* was cloned into the B*am*H I and N*de* I sites of pET28a (Novagen, Madison, WI). Expression and purification of glutathione-S-transferase (GST) and poly His-tagged fusion proteins were performed as described by Li et al. (*20*). Pre-cleared bacterial protein extracts (200 μL) containing GST−PC2C or GST alone were incubated with 20 μg of purified poly His−TnI3 fusion protein in the binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$). The mixture was incubated overnight at 4 °C with gentle shaking. It was then loaded onto glutathione−agarose affinity column (Sigma-Aldrich Canada) and incubated at room temperature (RT) for 30 min. The column was then washed four times with 140 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5, and eluted using 10 mM glutathione, 50 mM Tris, pH 8.0. The protein samples were prepared for Western blot. The membranes were blocked with 3% skim milk powder in 50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20, incubated with a poly His antibody (1:1500, Sigma-Aldrich Canada), and visualized with enhanced chemiluminescence (Amersham, Baie d'Urfe, Canada).

Microtiter Plate Assay. The microtiter plate assay was carried out as described (*29*) with some modifications. In short, purified poly His−TnI3 protein (0.2 μg/well) was covalently immobilized onto 96-well microtiter plates by incubating the protein in wells with 1.25 mg mL^1 of 1-cyclohexyl-3-(2-morpholinoethyl) carbodimide metho-*p*-toluenesulfonate (Sigma-Aldrich Canada) in a solution containing 150 mM NaCl, 100 mM Na_2HPO_4, pH 6.0, for 30 min at RT. The wells were washed three times with the PBS buffer and blocked for 2 h at RT in the PBS buffer supplemented with 2% BSA. After washing twice with an antibody buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM Ca^{2+}, 1% BSA, 0.05% Triton X-100), the wells were incubated with various concentrations (0−1600 nM) of purified GST or GST−PC2C, overnight at RT. Bound proteins were detected by incubating the wells with a monoclonal GST antibody (1:1500, a kind gift of Larry Fliegel) and a horseradish-peroxidase-coupled IgG secondary antibody (1:1500, Chemicon International, Temucula, CA). This was followed by incubation with the peroxidase substrate *o*-phenyldiamine (Sigma-Aldrich Canada) for 5 min, addition of 3 M sulfuric acid to stop the reaction, and detection of enzymatic activity at 450 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Reciprocally, GST−PC2C (1 μg/well) was incubated with various concentrations (0−1200 nM) of poly His−TnI3, and the association was detected with a cardiac troponin I antibody (Fitzgerald, Concord, MA). Binding curves were fitted to the Michaelis−Menten Equation ($y = y_0 + (y_{max} \cdot x)/(x + K_{1/2})$) using Sigmaplot 8 (Jandel Scientific Software, San Rafael, CA). Values were expressed as mean ± standard error (SE).

Expression in Mouse Fibroblast Cells and Xenopus oocytes. Full-length *PKD2* and three *TnI* isoforms were cloned into pCDNA3.1 (Invitrogen, Toronto, Canada) for mammalian cell expression or pTLN2 (*30*) for oocyte expression. Mouse fibroblast NIH 3T3 cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. Cells of less than 20 cycles were

cultured to 40% confluency for transient transfection using Effectene Transfection kit (Qiagen, Mississauga, Canada). For expression in *Xenopus* oocytes, capped synthetic RNAs were obtained through in vitro transcription using the mMESSAGE mMACHINE Kit (Ambion, Austin, TX). *Xenopus* oocytes were prepared as described (*31*). In short, stage V−VI oocytes were extracted from *Xenopus laevis* and treated at RT with collagenase I (2 mg/mL) (Sigma-Aldrich Canada) in a Ca^{2+}-free solution for about 2.5 h to remove the follicule. Oocytes were then injected with 50 nL H_2O containing 40 ng of each RNA 3−24 h following defolliculation. Oocytes were then incubated at 18 °C in the Barth's solution supplemented with antibiotics for 3 days prior to immunoprecipitation assays.

Co-Immunoprecipitation. Co-immunoprecipitation (Co−IP) using NIH 3T3 cells transfected with *PKD2* and/or *TnI* was performed 48 h after transient transfection. Cell monolayers in 100 mm dishes were washed twice with PBS. Cells ($\sim 10^6$) were lysed in 500 μL of ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, and proteinase inhibitor cocktails (Sigma-Aldrich Canada). Supernatant was collected following centrifugation. Equal amounts of total proteins (400 μL each) from postnuclear supernatant were incubated with the PC2 antibody, raised from GST−PC2C fusion protein, or TnI antibodies (skeletal, Biodesign, Saco, ME; cardiac, Fitzgerald) (1:70 dilution) on ice for 1 h. After addition of 50 μL protein G-sepharose (Sigma-Aldrich Canada), the mixtures were further incubated for 1 h at RT with gentle shaking. The immunoprecipitates absorbed to protein G-sepharose were washed three times with the lysis buffer. The resultant immunoprecipitates were resuspended in 50 μL of Laemmli's sample buffer. A 25 μL aliquot of the extract was subjected to SDS−PAGE, followed by immunoblotting with the TnI or PC2 antibody. A similar method was employed for co-IP using *Xenopus* oocytes expressing PC2 and/or TnI. Reciprocal co-IP of native PC2 and TnI was performed as described above using human adult heart tissues (10 mg total protein), which were collected from the University of Alberta Hospital. Total protein lysates were treated with desoxyribonuclease (DNase) I (100 μg/mL, Sigma-Aldrich Canada) (*32*) for 4 h at RT prior to the co-IP experiment to examine the modulation of the PC2−TnI interaction by the actin microfilament.

RESULTS

Identification of TnI3 as a PC2 Interacting Partner. The yeast two-hybrid system was used to screen proteins that interact with the putative intracellular C-terminus of PC2, PC2C. This region contains several structural motifs such as an EF-hand, a putative endoplasmic reticulum (ER) retention domain, and phosphorylation sites, indicating that it may mediate interaction with other proteins. A bait construct, pGBKT7−PC2C, was used to screen a human heart cDNA library. Four out of five independent plasmids isolated from the library encoded the full-length cardiac troponin I (TnI3). No β-galactosidase activity was detected by the colony lift assay in the absence of PC2C or when PC2C was replaced by an unrelated protein: the PC1 C-terminus (PC1C), human lamin C or murine p53 (Figure 1A). To evaluate the relative strength of this interaction, we quantified the β-galactosidase activity by a liquid culture assay using ONPG and compared with the positive control

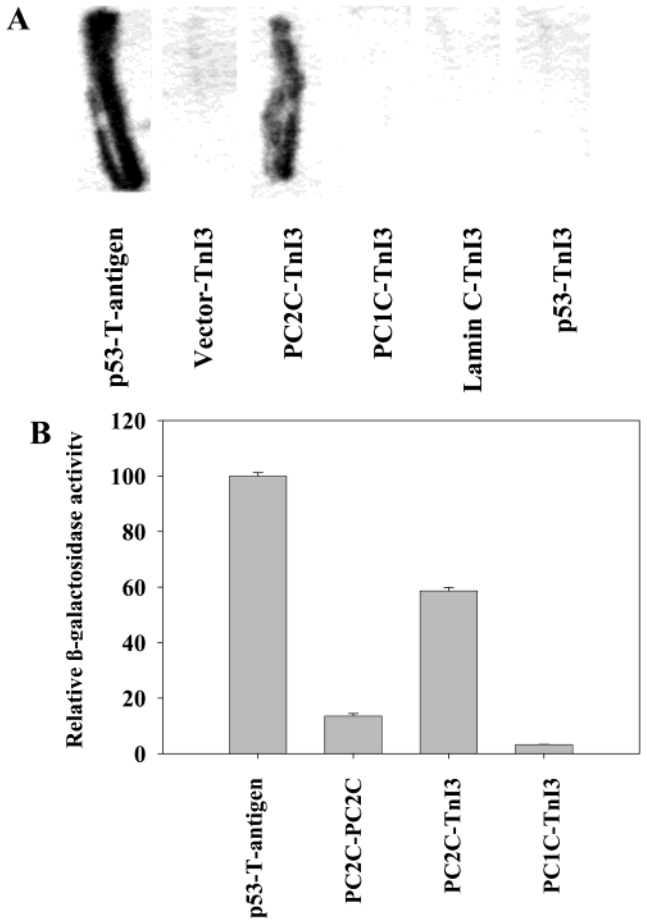

FIGURE 1: PC2C-TnI3 interaction identified by the yeast two-hybrid approach. (A) β-Galactosidase activity in the yeast strain Y187 visualized by colony lift assay to detect the interaction of p53 with T-antigen (positive control) and TnI3 with empty vector, PC2C, PC1C, human lamin C, and murine p53. (B) Quantification of interaction between PC2C and TnI3 in a yeast liquid β-galactosidase assay. The data were initially expressed as A_{420}/A_{600} and then normalized to the level of the p53-T-antigen interaction. PC2C−PC2C and PC1C−TnI3 interactions were evaluated as controls. The histograms represent the means ± SE from five independent experiments.

(p53 to T-antigen association). As illustrated in Figure 1B, the strength of the PC2C−TnI3 interaction was approximately 58% of the positive control level and 4.5-fold higher than that of PC2C−PC2C homodimeric interaction (*15*).

We continued to use the yeast two-hybrid system to delineate the domains of PC2C and TnI3 that mediate the association. Using PCR-based amplification, truncated fragments of PC2C and TnI3 were expressed as fusion proteins in yeast strain Y187 using the pGBKT7 and pGADT7 vectors, respectively, and tested for interaction. As shown in Figure 2, TnI3 interacted with the fragment R872−H927 of PC2C but not with the amino terminus of PC2. On the other hand, interestingly, PC2 interacted with two separate regions of TnI3, M1−V107 and K106−L158.

In vitro Interaction Between PC2 and TnI3. In vitro biochemical methods were applied to further demonstrate the interaction between PC2 and TnI3. We first used a GST fusion protein affinity binding method. For this purpose, the PC2C fragment was fused in frame with a GST epitope and purified from *E. coli*. TnI3 was subcloned into the vector pET28a harboring a poly His epitope. Bacterial cell extracts

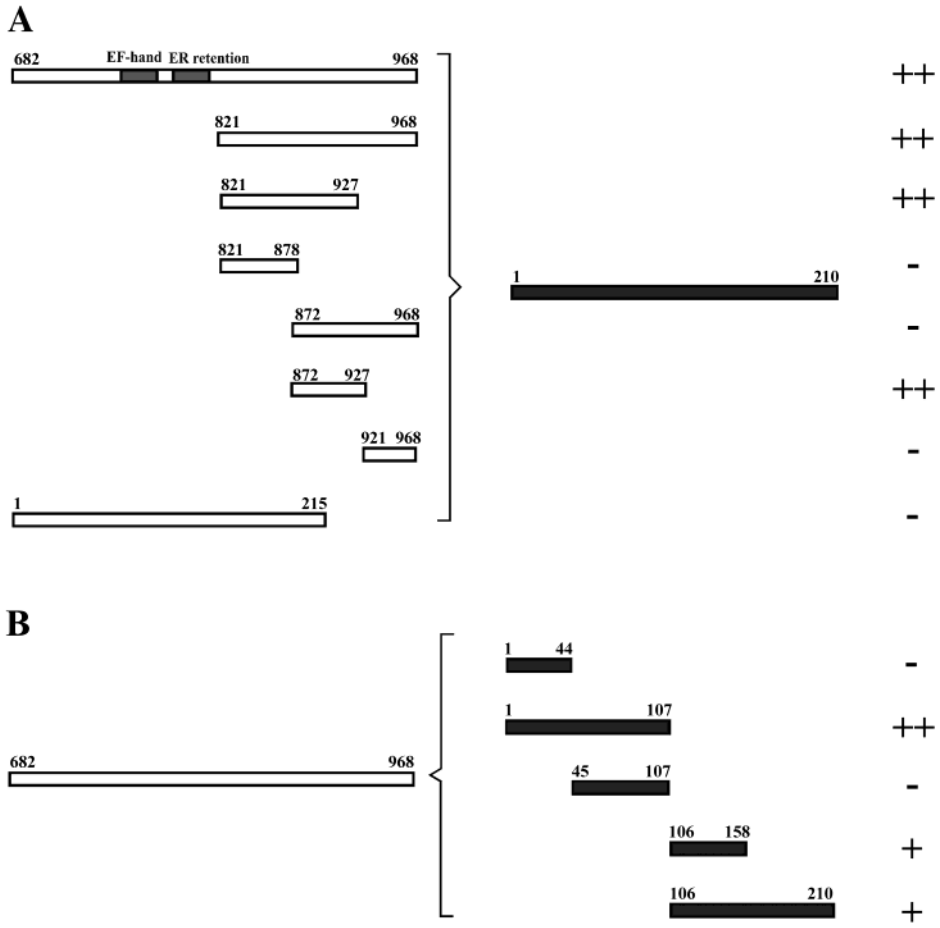

FIGURE 2: Domains of PC2 and TnI3 tested for interaction. PC2 and TnI3 domains were amplified by PCR and constructed in the vectors pGBKT7 and pGADT7, respectively, for the yeast two-hybrid analysis. The transformants were plated on the SD medium lacking leucine and tryptophan in yeast strain Y187. β-Galactosidase activity was analyzed by colony lift assay at 30 °C using X-gal as substrate. The negative control was not shown. The start and end amino acid residue numbers for each domain are indicated. "++", "+", and "−" indicate strong, weak, and no interaction, respectively.

containing GST−PC2C fusion protein were incubated with purified poly His−TnI3, and the mixtures were loaded onto glutathione−agarose affinity column. GST-tagged proteins and their binding partners were coupled to agarose beads. After extensive washes, retained proteins were eluted with reduced glutathione and prepared for Western blot probed with a monoclonal poly His antibody. Figure 3A showed that GST−PC2C but not GST alone (control) coprecipitated with poly His−TnI3.

A microtiter assay was performed to determine binding characteristics between PC2C and TnI3. Purified poly His−TnI3 protein immobilized on microtiter plates was examined for the capacity of GST−PC2C or GST alone to bind. GST−PC2C bound to poly His−TnI3 (0.2 μg, 150 nM) in a dose-dependent and saturable manner after the subtraction of GST alone reading background (Figure 3B, top). The sigmoidicity of the dose dependence provides information on the PC2C:TnI3 binding stoichiometry. We used the Hill equation ($y = y_0 + (y_{max} \cdot x^n)/(x^n + (K_{1/2})^n)$ to fit the data shown in Figure 3B (top), and no sigmoidal relationship was found. This result indicates that the data obey a Michaelis−Menten relation (with $K_{1/2} = 735 \pm 210$ nM, $N = 13$), which suggests that only one PC2C molecule participated in the association with one (or more) TnI3 molecule(s). Conversely, poly His−TnI3 also bound to the immobilized GST−PC2C (1 μg, 340 nM) in a Michaelis−Menten manner ($K_{1/2} = 96 \pm 10$ nM, $N = 4$) (Figure 3B, bottom), which suggests that only one TnI3 molecule binds to one (or more) PC2C molecule(s). Taken together, our data indicated that the PC2C:TnI3 binding stoichiometry was 1:1.

Co-Immunoprecipitation of PC2 with TnI3. To demonstrate in vivo interaction between PC2 and TnI3, we transiently cotransfected full-length *PKD2* and *TnI3* in mouse fibroblast NIH 3T3 cells and analyzed their ability to co-immunoprecipitate. Expression of both proteins in 3T3 cells was examined by immunoblotting. Cell extracts were incubated with the PC2 antibody and protein G-sepharose. Unbound proteins were removed by extensive washes, and the recovery of coprecipitated TnI3 protein was assessed by immunoblotting with the cardiac TnI antibody. TnI3 signal was observed with cells cotransfected with *PKD2* and *TnI3* but not with cells transfected with *PKD2* alone or nontransfected cells (Figure 4A). Likewise, in a reciprocal co-IP experiment, PC2 was detected only with cells cotransfected with *PKD2* and *TnI3* (Figure 4B).

Similar co-IP experiments were conducted using *Xenopus* oocytes as the expression system. Complementary RNAs of *PKD2* and *TnI3* were generated by in vitro transcription and injected into oocytes. PC2 also coprecipitated with TnI3 in oocytes (Figure 4C), with a slightly larger molecular mass than in 3T3 cells based on slower migration on the SDS−PAGE gel, possibly due to different posttranslational modi-

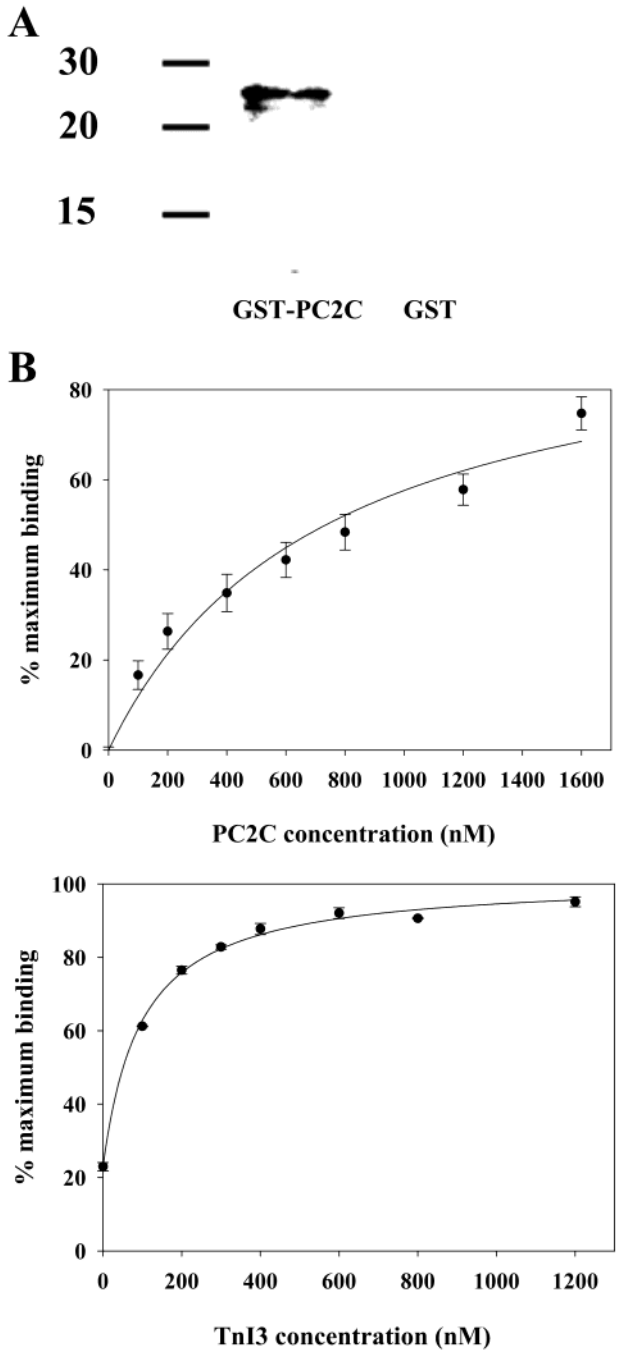

FIGURE 3: In vitro interaction of PC2C with TnI3. (A) Specific interaction detected by the GST pull-down assay. *E. coli* cell extracts expressing GST−PC2C or GST alone were incubated with purified TnI3 at 4 °C for overnight. Glutathione−agarose beads were used to precipitate GST epitope binding proteins. The resultant protein samples were immunoblotted with the poly His antibody. (B) Dose-dependent binding of GST−PC2C with poly His−TnI3 using reciprocal microtiter binding assay. Poly His−TnI3 protein (0.20 μg/well) immobilized on 96-well plates was incubated with various concentrations of GST−PC2C or GST. Bound proteins were incubated with the GST antibody and substrate *o*-phenyldiamine, followed by detection of OD values at 450 nm. Error bars represent the SE of the mean from thirteen replicates. The GST alone background reading was subtracted. The curves were generated by a Michaelis−Menten fit (see Experimental Procedures) to the normalized data (top). Reciprocally immobilized GST−PC2C (1 μg/well) was incubated with various concentrations of poly His−TnI3. Bound proteins were incubated with the cardiac TnI3 antibody, detected, and fitted with the Michaelis−Menten equation (bottom). Error bars represent the SE of the mean calculated from four replicates.

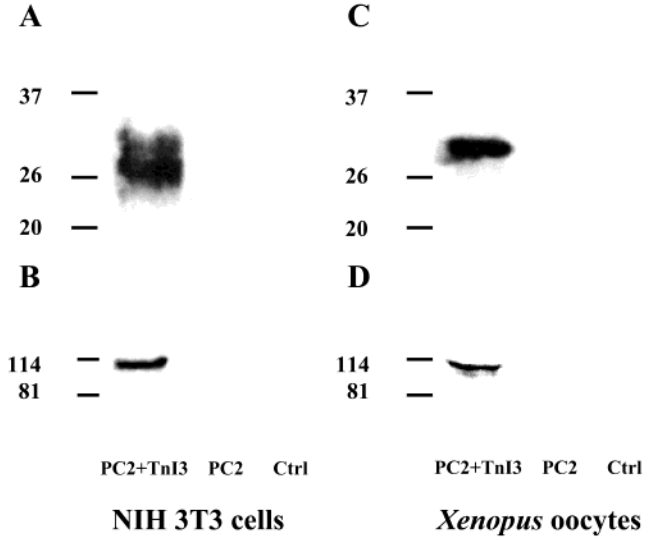

FIGURE 4: Interaction of PC2 with TnI3 in overexpressed NIH 3T3 cells and *Xenopus* oocytes by reciprocal co-IP. (A) Protein extracts from 3T3 cells were incubated with the PC2 antibody. Immunoprecipitated proteins were analyzed by immunoblotting using the cardiac TnI antibody. Results from 3T3 cells transiently expressing PC2 and TnI3, PC2 alone, or nontransfected (Ctrl) were shown. (B) Reciprocal co-IP data. Protein extracts from 3T3 cells were incubated with the cardiac TnI antibody. Immunoprecipitated proteins were analyzed by immunoblotting using the PC2 antibody. Panels C and D are similar to A and B, respectively, but using *Xenopus* oocytes. Molecular mass markers (in kDa) are shown.

fications such as glycosylation. Conversely, like in 3T3 cells, TnI3 was able to coprecipitate with PC2 in oocytes (Figure 4D). Taken together, our data demonstrated that PC2 and TnI3 associate with each other in vivo in both 3T3 cells and oocytes, in agreement with the in vitro data obtained from the microtiter binding and GST pull-down assays.

Association of PC2 with Skeletal TnIs. Three troponin I isoforms, i.e., skeletal TnI1 and TnI2 and cardiac isoform TnI3, have so far been reported and share substantial sequence homology. We employed the co-IP method to determine whether PC2 also associates with the two skeletal TnIs. Indeed, when coexpressed in 3T3 cells or *Xenopus* oocytes, PC2 co-immunoprecipitated with both TnI1 and TnI2 (Figure 5A,B), suggesting that the PC2−TnI interaction may be commonly required in different types of muscle.

Interaction between PC2 and TnI3 in Human Heart Tissues and Effects of DNase I on the Interaction. To examine whether native PC2 and troponin I interact with each other, we performed co-IP experiment using human cardiac tissues. Immunoprecipitation of tissue lysates from human heart with the PC2 antibody precipitated the TnI3 protein (Figure 6A, lanes 1 and 2). Reciprocally, TnI3 was able to precipitate the native PC2 protein from the same tissues (Figure 6B, lanes 1 and 2). To examine whether the PC2−TnI3 interaction is affected by the actin microfilament, we treated tissue lysates with DNase I before reciprocal co-IP assays. It is well-known that DNase I tightly forms a 1:1 molar complex with monomer G-actin and prevents actin polymerization (*32*). We found that, following the DNase I treatment, the PC2−TnI3 association persisted but with significantly reduced interaction intensity (Figure 6A,B, lanes 3 and 4), which suggests that filamentous actins modulate the interaction between PC2 and TnI3, possibly via the known association between troponin I and actin.

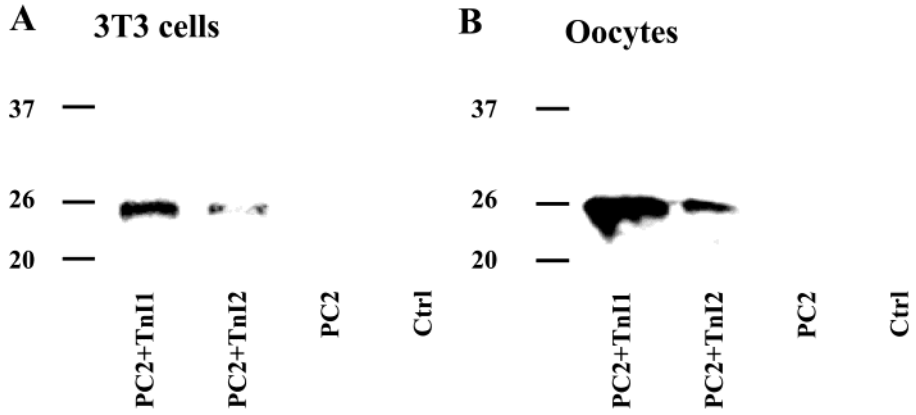

FIGURE 5: Interaction of PC2 with TnI1 or TnI2 in 3T3 cells and oocytes by co-IP. (A) Protein extracts from 3T3 cells were incubated with the PC2 antibody. Immunoprecipitated proteins were analyzed by Western blot using the skeletal TnI antibody. Signals from 3T3 cells transiently expressing PC2 and TnI1, PC2 and TnI2, PC2 alone, or nontransfected (Ctrl) were shown. (B) Similar to A, but using *Xenopus* oocyte as expression system.

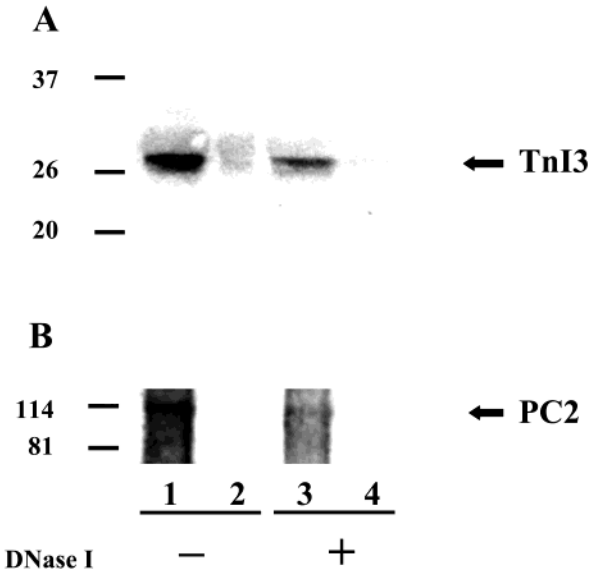

FIGURE 6: Association between PC2 and TnI3 in human adult heart tissues and effects of DNase I on the association. (A) Tissue lysates from human adult heart were precipitated with the PC2 antibody (lane 1) or nonimmune serum (lane 2) and detected by the cardiac TnI antibody. Lanes 3−4 showed the same experiment except for 100 μg/mL DNase I treatment 4 h before co-IP. Both lanes 1 and 3 confirmed the specific precipitation of TnI3. (B) Tissue lysates from human adult heart were precipitated by the cardiac TnI antibody (lane 1) or nonimmune serum (lane 2) and probed with the PC2 antibody. Lanes 3 and 4 showed the same experiment as lanes 1 and 2, respectively, except that lysates were treated by 100 μg/mL DNase I for 4 h prior to co-IP experiment. These experiments have been performed three times.

DISCUSSION

In the present study we have used a yeast two-hybrid method to identify PC2−interacting partners in heart in order to understand the modulation of PC2. We have produced several independent lines of evidence which supports that the intracellular C-terminus of PC2 associates with cardiac and skeletal isoforms of troponin I.

The C-terminus of PC2 specifically associated with TnI3, as the N-terminus of PC2 and the C-terminus of PC1 did not associate with TnI3. This specific interaction was also documented by in vitro approaches, including GST pull-down and microtiter binding, and in vivo reciprocal co-IP experiments using overexpressed mouse fibroblast NIH 3T3 cells and *Xenopus* oocytes and native cells from human adult heart tissues. The domain of PC2 responsible for this interaction was located to the segment R872−H927, which is different from the EF-hand (E754−D781), the ER-retention domain (E787−S820) (*33*), and the domain (G821−R878) for association with TM-1 (*20*).

PC2 is localized in the kidney, liver, lung, heart, brain, pancreas, intestine, and reproductive organs (*4*, *11*, *34*). In kidney, prominent expression of PC2 is observed in maturing proximal and distal tubules and collecting ducts but more pronounced distal localization is seen in adults. In nonrenal tissues the expression of PC2 is especially notable in the developing epithelial structures of the pancreas, liver, lung, intestine, brain, reproductive organs, placenta, and thymus (*11*). PC2 is also expressed in nonepithelial cells, such as smooth muscle, skeletal muscle, myocardial and endothelial cells, nucleated red cells, and neurons (*11*, *35*). In addition to cyst formation in kidney, *PKD2* null mouse embryos exhibit progressive total body edema and focal haemorrhage. These features are associated with functional cardiovascular failure. Further examinations disclosed defective cardiac phenotypes, including formation of the interventricular and atrial septum, atrio-ventricular canal, and pericardial effusions, which often result in mid-gestational embryonic lethality (*36*). Pathogenic mutations in *PKD1* and *PKD2* are associated with largely indistinguishable phenotypes. Apart from cystogenesis in organs such as kidney and liver, cardiac defects have been reported in the mouse line carrying targeted disruption of the *PKD1* gene (*37*). Abnormal vascular, skeletal, and cartilage developments have also been described in mutant *PKD1* mice (*37*, *38*). These seem to be consistent with the observation that PC1 and PC2 interact with each other at their C-terminus and confer a common signaling pathway.

TnIs were initially believed to be muscle-specific. In adult, TnI1, -2, and -3 are exclusively expressed in slow, fast skeletal muscle fibers, and cardiac muscle, respectively (*26*, *39*). However, TnI1 is also present in cardiac muscle during embryonic and fetal development (*26*, *40*). In the past decade, reports from different groups seem to support the idea that TnI proteins are more widely distributed. For example, Zanellato et al. (*41*) found that TnI3 antibodies specifically recognized a component in aortic or coronary smooth muscle extracts with electrophoretic properties identical to the TnI3

protein and further confirmed localization in these tissues by immunofluorescence. Messner et al. (*42*) discovered that TnI3 mRNA was present in serum samples of some patients with skeletal muscle myopathies. TnI2 has even been found in nonmuscle cells, such as cartilage (*27*) and corneal epithelium (*43*). Thus, together with our observation that PC2 also associates with the two skeletal isoforms, TnI1 and TnI2, it seems that the interaction of PC2 with TnIs may be present in all three types of muscle cells and nonmuscle cells, and that altered interaction due to *PKD2* mutations may be associated with ADPKD abnormalities in tissues in which they co-localize.

TnIs are important components of the actin microfilament system. It is well-known that TnI along with troponin C (TnC) and T (TnT) makes up the troponin complex, which regulates muscle contraction in a Ca^{2+}-dependent fashion in concert with tropomyosin (*21*, *22*). As the inhibitory subunit of the troponin complex, TnI binds to actin, tropomyosin, TnC, and TnT through different domains. It also inhibits actomyosin Mg^{2+}-ATPase in the presence of tropomyosin and the inhibition is abolished by TnC (*21*, *22*). In addition to this well-defined model in muscle, TnI has been demonstrated to play novel roles distinct from its conventional functions. For example, TnI2 has been shown to convert skeletal muscle ryanodine receptor into a rectifying Ca^{2+} release channel (*28*). More interestingly, TnI2 is also found in nonmuscle cells and is an angiogenesis inhibitor isolated from cartilage (*27*, *44*). Further evidence demonstrates that this inhibition occurs, at least in part, via a cooperative interaction of TnI2 with the cell-surface basic fibroblast growth factor receptor in both endothelial and nonendothelial cells (*45*). These results suggest that TnI may have the potential to be a useful inhibitor of a large number of serious diseases characterized by deregulated angiogenesis (*27*, *44*). Bello-Reuss et al. (*46*) presented a convincing body of evidence indicative of angiogenesis in ADPKD, a process that may be necessary for cystic cells to grow and responsible for increased vascular permeability.

The physiological implications of the interaction of TnI with PC2 are currently unknown. It is possible that the function of PC2 or the PC2−PC1 complex is modulated by the actin cytoskeleton system through direct association of PC2 with troponin I and tropomyosin-1 (*20*), which is important for cell growth and capillary morphogenesis. Because TnI serves as an angiogenesis inhibitor and TM-1 as a suppressor of neoplastic growth, altered TnI−PC2 and TM-1−PC2 interactions due to pathogenic PC2 or PC1 mutations may lead to undifferentiated cyst growth and angiogenesis in and around cysts in ADPKD. Thus, the inhibitory effects of exogenous TnI and TM-1 may rely on that these inhibitors compensate to certain extent for the altered interaction.

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