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Receptor protein tyrosine phosphatases are novel components of a polycystin complex

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Abstract

Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutation of *PKD1* and *PKD2* that encode polycystin-1 and polycystin-2. Polycystin-1 is tyrosine phosphorylated and modulates multiple signaling pathways including AP-1, but the identity of the phosphatases regulating polycystin-1 are previously uncharacterized. Here we identify members of the LAR protein tyrosine phosphatase (RPTP) superfamily as members of the polycystin-1 complex mediated through extra- and intracellular interactions. The first extracellular PKD1 domain of polycystin-1 interacts with the first Ig domain of RPTP σ , while the polycystin-1 C-terminus of polycystin-1 interacts with the regulatory D2 phosphatase domain of RPTP γ . Additional homo- and heterotypic interactions between RPTPs recruit RPTP δ . The multimeric polycystin protein complex is found localised in cilia. RPTP σ and RPTP δ are also part of a polycystin-1/E-cadherin complex known to be important for early events in adherens junction stabilisation. The interaction between polycystin-1 and RPTP γ is disrupted in ADPKD cells, while RPTP σ and RPTP δ remain closely associated with E-cadherin, largely in an intracellular location. The polycystin-1 C-terminus is an *in vitro* substrate of RPTP γ , which dephosphorylates the c-Src phosphorylated Y4237 residue and activates AP1-mediated transcription. The data identify RPTPs as novel interacting partners of the polycystins both in cilia and at adhesion complexes and demonstrate RPTP γ phosphatase activity is central to the molecular mechanisms governing polycystin-dependent signaling.

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Keywords

polycystins; tyrosine kinase; tyrosine phosphatase; adherens junctions; primary cilium; G-protein coupled signaling

Introduction

Autosomal dominant polycystic kidney disease is a common hereditary disorder caused by mutation of the *PKD1* or *PKD2* genes [1–4]. The ensuing disease is characterized by the proliferation of renal tubular epithelia to form fluid-filled cysts and in the long-term results in kidney failure. The polycystin proteins (polycystin-1 and polycystin-2) encoded by the *PKD* genes have regulatory functions in cell-cell adhesion, ciliary calcium signaling, transcription and cell differentiation [5–10].

Polycystin-1 is a large cell surface, multi-membrane spanning glycoprotein and is regulated through tyrosine phosphorylation [11–13]. Polycystin-1 is comprised of a novel array of evolutionary conserved protein domains that form potential interaction sites for multiple extracellular and intracellular ligands and are speculated to be important for adhesion and signaling [11,14]. The 218 amino acid cytoplasmic C-terminus contains a coiled-coil domain for interaction with polycystin-2 and is modified by tyrosine phosphorylation [12,13,15,16]. Nearly 50% of the extracellular region of polycystin-1 is constituted of 16 copies of the PKD domain. The first PKD domain is the prototypic member of a new class of immunoglobulin (Ig) fold and has been shown to have unique structural and biophysical properties compatible with a role in mechanotransduction [17,18]. The polycystin-1 ectodomain is also multiply glycosylated [11,19]. Polycystin-1 is widely expressed during development and in adult tissues with expression seen in kidney, liver, pancreas, vascular, skeletal and other epithelial-derived tissues [20,21]. In renal epithelia, polycystin-1 localises to intracellular membranes, as well as to specialized plasma membrane domains (including lamellipodia of spreading cells, cell-matrix and cell-cell adhesion complexes, and the primary cilium) in a spatially and temporally regulated manner [22–24]. A better understanding of polycystin-1 function depends on elucidating its extracellular ligands and establishing how phosphorylation may regulate its dynamic subcellular localisation and signaling.

Polycystin-1 forms a heteromeric complex with polycystin-2, a ciliary mechanosensitive cation channel that is activated by fluid flow *in vitro* [5,25,26]. Polycystin-2 (TRPP2) is related to a large family of channel proteins, mutations in which are closely associated with kidney disease [27]. Flow responses can be blocked by antibodies to the first extracellular PKD domain of polycystin-1 identifying an important role for this domain and the extracellular region of polycystin-1 in mechanotransduction [5]. Coiled-coil domains within the cytoplasmic C-terminal sequences of both polycystins play an important role in the interaction of the two polycystins, but not in ciliary localisation [6,15]. As yet, the connections between flow-induced channel activation and intracellular signaling remain to be fully elucidated.

Together the polycystins interact with ciliary proteins, focal adhesion proteins, E-cadherin and desmosomal proteins [12,16,23,28–31]. These interactions are continuously remodelled as a function of cellular differentiation and modified by both calcium concentration and tyrosine phosphorylation [12,13,32,33]. Both polycystins are tyrosine phosphorylated, most likely through the actions of c-Src and EGFR [12,13,34,35]. EGFR-dependent phosphorylation is in keeping with the critical role of the EGFR signaling axis in the development and progression of both ARPKD and ADPKD [36–40]. *In vitro*, the tyrosine

phosphorylation of polycystin-1 regulates G-proteins and several different signal transduction pathways including activator protein-1 (AP-1), calcineurin/NFAT, phosphatidylinositol 3-kinase/Akt and mTOR [8,13,41–43]. AP-1 and mTOR are dysregulated in ADPKD tissues [7,43,44] and the mTOR inhibitor, rapamycin, is beneficial in ameliorating disease progression in animal models of PKD [45,46]. Polycystin-2 calcium channel function is principally regulated through ser/thr phosphorylation [47,48], though interaction with regulators of channel activity (Kim1 and EGFR) depends on tyrosine phosphorylation [35,49,50]. Thus, tyrosine phosphorylation plays important roles in the regulation of the polycystins.

Tyrosine phosphorylation is normally continuously turned over through the action of specific phosphatases to terminate signaling and allow dynamic regulation of interactions. In ADPKD, primary patient derived PKD cells exhibit increased polycystin-1 phosphorylation [29]. Excessive tyrosine kinase activation has also been linked to cyst formation in ADPKD by triggering hyperproliferation, apoptosis, disruption of planar cell polarity and aberrant fluid secretion [38–40,51–53]. Abnormal composition and localisation of cell adhesion complexes, adherens junctions and desmosomes, and altered phosphorylation of polycystin-1 are also a feature of cystic epithelial cells suggesting that defects in phosphorylation may be inextricably linked to the changes in cell adhesion and ciliary signaling that contribute to the development of renal cysts in this disease [23,29]. Although phosphatase activity has been found associated with the polycystins [29,34], the identity of the phosphatase(s) remains to be identified.

Here we identify members of the classical receptor protein tyrosine phosphatase (RPTP) family as regulatory, interacting partners of a multimeric polycystin protein complex. RPTPs are modular proteins containing variable extracellular domains, a single transmembrane domain and two intracellular protein tyrosine phosphatase (PTP) domains [54,55]. Substrate specificity and functional diversity of the RPTPs is achieved by alternative splicing, proteolytic cleavage and the formation of large homo- and hetero-multimeric complexes [55–57]. The first membrane proximal PTP domains (D1) are catalytically active whilst the second structurally identical membrane distal PTP domains (D2), have little or no activity but are proposed to have a regulatory role [58]. Co-immunoprecipitations and substrate-trapping mutants have been used to demonstrate interaction of RPTPs with interacting partners, such as the cadherins and focal adhesion proteins which are also partners of the polycystins [12,16,29,34,59–62]. We show that the first extracellular PKD domain of polycystin-1 interacts with the first extracellular Ig domain of RPTP σ (E-subunit), a member of the LAR family of RPTPs, whilst the intracellular cytoplasmic tail of polycystin-1 interacts with the intracellular phosphatase domains (P-subunit) of RPTP γ . RPTP γ is shown to dephosphorylate a c-Src phosphorylated residue, Y4237, in the C-terminus of polycystin-1. This residue, together with Y4127 is able to regulate AP-1 signaling. The combined data suggest that RPTPs and tyrosine phosphorylation, play a central role in modulating the mechanical and signaling functions of the polycystin complex.

Material and methods

Cloning and expression

The following reference sequences were used *PKD1* (nm_000296), *PKD2* (nm_000297), *PTPRS* (nm_002850), *PTPRG* (nm_002841) and *PTPRD* (nm_002839). The first PKD domain of human polycystin-1 (residues 268–356) was expressed and purified as previously described [17]. Specific RPTP domains were amplified by PCR from human fetal kidney marathon-ready cDNA (Clontech) and verified by direct sequencing. RPTP γ D1 (Ile828–Asn1130), substrate-trapping RPTP γ D1 with Asp1028Ala mutation, and RPTP σ D1 (Ile1373–Arg1659) were cloned into pET30a in frame with the N-terminal His-tag. Site

directed mutagenesis was carried out using the Quikchange® kit (Stratagene). The CD44-CTD expression construct consisting of the extracellular and transmembrane domains of CD44 (Met1-Ser291) in-frame with the C-terminus of polycystin-1 (Leu4105-Thr4302) was cloned into pcDNA3.1 (Clontech).

Peptide ‘phage display’

A Ph.D.-7™ ‘phage display random peptide library (New England Biolabs) was screened to identify short peptide sequences that bound to PKD domain 1 according to the manufacturer’s protocol. Folding of PKD domain 1 was confirmed using circular dichroism and NMR spectroscopy. After three rounds of biopanning 26 independent ‘phage plaques and an aliquot of the eluted pool of ‘phage were sequenced. Peptide sequences were searched against public protein databases using BLAST (<http://www.ncbi.nih.gov/BLAST/>) and PEPSEARCH (<http://www.sanger.ac.uk/Users/agb/Pepsearch/>). An enzyme-linked immuno-sorbant assay (ELISA) was used to confirm binding between PKD domain 1 and individual ‘phage clones. Additional ‘phage clones expressing protein specific peptide sequences were generated using the Ph.D.-7 peptide display cloning system according to the manufacturer’s instructions.

Structural modelling

A structural model of the first Ig domain of mouse RPTP σ (residues 31–130) was generated using Phyre, a successor to 3D-PSSM.

Yeast-2-hybrid assays

The CytoTrap® 2-hybrid system (Stratagene) was used to screen for proteins that interact with the C-terminus of polycystin-1 (CPC-1). CPC-1 (Leu4105-Thr4302) expressed in pSOS was used to screen 1.2×10^6 clones of a testis pMyr cDNA library (using the manufacturer’s protocol).

Potential interactions between the polycystin-1 and polycystin-2 C-termini and the RPTP PTP domains were investigated by yeast 2-hybrid mating assays using CytoTrap® and Matchmaker™ standard protocols. RPTP σ D1 (Ala1376-Val1656), substrate-trapping RPTP σ D1 with Asp1557Ala mutation, RPTP σ D2 (Arg1659-Ala1947), RPTP γ D1 (Lys831-Val1127), substrate-trapping RPTP γ D1 with Asp1028Ala mutation, RPTP γ D2 (Asn1130-Asn1418) and RPTP δ D2 (Ser1654-Thr1912) were used. The C-terminus of polycystin-2 (CPC-2) (Asp690-Val968) was also used. For the Matchmaker™ system, high stringency interactions were detected by growth on SD glucose/-Ade/-His/-Leu/-Trp/X- α -gal plates and medium stringency interactions were detected by growth on SD glucose/-His/-Leu/-Trp plates.

Antibodies

The following primary antibodies against the RPTPs were used: anti-RPTP σ (C19), anti-RPTP γ (C18), anti-RPTP γ (M18) and anti-LAR (7/LAR mouse mAb IgG2a raised against aa 24–196 BD-Biosciences), and monoclonal anti-RPTP σ (17G7.2) [63]. A new rabbit polyclonal anti-RPTP σ (KTPTP σ) was raised and affinity purified against peptide sequence NH₂-Cys-KPDADGFIMVYLPDGQ -CONH₂. A new rabbit polyclonal anti-RPTP δ (KTPTP δ) was raised and affinity purified against peptide sequence NH₂-Cys-GKFIKPWESPDEMEL-CONH₂. A new rabbit polyclonal anti-RPTP γ (CBPTP γ) was raised and affinity purified against peptide sequence NH₂-Cys-STKENGNGPMTVDKN-CONH₂. Supplementary Fig. 1 shows locations of peptides within RPTP sequences and immunoblots for newly prepared antibodies ± peptide blocking.

The following antibodies against the polycystins were used: rabbit polyclonal anti-polycystin-2 PKD2-CFP [64], mouse monoclonal anti-polycystin-1 (7e12) [65], rabbit polyclonal anti-polycystin-1 (PWPC-1) [16], rabbit polyclonal anti-polycystin-1 (NM005) [6,29]. Rabbit polyclonal anti-polycystin-1 (LRR) was raised against LRR residues 27–360 and rabbit polyclonal anti-polycystin-1 PKD domain-1 (Dom-1) was raised against residues 268–356 using standard immunization protocols. Antiserum was purified using protein-A Sepharose and the specificity of the antibody confirmed by ELISA, western blotting and immunochemistry with and without preadsorption with the immunizing protein (data not shown).

Other primary antibodies were: anti-acetylated α -tubulin (Sigma), mouse monoclonal anti-CD44 (E1/2) [66], rabbit polyclonal anti-GFP (Ab6556; AbCam), mouse monoclonal anti E-cadherin (Transduction Laboratories), rabbit polyclonal anti-E-cadherin (Santa Cruz SC-7870), normal rabbit IgG (Santa Cruz SC-2027) mouse monoclonal anti pan-cadherin (Sigma), mouse monoclonal anti-actin (MP Biomedical, Aurora, OH).

The following secondary antibodies for immunofluorescence were obtained from Molecular Probes: Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 555 donkey anti-rabbit IgG, Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor®, Alexa Fluor® 555 donkey anti-mouse IgG, 488 donkey anti-goat IgG and Alexa Fluor® 546 donkey anti-goat IgG.

Cell lines and reagents

Hela, HEK293, MDCK, A431, mCCD and IMCD cells were maintained in DMEM supplemented with 10% fetal calf serum, 2mM glutamine, 10g/L streptomycin and penicillin. NHCT and NFCT cells have been previously described [13]. Normal primary human kidney and primary human ADPKD cells were isolated and cultured as described [29]. Immortalized human wild-type (wt) renal cortical tubular epithelial cells (RCTEC) and PKD9–12 cells were cultured as described [67,68]. PKD9–12 cells are homozygous for a truncating mutation in PKD1, Q2556X. Tissue and cell lysates were prepared according to standard protocols.

Immunoprecipitation and immunoblot analyses

Polycystin-1 and E-cadherin immunoprecipitation and immunoblotting were performed as previously described with attention to the following details [16,29,69]. For immunoprecipitation and immunoblot experiments using immortalized (RCTEC) or primary normal kidney and ADPKD cells, cells were plated on 100 mm culture dishes and grown to confluence. Cell lysates were prepared by washing with PBS buffer to remove growth medium and addition of 1 ml Lysis Buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 1% (vol/vol) TX-100 and a protease inhibitor cocktail) or 1 ml OG Lysis Buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 1% (wt/vol) n-octylglucoside (BD) and a protease inhibitor cocktail CLAP). Lysates were clarified by high-speed centrifugation and an aliquot of the total cell lysates was reserved for direct immunoblot analysis. Actin was used to confirm equal protein concentrations in cell lysates. Several measures were used to assess the specificity of the observed protein interactions: IgG isotype control immunoprecipitations were negative for the polycystin and E-cadherin complexes, membrane proteins associated with endosomes and cytoskeletal proteins were absent from immunoprecipitates, and similar results were obtained using two different detergents. Because ADPKD cells express variable levels of E-cadherin and some N-cadherin ([29,69] and Fig. 5), immunoprecipitations were repeated at least 4 times for both detergents using monoclonal or polyclonal antibodies directed against E-cadherin, a monoclonal antibody directed against N-cadherin or a monoclonal pan-cadherin antibody to assure consistency of results. Immunoprecipitates were collected using protein G-Sepharose, resolved on 4–15%

SDS-PAGE gradient gels and transferred to nitrocellulose. Blots were probed for RPTP σ (C19), RPTP δ (KTPTP δ), polycystin-1 (NM005) or E-cadherin (pAb or mAb), N-cadherin (mAb) or pancadherin (mAb).

Confocal microscopy

Cells were grown on polycarbonate membranes (Transwells, Costar) until confluent. For cilia growth, IMCD cells were grown for 7 days. Cells were then washed in PBS, fixed 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton-X 100 prior to incubation with primary antibody for 1 h at room temperature. After washing with PBS, the cells were incubated for 1 h with secondary antibody. After further washes in PBS membranes were excised from the transwell and mounted onto slides with vector shield DAPI mounting media (Vector Laboratories). Primary normal kidney and ADPKD cells were grown to subconfluence on glass coverslips, fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton-X 100, stained and mounted in Mowiol [23,29]. RCTEC and PKD9–12 cells were grown on coverslips or 0.4 μ m pore size 6-well Culture insert (Falcon 353090). Cells were washed with PBS $^+$ and pre-treated with 0.01% saponin in Pipes buffer, pH 6.8 (80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂) for 5 min. Cells were subsequently washed with PBS $^+$, fixed in 3% PFA, quenched with NH₄Cl in PBS $^+$ and permeabilized with 0.1% Triton-X 100 in PBS $^+$ for 5 min. Cells were blocked with 0.4% fish skin gelatin in PBS $^+$ and incubated 1 h with primary and secondary antibodies in PBS $^+$ containing 0.4% fish skin gelatin. Stained cells were mounted in Mowiol. For the IMCD cells, A431 cells, and MDCK cells confocal images were taken on a Zeiss LSM510 META confocal microscope using 63 \times objective. Images were further processed using Adobe Photoshop. For the HFCT cells microscopy was carried out on a Leica TCS-SP laser scanning confocal microscope equipped with an Ar-UV laser (350 nm), Argon laser (488 nm) and a diode laser (561 nm). Step size between optical sections was determined by the Nyquist limit. Stacks were volume rendered using Volocity software (Improvision, Inc, Lexington, MA). Primary normal kidney and ADPKD cells were imaged using a Zeiss LSM510 or a Zeiss META fitted and a 63 \times objective. For the RCTEC and PKD9–12 cells images were captured using the BioRad Radiance 2100 Microscope (<http://hsc.unm.edu/crc/microscopy/instru.html#four>) using a 60 \times oil objective. Image stacks were acquired and volume renderings were generated using Voxx, a PC-based image analysis program developed at the Indiana Center for Biological Microscopy [70]. Volumes are depicted beginning at the top of the nucleus through the basal membrane. Voxx is available at no charge (<http://nephrology.iupui.edu/imaging/voxx>).

Kinase and phosphatase assays

Phosphatase activity was determined using *p*NPP as substrate [71]. Briefly, varying amounts of recombinant RPTP σ D1, RPTP γ D1 and LAR-D1 were assayed in a microtiter plate at 30°C in 150 μ l of buffer (50 mM Tris, pH 6.8, 2 mM DTT) with 20 mM *p*NPP (Sigma). Absorbance readings were taken at 405 nm after 10 and 20 min of incubation. These values were used to calculate the relative unit activity compared to purified LAR-D1 protein (Sigma).

The activity of the recombinant RPTP phosphatase D1 domains against the C-terminus of polycystin-1 was measured as follows. CPC-1 was tyrosine phosphorylated as previously described [13]. CPC-1 (residues 4105–4302) was expressed as a His-tagged fusion protein in pET28a (Novagen). Protein was purified using Ni-NTA affinity chromatography and dialysed into 50 mM Tris (pH 7.4), 0.5% N-laurylsarcosine. Based on a series of protein refolding experiments, N-laurylsarcosine was found to be the best detergent for refolding CPC-1 following purification. However, the detergent is not essential for Src-dependent phosphorylation of polycystin-1 as it was not included in the initial c-Src assays performed by [13]. Ten μ g of fusion protein was incubated at 37°C for 30 min with 500 ng c-Src (150

units) in Kinase Buffer (10 mM HEPES, 2 mM MnCl₂, pH 7.5) and 1 μl [γ -³²P]ATP (3000 Ci/mmol, Amersham) and subsequently heat inactivated at 65°C for 20 min (control reactions confirmed that no kinase activity could be detected following this treatment). Reaction products were then incubated with 10 units of phosphatase domains at 30°C for 20 min in 10 mM imidazole, pH 7.0 containing 2 mM EDTA, 5 mM DTT, 100 mM NaCl and resolved by 10% SDS-PAGE prior to autoradiography.

AP-1 luciferase assay

Site-directed mutagenesis was used to generate Y4127F, Y4237F and double mutant constructs of pMyr-EGFP-CTD [72]. Hek293 cells were co-transfected with each pMyr-EGFP-CTD and 5xjun:ATF firefly luciferase reporter construct by the CaCl₂ precipitation method and grown in serum-free medium [7]. Cell lysates were collected 48 h after transfection, and luciferase activities were determined using a luminometer (Luciferase Reporter Assay; Promega). Comparisons were made with pMyr-EGFP-CTD4147X co-transfected with the 5x-jun reporter construct and a negative control transfected with the 5x-jun reporter construct only. Cell lysates were Western blotted with anti-GFP to confirm expression of chimeric protein. Western blots were scanned and the density of each band measured using ImageJ. Means, standard deviations and student t-tests were calculated using Excel.

Results

The first polycystin-1 PKD domain interacts with the RPTP σ Ig domain

Based on its importance in regulating mechanotransduction, we used the first PKD domain of polycystin-1 to screen 2×10^{11} bacteriophage clones from a random 7-mer peptide bacteriophage display library (New England Biolabs). The peptide library was used to overcome potential biases in gene expression that may occur with the use of tissue and temporal specific cDNA libraries. After three rounds of biopanning a single peptide sequence, VASFHRQ, represented 73% of the total number of bacteriophage clones sequenced. Bacteriophage clone sequences that matched known extracellular or membrane-associated proteins included integrin $\alpha 8$, members of the leukocyte common antigen related (LAR) family of receptor protein tyrosine phosphatases (RPTPs) and the RPTP interacting liprin proteins, among others (Table 1). The known association of polycystin-1 with adhesion molecules and its regulation through phosphorylation prompted us to clone peptide sequences corresponding to integrin $\alpha 8$, RPTP σ , LAR and RPTP δ into a bacteriophage display vector (M13KE gIII). Binding was assessed by ELISA to determine which of the cloned sequences with similarity to VASFHRQ bound specifically to the PKD domain 1 (Fig. 1A and B). Specific binding only occurred with the original library sequence, VASFHRQ (expressed with a GGGS spacer sequence that was present in all bacteriophage clones), and a sequence that is identical in RPTP σ and LAR, VASFVCQ. The VASFVCQ sequence corresponds to amino acids 49–55 of RPTP σ and 39–45 of LAR. These residues are predicted to form a β -strand in the first extracellular immunoglobulin (Ig) domain of RPTP σ and LAR based on secondary structure predictions and structural modelling (Fig. 1C). Somewhat surprisingly, PKD domain 1 did not directly interact with the highly homologous VASFICQ sequence derived from RPTP δ .

Polycystin cytoplasmic C-terminal domains of the polycystins interact with RPTP γ

The cytoplasmic polycystin-1 C-terminus is reversibly tyrosine phosphorylated and the modification is demonstrated to regulate interactions with proteins involved in cell adhesion [12,13,16,29,34]. Therefore, we also analyzed the interactions between the cytoplasmic regions of the RPTPs and the polycystin-1 C-terminal domain using two different yeast two-hybrid systems (CytoTrap® and Matchmaker™) (Fig. 1D–F). RPTP σ contains two

phosphatase domains, D1 and D2. The RPTP D1 domains are considered to be the active site domains, while the D2 domains are regulatory in nature [55]. A strong interaction was observed using the CytoTrap system between the C-termini of polycystin-1 and polycystin-2, thereby confirming the sensitivity of the system for detecting known protein-protein interactions (Fig. 1E). In addition, the Cytotrap screen identified novel interactions between the intracellular RPTP γ D2 regulatory domain (Ala1062-term) and the polycystin-1 C-terminus, as well as between the RPTP γ D1 catalytic domain and the polycystin-2 C-terminus. The RPTP γ D2/polycystin-1 interaction was confirmed in the Matchmaker system, though the RPTP γ D1/polycystin-2 was undetectable in this system (Fig. 1F). The Matchmaker system also enabled detection of several homo- and heterotypic interactions between RPTP wild-type and substrate-trapping mutant subunits; including homotypic interactions between RPTP σ D1 domains, and heterotypic interactions between RPTP σ D1/RPTP γ D2 and RPTP σ D1/RPTP δ D2 (Fig. 1F). The observed homotypic RPTP σ D1 and RPTP σ D1/RPTP δ D2 interactions agree with the published literature [58,73]. Therefore, the RPTP σ , RPTP δ , RPTP γ phosphatases are capable of forming a heteromultimeric complexes with one another and with the polycystins, poised them to play regulatory roles in the function of the polycystins (see graphical abstract and Fig. 7 for interaction summary).

Polycystin-1 and RPTP σ co-immunoprecipitate and co-localise in the cilia of renal tubular epithelial cell lines

The RPTPs have been best characterized in the brain and in neurons [55]. Immunoblots using several mAb and pAb directed against individual RPTPs demonstrated that RPTP σ , RPTP δ , RPTP γ , and LAR are also highly expressed in kidney tissue extracts and cultured renal cell lysates, among others (Supplementary Fig. 1). Likely due to the known furin and calcium activated proteolytic cleavage of the RPTPs [55] (see Supplementary Fig. 1A–cartoon), multiple bands were detected with most of the antibodies (Supplementary Fig. 1B–J). Molecular weights were as expected and peptide blocking experiments further confirmed antibody specificity. These RPTP antibodies were used for IP/Western assays to confirm the interaction between endogenous polycystin-1 and the LAR-family RPTPs.

Immunoprecipitation of polycystin-1 (with antibody PWPC-1) from immortalized normal human kidney and ADPKD cell lysates co-precipitated RPTP σ , but not LAR (Fig. 2A). RPTP γ , which interacts with polycystin-1 only via the cytoplasmic C-terminal sequences was only co-precipitated from normal kidney cell lysates but not from ADPKD cell lysates (Fig. 2A), and is consistent with frequent PKD1 mutations that result in C-terminal truncation of polycystin-1 [52]. RPTP δ though not a direct interacting partner of the polycystins (Fig. 1E–F) was also co-precipitated with polycystin-1 (Fig. 2B), most likely through known interactions between RPTP σ and RPTP γ (Fig. 1F and [58]). Fig. 2B further demonstrates the specificity of the co-immunoprecipitations in that no irrelevant bands were detected over the length of the blot and the specific bands were absent when an irrelevant control antibody was used for the immunoblot.

In fully polarized renal tubular epithelia, polycystin-1 is expressed in primary cilia [30]. Using rat kidney inner medullary collecting duct cell line (IMCD) cells, RPTP σ , RPTP γ and RPTP δ were all found co-localised with acetylated tubulin, a ciliary marker, as well as with polycystin-1 (Fig. 3A–B). The ciliary localisation of RPTP σ , RPTP δ and RPTP γ was further confirmed by peptide blocking experiments and through the use of two different antibodies for RPTP σ (KTPTP σ ; C19 antibodies) and RPTP γ (C18; CBPTP γ antibodies) (Fig. 3C–D).

RPTPs co-localise with polycystin-1 and E-cadherin at the basolateral membrane

Polycystin-1 functions in cell-cell adhesion, cell-matrix adhesion, calcium channel regulation and signal transduction and is regulated both temporally and spatially [24].

During the early phase of renal cell polarization, polycystin-1 localises to focal adhesions [16], followed by polycystin-1 presences at basolateral membrane junctions, such as desmosomes [23,31] and adherens junctions, where polycystin-1 is known to play a role in E-cadherin localisation to the adherens junction [29,69,74]. Upon the formation of stable junctions, polycystin-1 retreats from the lateral membrane [23] and is found in the apical membrane and primary cilia [5,30,75]. LAR and RPTP σ also have demonstrated roles in regulating cadherins and cell-cell adhesion, as well as integrins and cell-matrix adhesion [62]. Like polycystin-1, the RPTPs are also closely associated with tyrosine kinases such as EGFR and src [13,32,34,55,59,76,77]. EGFR is also found in cilia where it regulates polycystin-2 activity [35]. Based on the multitude of interconnections, it was also of interest to analyze the co-localisation of polycystin-1 and RPTPs with the lateral cell membrane and adherens junctions marked by E-cadherin.

Analysis of IMCD cells stably expressing RPTP σ ::GFP (corresponding to a major RPTP σ isoform isolated from a kidney cDNA library, lacking the FNIII domains 4–7) was exclusively localised to the lateral membrane (Fig. 4A). Immunolabeling of endogenous RPTP σ also showed RPTP σ at the lateral plasma membrane where it co-localised with polycystin-1 and E-cadherin in confluent, non-ciliated IMCD cells, (Fig. 4B–C).

To test the functional significance of the RPTP localisation with E-cadherin at lateral membranes, their localisations were comparatively evaluated in normal kidney and ADPKD cell lines (using immortalized lines and primary cells). Both RPTP σ and RPTP δ co-localised with E-cadherin at the lateral membrane and intracellular membranes of normal immortalized kidney cells (RCTEC) and were exclusively intracellular in immortalized PKD9–12 cells (Fig. 4D–G), as previously shown for E-cadherin [29,69]. In more confluent, polarized RCTEC and PKD9–12 cells, there was limited co-localisation between E-cadherin and RPTP σ (Fig. 4E). Similar trends were observed in primary cells (Fig. 4H). Filter-grown, polarized RCTEC cells revealed that RPTP δ co-localised at the lateral membrane with E-cadherin of normal cells. Renal epithelial PKD cells fail to traffic E-cadherin to adherens junctions and exhibit significantly less intracellular E-cadherin compared to normal renal epithelial cells [34]. The lateral membrane localisation of RPTP δ was lost in the PKD9–12 cells, where the levels of E-cadherin were also significantly reduced, and RPTP δ and E-cadherin were confined to the intracellular compartment (Fig. 4F–G). In subconfluent primary cells, RPTP δ was often seen at the leading edge of membrane ruffles in spreading normal kidney cells, a pattern that was not apparent in primary PKD cells where the staining was diffusely localised across the cell membrane and in concentrated in the Golgi region (Fig. 4I).

RPTPs interact with E-cadherin

In previous studies, we identified a multimeric complex between the polycystins, E-cadherin and β -catenin that was disrupted in ADPKD cells [34]. The complex was also associated with tyrosine kinase and phosphatase activities and polycystin was increasingly phosphorylated in ADPKD cells heterozygous for the Q2556X mutation in PKD1. Therefore, co-immunoprecipitation experiments were carried out using primary normal human kidney and ADPKD cells to determine if RPTPs might represent the phosphatase activity that is part of a polycystin/E-cadherin complex. We also wanted to comparatively evaluate ADPKD related changes that would lend functional significance to any identified interactions. E-cadherin was immunoprecipitated from three different normal (67F05, 59M04, 57M03) and three different ADPKD (51M06, 46M06, 46F04) patient derived primary cells. The immunoprecipitates were subsequently immunoblotted for E-cadherin, RPTP σ , RPTP δ and polycystin-1 (PC1) (Fig. 5A). (NB: Since a polycystin-1:RPTP γ complex appeared to be present only in the immortalized normal human kidney cells and not the ADPKD cells (Fig. 2), it was not retested in the primary cell lines). RPTP σ expression

was modestly increased in ADPKD cell lysates relative to the actin loading control (graph not shown), while RPTP δ expression was uniform in normal and ADPKD cells. Both RPTP σ and RPTP δ were coimmunoprecipitated with E-cadherin and full-length polycystin-1 (460 kDa) from normal and ADPKD cell lysates, and were not statistically different (Fig. 5A graph). Smaller fragments of polycystin-1 at 250 kDa and 150 kDa correspond to known cleavage products [74,78]. All RPTPs and their subunits were found specifically co-precipitated and enriched with E-cadherin as compared to isotype control immunoprecipitations (Fig. 5B). The RPTP-E-cadherin interaction was also identified in immortalized cell lines using different detergent conditions serving as an additional specificity control (Fig. 5C). Actin was not recovered in the immunoprecipitated fractions indicating that the detected complexes were not due to a co-precipitating cytoskeletal network. Based on the composite data, the RPTPs are identified as complexed to E-cadherin, as well as the polycystins.

RPTP γ , but not RPTP σ , dephosphorylates polycystin-1 at Y4237

The Y4237 residue has been identified to be specifically phosphorylated in C-terminal tail constructs of polycystin-1 [13]. To determine if polycystin-1 can serve as a substrate of the RPTP phosphatases, Y4237 of recombinant polycystin-1 C-terminus was first phosphorylated by c-Src prior to incubation with the catalytic D1 domains of RPTP σ and RPTP γ in a dephosphorylation assay. The phosphatase activity of each RPTP domain was determined using p-nitrophenyl phosphate (pNPP) as a substrate. Measuring the loss of 32 P-label from the polycystin-1 C-terminus, demonstrated that with equal unit activity, RPTP γ D1 was able to specifically dephosphorylate Y4237 of polycystin-1, while LAR D1, RPTP σ D1, and a substrate-trapping inactive mutant of RPTP γ D1 were essentially inactive (Fig. 6A). An equimolar amount of the regulatory RPTP σ D2 was used as a negative control. Similar results were obtained using a kinetic assay measuring phosphate release, again showing that the RPTP γ D1 domain rapidly dephosphorylated the polycystin-1 C-terminus when compared to RPTP σ and LAR D1 domains (Fig. 6B). These data identify RPTP γ as a potential physiological regulator of polycystin-1 function.

Functional characterization of polycystin-1 Y4237

The location of Y4237 in the same C-terminal coiled-coil domain of polycystin-1 that is responsible for interacting with polycystin-2 and regulating its calcium channel activity suggests that this residue may be pivotal for regulating protein-protein interactions. Y4237 together with nearby residues Y4127 and Y4147 may also regulate signaling activity through reversible phosphorylation. In a yeast-2-hybrid interaction assay, the Y4237F mutation of polycystin-1 did not affect the interaction of the C-terminus of polycystin-1 with the C-terminus of polycystin-2 (Data not shown). By immunofluorescence, the wild-type and Y4237F mutant forms of a membrane targeted CD44-polycystin-1 C-terminus chimera (CD44-CTD) both localised to the basolateral membrane of MDCK cells (Fig. 6C).

The signaling functions of Y4127, Y4147 and Y4237 were assessed individually and in combination using an AP-1 reporter assay. A myristylated polycystin-1 C-terminal construct, pMyr-PKD1, was used to assay the modulation of AP-1 signaling in HEK293 cells. Expression of the myristylated polycystin-1 C-terminus produced a highly reproducible activation of AP-1 (Fig. 6D). Mutation of the conserved tyrosine residues, Y4127 (in the G-protein activation domain of polycystin-1) and Y4237 demonstrated that both tyrosine residues were essential for AP-1 activation producing a response equivalent to a 4147X truncating mutation lacking the coiled-coil domain (Fig. 6D). Equal expression of all non-truncating constructs was confirmed using western blotting and densitometry (Fig. 6E).

Discussion

In this study we identify RPTP σ , RPTP δ and RPTP γ as novel interacting partners of polycystins (Fig. 7). Direct interactions between RPTP γ and both polycystin-1 and polycystin-2 are demonstrated and the first Ig repeat of RPTP σ (but not LAR) interacts with the first PKD1 domain of polycystin-1. RPTP δ is most likely complexed with the polycystins indirectly through interactions between RPTP σ and RPTP δ . The RPTPs are components of a known polycystin/E-cadherin protein complex and are co-localised at the lateral membranes of renal epithelia. All of the RPTPs also co-localise with polycystin-1 in cilia. The levels of RPTPs detected at the plasma membrane of renal epithelial cells were low, while a larger pool was present on intracellular membranes. Strong plasma membrane staining and limited intracellular staining was observed in A431 cells. This suggests that plasma membrane expression may be dependent on cell type and in flux with an intracellular pool. In ADPKD cells, the polycystin/RPTP complexes were altered with RPTP γ being absent and the proteins exhibited different localisations. Finally, functional studies show that polycystin-1 regulated AP-1 activity required RPTP γ . Together the data suggest that several members of the LAR subfamily (type IIA) of RPTPs are key regulators of the polycystins in multiple cellular locations.

The associations of the RPTPs with the polycystins in cilia and at cell-cell adherens junctions are in keeping with their known functions in the kidney and other systems. RPTP σ and RPTP δ have adhesion molecule-like Ig repeats and along with RPTP δ have multiple fibronectin-like domains [57,79]. RPTP σ regulates diverse cellular functions including signal transduction, mechanotransduction, ion channel regulation and adhesion complex formation [80–84]. RPTP γ inactivation in mice gives rise to only minor behavioral disturbance, suggesting functional redundancy, and has recently been shown to play a role in neural adhesion [85,86]. Functional redundancy among the RPTPs is further evidenced by the necessity for double knockout mice to reveal tissue specific functions [87]. In support of this hypothesis, previous observations identified an additional RPTP isoform (RPTP α) as a binding partner of RPTP σ D1 [58], which we also observed in our yeast two-hybrid screens (not shown). Although RPTP σ , RPTP δ and RPTP γ are all expressed in the kidney (reviewed in [55]), renal cyst formation has not been described in single RPTP σ , RPTP δ or RPTP γ knock-out models. However, RPTP δ has been identified as a candidate modifier of polycystic kidney disease in the *cpk* mouse suggesting that different alleles of RPTPs may act as genetic modifiers of polycystic kidney disease severity [88]. While it is evident that RPTPs are likely important in kidney cell differentiation, signaling and adhesion, a more complete understanding of the regulatory roles of RPTPs in kidney (paralleling the in depth studies that have been done for brain development) will depend on long-term survival studies and double knock-out animals.

Polycystin-1 localises to basolateral and ciliary membranes and is implicated in the regulation of processes such as cell migration, adhesion, proliferation and ciliary signalling, much like the RPTPs. The formation of a polycystin-1/RPTP/E-cadherin complex suggests that the RPTPs play a role in cell-cell adhesion and identifies the tyrosine phosphatases shown previously to co-precipitate with a flotillin-2/polycystin-1/E-cadherin complex [34]. RPTP σ and RPTP δ were also found to coimmunoprecipitate with N-cadherin (data not shown). These data agree with the known localisation of RPTP σ to adhesion complexes where it can functionally associate with N-cadherin/ β -catenin and dephosphorylate β -catenin and other desmosomal catenins such as plakoglobin (γ -catenin) [32,61,89]. Further support for a role of regulated tyrosine phosphorylation in adhesion complex formation comes from the observation that phosphatase inhibitors result in tyrosine phosphorylation of adherens junction proteins and deterioration of junctional structures [33]. Inhibition of tyrosine phosphorylation also disrupts polycystin-1-FAK interactions [12]. In ADPKD epithelia

polycystin-1 has been shown to be hyperphosphorylated and suggested to disrupt normal interactions with E-cadherin [29]. Thus, the identified polycystin/RPTP complex is likely important for the regulation of the polycystins in multiple cellular locations.

Abnormalities in the structure and function of the renal primary cilium have been identified in human and murine polycystic kidney disease [90,91]. Deflection of the primary cilium results in increased intracellular calcium, mediated by the polycystin channel complex and ciliary signaling is abrogated in ADPKD cells [5,6,68]. However the mechanisms underlying the activation and regulation of the polycystin complex in adhesion complexes and the primary cilium are poorly characterized. The importance of the first PKD domain in mechanotransduction has been demonstrated by the ability of antibodies to this domain to block the cilia dependant flow-induced increase in intracellular calcium in renal epithelial cells [5,6,68]. This domain also demonstrates unique force-induced biophysical properties further supporting its central role in mechanotransduction [18]. The ‘phage display’ data identified a potential interaction between the first PKD domain of polycystin-1 and the first Ig domain of RPTP σ . The first Ig domain of RPTPs has been shown to be a major ligand-binding site recognizing heparan sulphate proteoglycans and chondroitin sulphate proteoglycans [92,93]. Therefore the identification of an RPTP-polycystin complex that localises to the primary cilium suggests that mechanosensing and signaling properties are regulated by tyrosine phosphorylation. RPTP δ has been identified in the mouse photoreceptor sensory cilium proteome, which suggests a more general role for RPTPs in cilia function [94]. RPTP α , the LAR-interacting proteins Trio and liprin, and members of the cadherin and catenin family were also identified in the same screen, suggesting that both RPTP and cadherin functions are required for normal ciliary signaling.

RPTP ζ , which has the same domain architecture as RPTP γ , has been shown to interact extracellularly and intracellularly with voltage-gated sodium channels where dephosphorylation leads to increased channel activity [83,95]. Recently the extracellular carbonic anhydrase domain of RPTP γ (residues 56–320) has also been shown to interact with the Ig domains of members of the contactin family of proteins involved in neural recognition [86]. Thus, the dual interaction between both the extracellular and intracellular domains of the polycystin-1 and RPTP σ and RPTP γ , is likely to enable both ‘outside-in and inside-out’ signaling (as described for the integrins) and convert changes in adhesion to changes in cell behavior. A dual binding site model predicts that an extracellular polycystin-1-RPTP interaction serves as a sensor that regulates the function of polycystin-1 or polycystin-2 by tyrosine phosphorylation. Therefore the intracellular regions of the polycystins may regulate, or be substrates for, RPTP σ , RPTP γ or RPTP δ . This model is supported by several observations. Firstly, the C-terminus of polycystin-1 can interact with the RPTP γ D2 and secondly, tyrosine phosphorylated polycystin-1 is a substrate of RPTP γ D1 in vitro. RPTP γ D2 also interacts with RPTP σ D1 suggesting that RPTP γ may be capable of regulating the dephosphorylation of E-cadherin/ β -catenin with RPTP σ . More intriguingly, RPTP γ D1 also interacts with the C-terminus of polycystin-2 in our yeast two-hybrid assay suggesting that tyrosine phosphorylation may regulate polycystin-2 channel activity. Neural network predictions using Netphos 2.0, identified Y684 and Y836 as potential tyrosine phosphorylation sites in polycystin-2. However, we were unable to detect endogenous tyrosine phosphorylation of polycystin-2 in IMCD cells by immunoprecipitation (unpublished data) although other TRP channels can be regulated by this mechanism. Additional work will be required to elucidate how dynamic changes in polycystin protein interactions during cell differentiation are modulated by the RPTPs.

The signaling functions of polycystin-1 are mediated by the short intracellular cytoplasmic tail, which contains serine and tyrosine phosphorylation sites, a G-protein activation domain and a coiled-coil protein interaction domain [13,96,97]. Studies have shown that the C-

terminal tail of polycystin-1 is able to regulate AP-1 signaling in a G-protein dependant manner [8,98]. Aberrant AP-1 activation has also been shown in ADPKD cells and tissues [7,44]. Using a Jun::ATF luciferase reporter assay we show that two tyrosine residues in the C-terminus of polycystin-1 (Y4127 in the G-protein activation domain and Y4237 within the coiled-coil domain) are critical for polycystin-1 mediated AP-1 activity. As Y4237 does not alter the interaction with polycystin-2 or the membrane localisation of polycystin-1, it suggests that it is crucial for the direct interaction with components regulating the AP-1 signaling pathway.

Conclusions

The cumulative data suggest that RPTPs can regulate the mechanosensitive, adhesive and signaling properties of the polycystin complex. We postulate that in the renal primary cilium an interaction between polycystin-1 with the extracellular region of RPTP σ forms the mechanosensing subunit of the complex that modulates activation of the intracellular phosphatase domains with the subsequent dephosphorylation of Y4237 of polycystin-1 and regulation of AP-1 signaling. Other subunits of the RPTP/polycystin-1 assembly in different localisations and in association with distinct partners (e.g. adherens junctions and E-cadherin/ β -catenin) may also be regulated by this mechanism resulting in altered composition and stability of cell-cell and cell-matrix adhesion complexes. Intriguingly, phosphatase activity is regulated by proteolytic cleavage in response to calcium influx with shedding of the extracellular domains of LAR and RPTP σ and redistribution of their intracellular phosphatase domains away from sites of cell-cell contact [89]. Consequently, such a calcium dependent mechanism could simultaneously regulate polycystin-1 and phosphatase activation and enable redistribution of polycystins from the lateral membranes to cilia as the cells differentiate. Therefore, disruption of the polycystin-RPTP complex, leading to altered phosphorylation of polycystin-1/adhesion complex proteins and/or aberrant AP-1 activation may be critical in establishing a renal cystic phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ADPKD	autosomal dominant polycystic kidney disease
AP-1	activator protein 1
CPC-1	C-terminus of polycystin-1
CPC-2	C-terminus of polycystin-2
LAR	leukocyte common antigen related
IMCD	inner medullary collecting duct cell line
IB	immunoblot
IP	immunoprecipitate
mAb	monoclonal antibody
mCCD	cortical collecting duct cell line
MDCK	Madin-Darby canine kidney cell line
LRR	leucine rich repeat

NFCT	normal fetal collecting tubule cell line
NHCT	normal human collecting tubule cell line
OG	octylglucoside
pAb	polyclonal antibody
PKD	polycystic kidney disease
RCTEC	renal cortical tubular epithelial cell line
RPTP	receptor protein tyrosine phosphatase
TX	Triton-X 100

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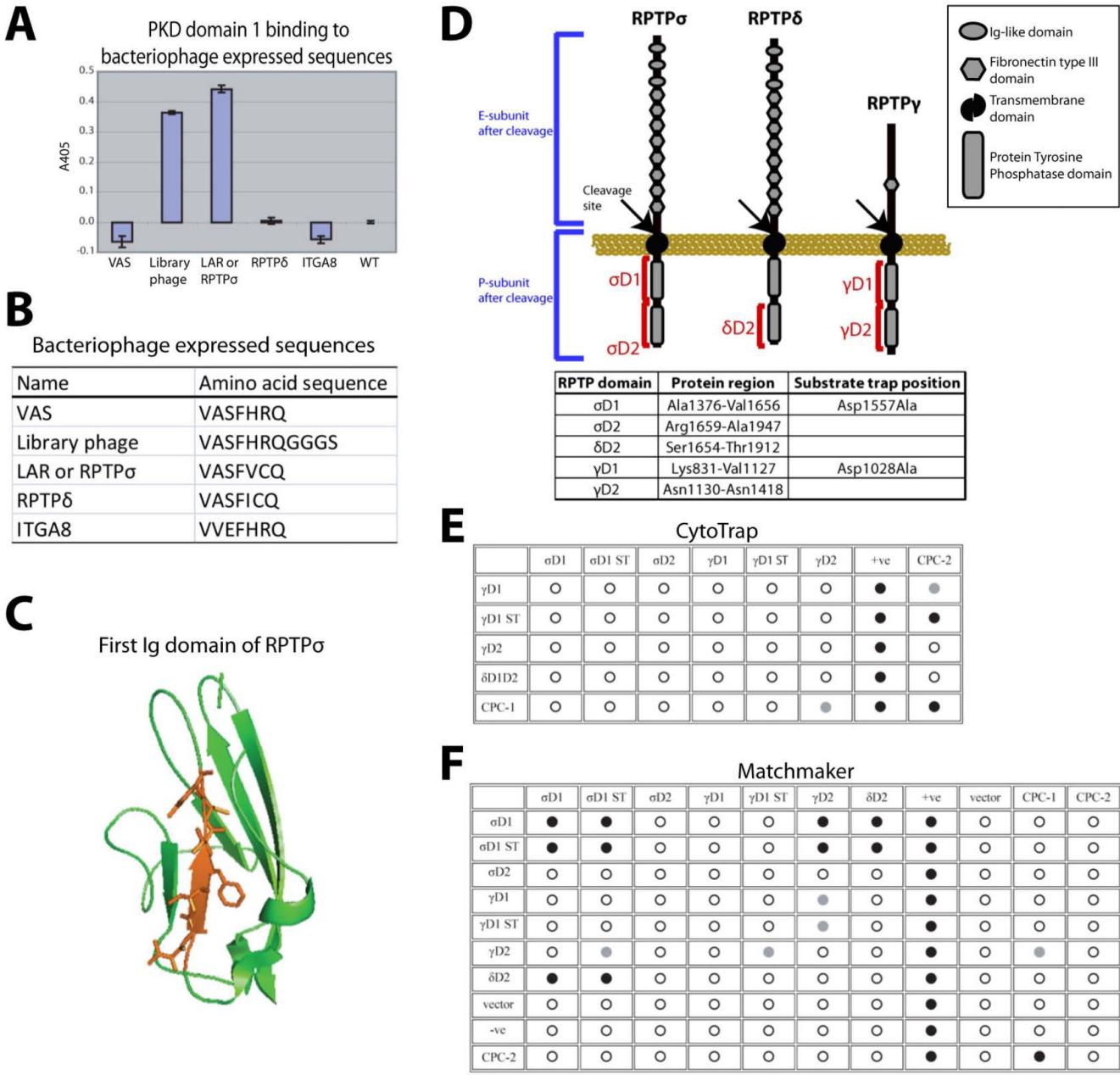
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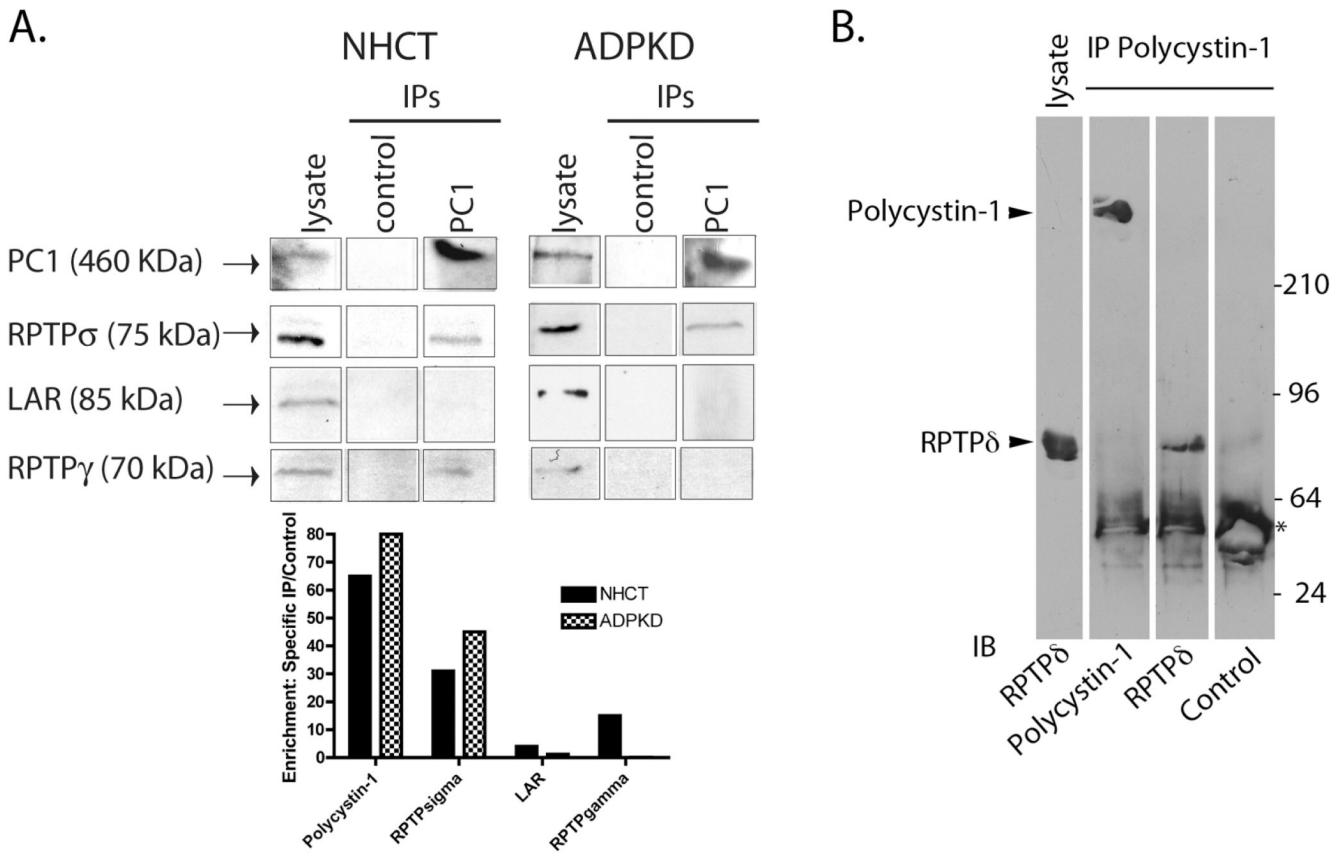
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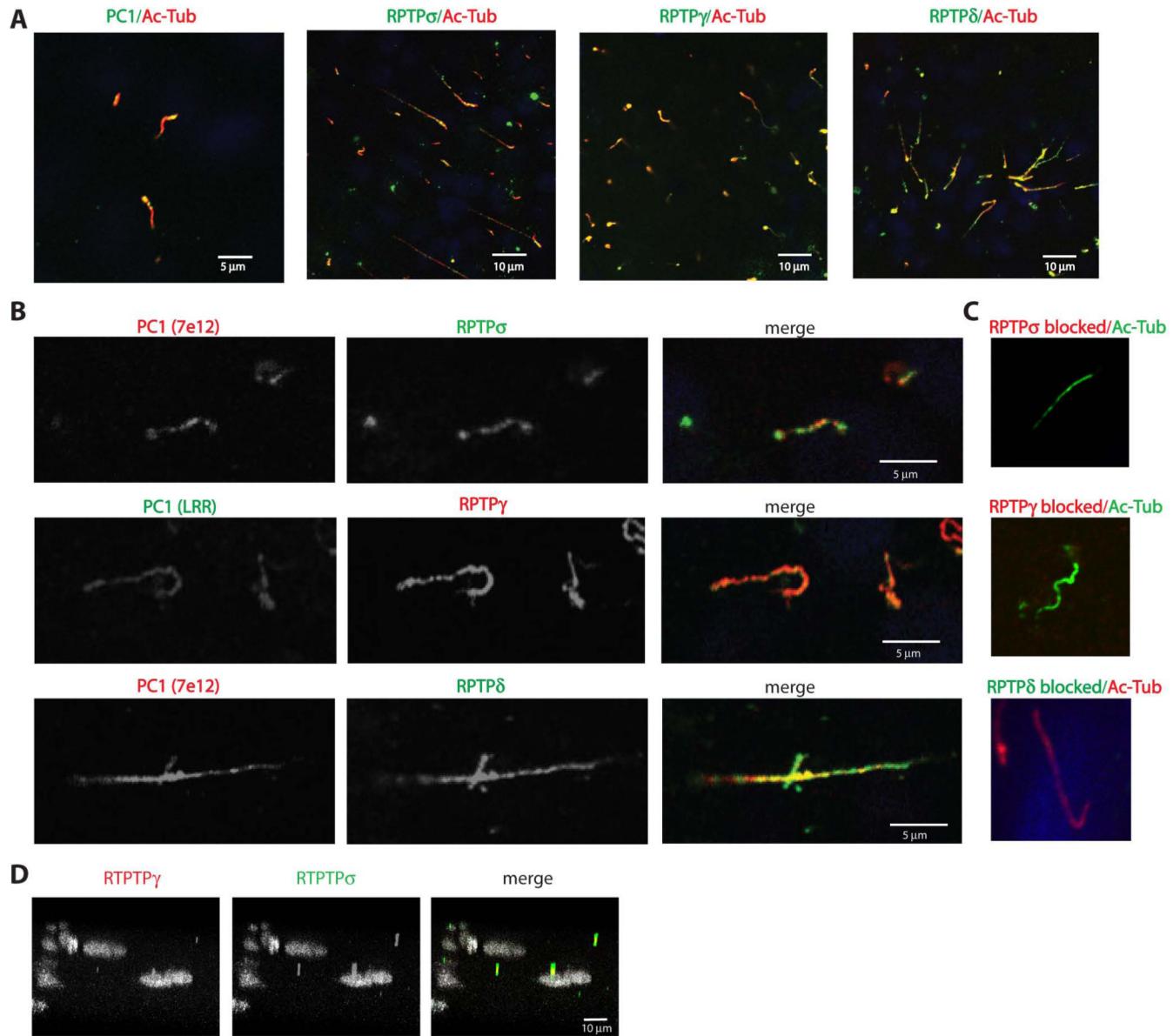
**Fig. 1.**

The first PKD domain of polycystin-1 binds specifically to a peptide sequence from RPTP σ /LAR. (A) Binding of wild-type PKD domain 1 to bacteriophage display clones expressing protein specific peptide sequences. WT corresponds to wild type empty vector. Each column represents the mean and standard error of three separate experiments. (B) Protein specific peptide sequences used in the bacteriophage display assay. (VAS = single peptide sequence represented in 73% of the total number of bacteriophage clones sequenced). (C) Structural model of the first Ig domain of RPTP σ generated using Phyre/3D-PSSM. The sequence VASFVCQ (orange) corresponds to a region containing the second β strand of an I type Ig domain (green). Residues are represented in ball and stick. (D) Cartoon representation of RPTP isoforms and RPTP domains. Full length RPTPs are proteolytically cleaved near the transmembrane domain by a furin-like endoprotease to produce an extracellular domain (E-

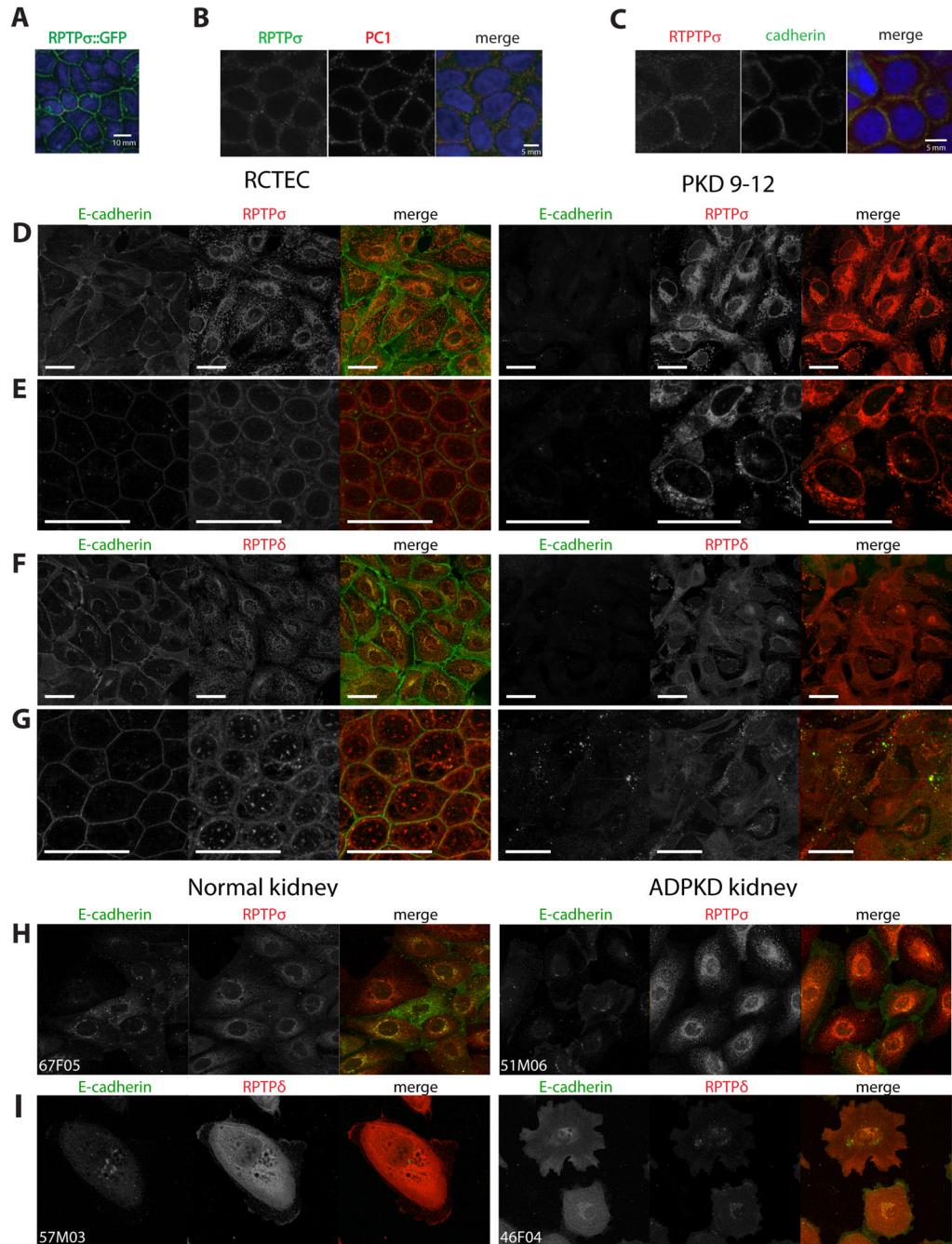
subunit) and tandem phosphatase domains (P-subunit), followed by calcium-dependent proteolytic cleavage to shed the extracellular domain. Regions marked in red correlate to sequences used for CytoTrap and Matchmaker assays that are listed in table below cartoon. (E) Summary of interactions between PTP domains from RPTP σ , RPTP δ and RPTP γ and the polycystins detected using the CytoTrap® yeast-2-hybrid system. The clones in the first row are expressed in pMyr and clones in the first column are expressed in pSos. (F) Summary of Matchmaker™ yeast 2-hybrid results. The clones in the first row are expressed in pGADT7 and the clones in the first column in pGBT7. Interactions are indicated as • = strong interaction, ● = weak interaction, ○ = no interaction. WT = wild-type, ST = substrate trapping, CPC-1 = polycystin-1 C-terminus, CPC-2 = polycystin-2 C-terminus. Positive (+ve) and negative control interactions were carried out according to the manufacturer's protocol.

**Fig. 2.**

RPTPs form a complex with polycystin-1. Polycystin-1 was immunoprecipitated from cell lysates using a specific antibody, PWPC-1, directed against the CPC-1. (A) Immunoblot analyses of whole cell lysates from immortalized normal human kidney (NHCT) and ADPKD cells, control IgG immunoprecipitates and polycystin-1 immunoprecipitates were performed to detect the presence of polycystin-1, RPTP σ , RPTP γ and LAR. Enrichment shown in graph was calculated as fraction of protein immunoprecipitated with PC1 antibody relative to the fraction of protein precipitated with control IgG. (B) Immunoblot analyses of whole cell lysates from immortalized normal human fetal kidney (NFCT) cells for polycystin-1 and RPTP δ . Fold enrichment of RPTP δ in PC1 immunoprecipitates was 5-fold (calculated as described in panel A). Asterisk denotes IgG heavy chain in the immunoprecipitates. Proteins were detected with the following antibodies: polycystin-1 (PWPC-1), RPTP σ (17G7.2), LAR (BD Biosciences), RPTP γ (CBPTP γ), RPTP δ (KTPTP δ), control panel A (isotype matched Ig), control panel B (irrelevant antibody against sprouty). N=4 for all panels.

**Fig. 3.**

RPTPs localise to the primary cilia of renal epithelial cells with polycystin-1. (A) Confocal immunofluorescence images show polycystin-1 (LRR mAb), RPTP σ (KTPTP σ pAb), RPTP γ (C18 pAb) and RPTP δ (KTPTP δ pAb) co-localise with acetylated α -tubulin (Ac-Tub, mAb) in the primary cilium of IMCD cells. (B) Confocal immunofluorescence images show RPTP σ (KTPTP σ pAb), RPTP γ (C18 pAb) and RPTP δ (KTPTP δ pAb) co-localise with polycystin-1 (7e12 mAb or LRR mAb) in a single cilium. (C) Confocal immunofluorescence images show the specificity of the antibodies to RPTP γ (C18), RPTP σ (KTPTP σ) and RPTP δ (KTPTP δ) when blocked with their respective antigen raising peptides. In peptide-blocked samples, only acetylated α -tubulin (Ac-Tub) staining of the primary cilium of IMCD cells was detected. (D) Confocal immunofluorescence images show RPTP γ (CBPTP γ) and RPTP σ (C19) co-localise in the primary cilium of HFCT cells. N \geq 3 for all panels.

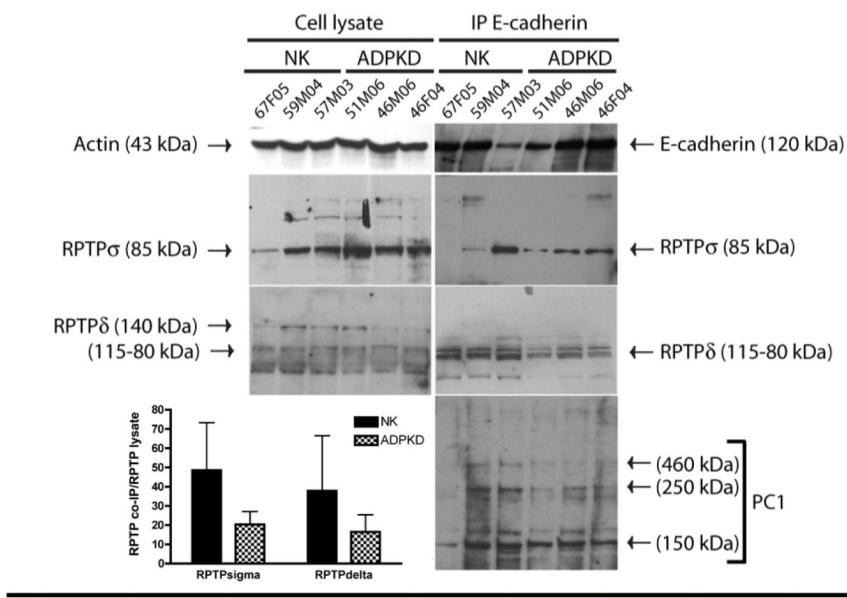
**Fig. 4.**

RPTP σ and RPTP δ localisation in renal epithelial cells. (A) Stably transfected IMCD cells expressed RPTP σ ::GFP construct (green) at the basolateral plasma membrane. Nuclei labeled with DAPI (blue). The expressed RPTP σ isoform is a major isolated from a kidney cDNA library, lacking FNIII domains 4–7. (B) Confocal immunofluorescence images of RPTP σ (17G7.2 mAb) and polycystin-1 (PKD domain 1, Dom1 pAb) at the basolateral membrane in IMCD cells. (C) Confocal immunofluorescence image of RPTP σ (17G7.2) and pan-cadherin at the lateral membrane in A431 cells. (D–E) Confocal immunofluorescence images of confluent RCTEC and PKD9–12 cells labelled with antibodies directed against RPTP σ (KTRRPTP σ) and E-cadherin (Transduction Laboratories). (F–G) Confocal

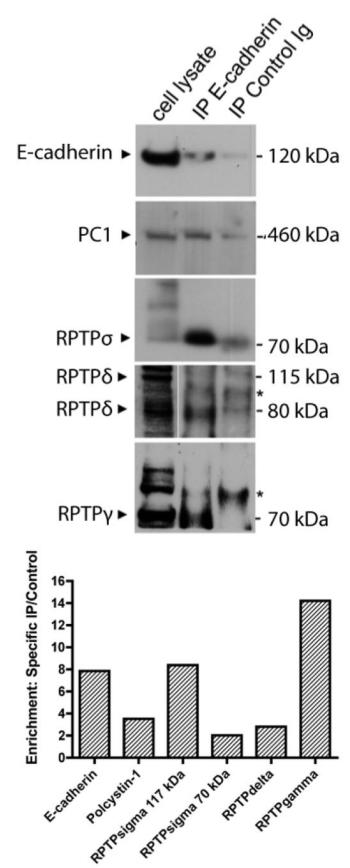
immunofluorescence images of confluent RCTEC and PKD9–12 cells labelled for RPTP δ (KTPTP δ) and E-cadherin. Panels D and F: cells were grown on glass coverslips. Panels E and G: cells were grown on 0.4 μ m filter supports. (H) Confocal immunofluorescence images of confluent normal (67F05) and ADPKD (51M06) primary kidney cells labelled with antibodies directed against RPTP σ (KTRPTP σ) and E-cadherin (Transduction Laboratories). (I) Confocal immunofluorescence images of subconfluent, spreading normal (57M03) and ADPKD (46F04) primary kidney cells labelled with antibodies directed against RPTP δ (KTPTP δ) and E-cadherin (Transduction Laboratories). N \geq 3 for all panels.

Co-Immunoprecipitation of RPTPs with E-cadherin

A. Primary Human Cells



B. Immortalized RCTEC



C. Immortalized RCTEC varying detergents

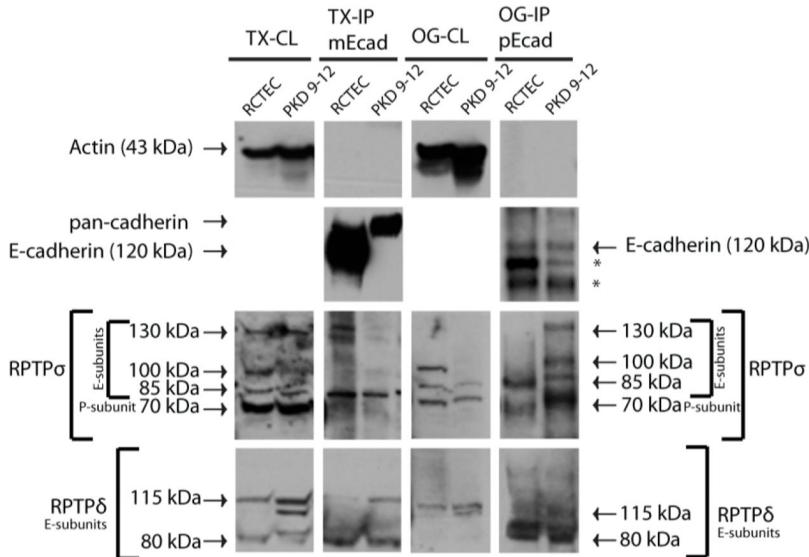
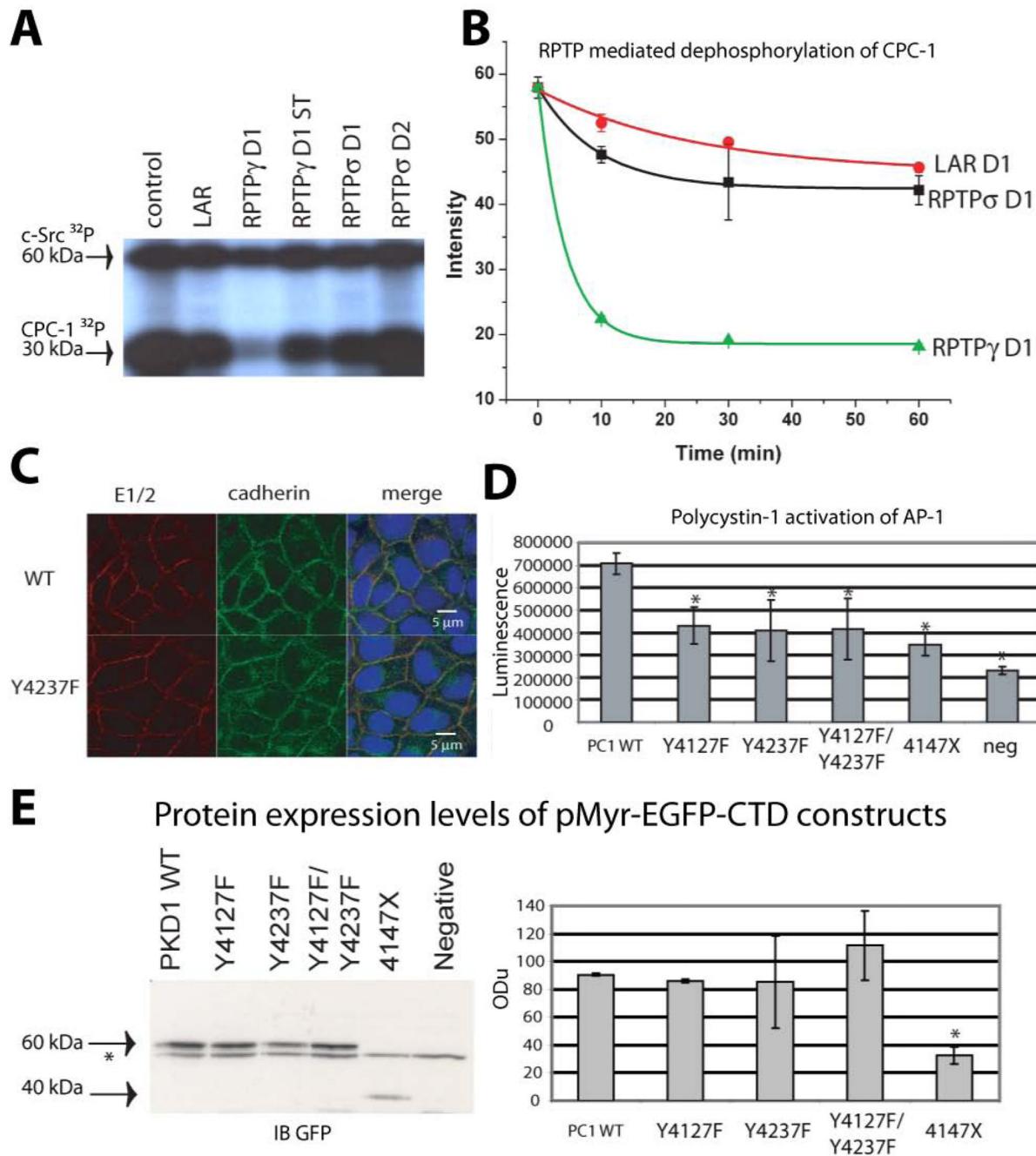


Fig. 5.

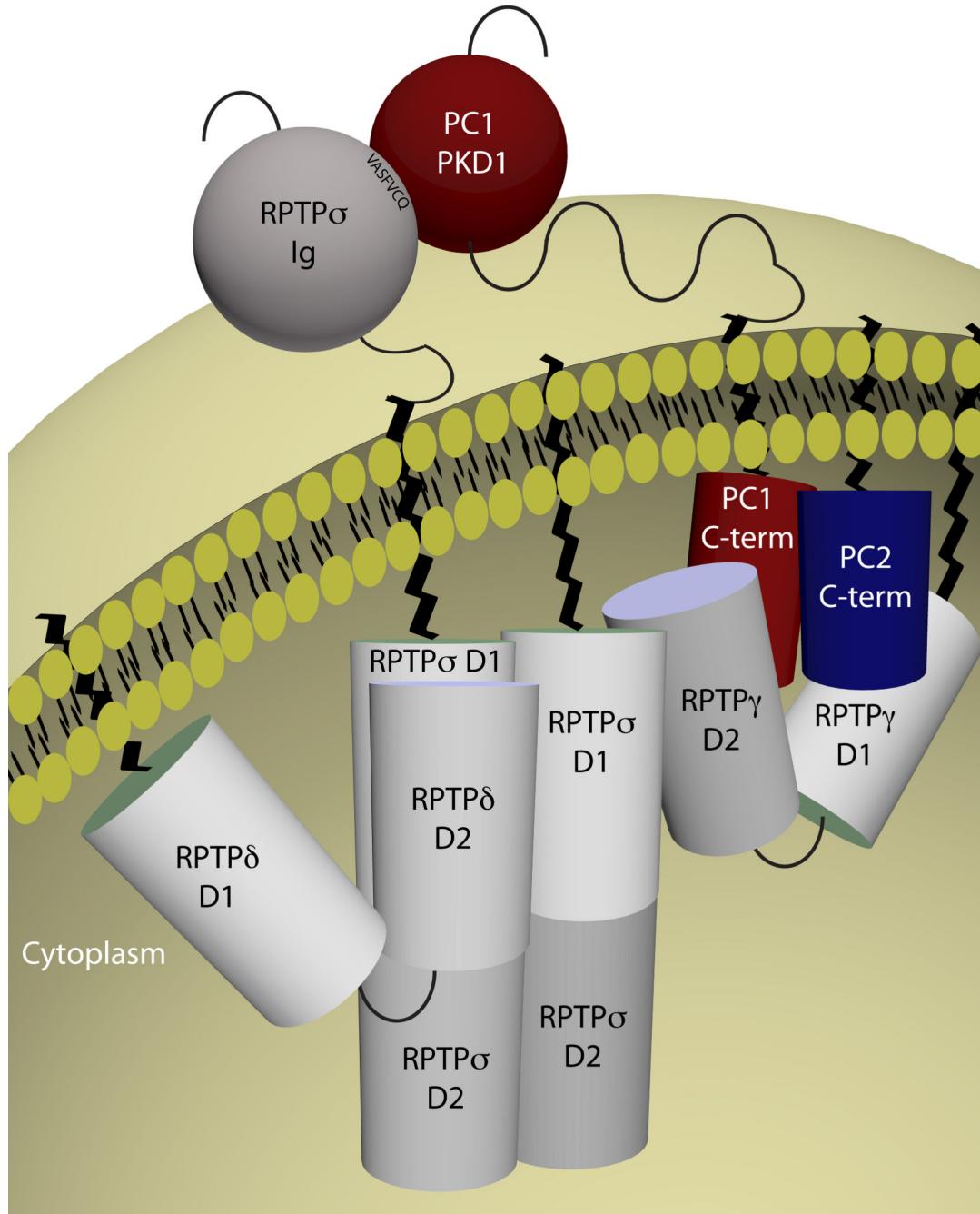
(A) RPTPs co-immunoprecipitate with E-cadherin and polycystin-1 in normal kidney (NK) and ADPKD primary cells. The expression of RPTP σ and RPTP δ was confirmed in normal and ADPKD cell lysates. E-cadherin was immunoprecipitated with a monoclonal antibody from cell lysates and immunoblot analysis was performed to confirm the presence of E-cadherin and polycystin-1 in the immunoprecipitates. Immunoblot analysis demonstrated the presence of RPTP σ and RPTP δ in the complex. Proteins were detected with the following antibodies: pan-cadherin used to detect E-cadherin on blots (Sigma), polycystin-1 (NM005), RPTP σ (KTPTP σ) and RPTP δ (KTPTP δ) and actin (MP Biomedical) as a loading control. Representative results from one of four independent trials. Three normal adult kidney

primary cell lines (67F05, 59M04, 57M03) and three ADPKD kidney primary cell lines (51M06, 46M06, 46F04) were used for these experiments. Arrowheads denote lower molecular weight bands seen on PC1 blot represent commonly observed cleavage products of 250 and 150 kDa. Graph shows densitometric quantification of two independent trials RPTP IP/E-cadherin IP:RPTP lysate/actin ± SEM. (B) RCTEC cell lysates were prepared and immunoprecipitated (IP) for E-cadherin (pAb) or using a normal rabbit IgG control antibody. Individual blots were probed for polycystin-1 (anti-LRR), E-cadherin (mAb), RPTP σ (pAb C19), RPTP δ (KPTP δ) and RPTP γ (pAb C18). Molecular weights are as indicated. Arrows indicate the co-precipitated proteins. Asterisks denote non-specific bands. Graph shows enrichment calculated as fraction of protein immunoprecipitated with E-cadherin antibody relative to the fraction of protein precipitated with control IgG. N=2. (C) E-cadherin-RPTP complexes form stable complexes that are resistant to dissociation in two different non-ionic detergents. Immortalized normal human kidney (RCTEC) or ADPKD cells (PKD9–12) were grown to confluence and lysed in Triton X-100 (TX) or n-octylglucoside (OG) containing buffer. Cell lysates (CL) and samples immunoprecipitated (IP) for E-cadherin using monoclonal (mEcad) or polyclonal (pEcad) were resolved via SDS-PAGE and immunoblotted with antibodies directed against actin, E-cadherin (arrowhead denotes Ig bands), pan-cadherin, RPTP σ and RPTP δ as indicated with respective molecular weights as indicated. N=4.

**Fig. 6.**

Y4237 phosphorylated polycystin-1 C-terminus is dephosphorylated by RPTP γ D1 and regulates AP-1 signaling. (A). Equal amounts of c-Src 32 P tyrosine phosphorylated polycystin-1 C-terminus were incubated with 10 units of each individual phosphatase domain and dephosphorylation determined by autoradiography. Upper bands (60kDa) represent autophosphorylated c-Src and lower bands CPC-1 (30kDa). (B). Time dependent dephosphorylation of 32 P-phospho-tyrosine in the polycystin-1 C-terminus by RPTP γ D1, LAR D1 or RPTP σ D1. (C). Confocal immunofluorescence images of MDCK cells labelled with antibodies directed against CD44 (E1/2) (labels polycystin-1 C-terminal fusion proteins: CD44-CTD-WT and CD44-CTD-Y4237F mutation) and pan-cadherin. (D) The C-

terminus of polycystin-1 activates AP-1 in a luciferase reporter assay. HEK293 cells were co-transfected with individual pMyr-EGFP-CTD and 5xjun:ATF firefly luciferase reporter constructs. Cell lysates were collected 48 h after transfection, and luciferase activities measured. Comparisons were made with pMyr-EGFP-CTD4147X co-transfected with the 5x-jun reporter construct and a negative (neg) control transfected with the 5x-jun reported construct only. Experiments were carried out in triplicate and the mean and standard deviation of relative luminescence plotted. A two-tailed paired Student t-test was performed for the luciferase activities of each construct compared with the wild type and * indicates statistical significance $p<0.05$ for each mutant when compared to wild-type activity. (E). Left Panel: Immunoblot probed with anti-GFP (Ab6556; AbCam) demonstrates equal protein expression of all constructs (arrows) used in AP-1 assay. A non-specific band detected by the anti-GFP antibody is seen in all lanes (*). Right Panel: Experiments were carried out in triplicate, densitometry was performed to measure the levels of expression of each chimeric protein (wild type, Y4127F, Y4237F and Y4127F/Y4237F pMyr-EGFP-CTD), and the mean relative expression and standard deviation plotted. A two-tailed paired Student t-test was performed for each construct compared with the wild type and * indicates statistical significance $p<0.05$. N= 3.

**Fig. 7.**

Diagrammatic illustration of the receptor protein tyrosine phosphatase (RPTP) type IIA subfamily and polycystin protein interactions. The cytoplasmic D1 domains of the RPTP proteins are membrane proximal and catalytic, while the D2 domains are regulatory, and serve to inactivate the phosphatase activity of the D1 domains and participate in substrate recognition. A novel interaction between RPTP γ D1 and D2 domains and the polycystins is identified and previously characterized interactions between RPTP isoforms are confirmed. The extracellular domains of the RPTP σ consist of Ig and fibronectin-like domains, which function as adhesion receptors. These domains are largely not shown except for the first Ig repeat of sigma, which serves as a ligand for the first PKD1 repeat of polycystin-1. The

polycystin/RPTP protein complex can also associate with the adherens junction protein E-cadherin (not shown) and the complex can be detected at the lateral membrane and in primary cilia of normal cells. Both the complex composition and protein localisations are altered in primary ADPKD cells suggesting an important regulatory role of the RPTP in polycystin function.

Proteins with sequence similarity to VASFHRQ identified using Pepsearch and BLAST.

Table 1

No.	Accession No.	Protein Name	Gene Name	Predicted localisation	Sequence
'phage display peptide sequence					
1	O43457	Hypothetical 9.2KDa Protein		intracellular	VASFHRQ
2	O60536	Antigen NY-CO-43		intracellular	VASFHRQC
3	Q13136	Liprin-alpha 1	<i>PPFIA1</i>	intracellular	VASFHRQ
4	Q96IY4	Carboxypeptidase B2 precursor	<i>CPB2</i>	intracellular	VASFHRN
5	Q13332	Receptor-type tyrosine protein phosphatase, S	<i>PTPRS</i>	extracellular	VASFLLR
6	P10586	Receptor-type tyrosine protein phosphatase, F	<i>LAR</i>	extracellular	VASFVCQ
7	P53708	Integrin Alpha-8	<i>ITGA8</i>	extracellular	VASFVCCQ
8	Q8WZ42	Titin	<i>TTN</i>	intracellular	VVEFHRQ
9	O15535	Zinc Finger Protein 193	<i>PRD51</i>	intracellular	VASFRIIQ
10	O75145	Liprin-alpha 3	<i>PPFIA3</i>	intracellular	VAHRRHRQ
11	O75335	Liprin-alpha 4	<i>PPFIA4</i>	intracellular	VDSFHHRV
12	Q9ULV5	Heat Shock Transcription Factor 4	<i>HSF4</i>	intracellular	VDSFHRT
13	Q03164	Zinc finger protein HRX	<i>MLL</i>	intracellular	ASFVRQ
14	O94874	KIAA0776 Protein	<i>KIAA0776</i>	Nuclear	VDSFFFRQ
15	Q12913	Receptor-type tyrosine-protein phosphatase eta	<i>PTPRA</i>	extracellular	AESFHKQ