A polycystin multiprotein complex constitutes a cholesterol-containing signalling microdomain in human kidney epithelia

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Polycystins are plasma membrane proteins that are expressed in kidney epithelial cells and associated with the progression of ADPKD (autosomal dominant polycystic kidney disease). A polycystin multiprotein complex, including adherens junction proteins, is thought to play an important role in cell polarity and differentiation. Sucrose gradient analyses and immunoprecipitation studies of primary human kidney epithelial cells showed the polycystins and their associated proteins E-cadherin and β -catenin distributed in a complex with the raft marker flotillin-2, but not caveolin-1, in high-density gradient fractions. The integrity of the polycystin multiprotein complex was sensitive to cholesterol depletion, as shown by cyclodextrin treatment of immunoprecipitated complexes. The overexpressed C-terminus of poly-

cystin-1 retained the ability to associate with flotillin-2. Flotillin-2 was found to contain CRAC (cholesterol recognition/interaction amino acid) cholesterol-binding domains and to promote plasma membrane cholesterol recruitment. Based on co-association of signalling molecules, such as Src kinases and phosphatases, we propose that the polycystin multiprotein complex is embedded in a cholesterol-containing signalling microdomain specified by flotillin-2, which is distinct from classical light-buoyant-density, detergent-resistant domains.

Key words: cholesterol, CRAC domain (cholesterol recognition/interaction amino acid domain), kidney epithelial cell, lipid raft, polycystin, reggie-1.

INTRODUCTION

Polycystins are integral membrane proteins, implicated in the pathogenesis of ADPKD (autosomal dominant polycystic kidney disease) and thought to play critical roles in kidney morphogenesis and epithelial differentiation [1,2]. The molecular composition of polycystin protein complexes is the subject of intensive investigation [3]. In our previous work, we identified a polycystin–E-cadherin– β -catenin complex formed at the cell plasma membrane of primary human kidney epithelial cells and disrupted in ADPKD, suggesting a possible role for the polycystin multiprotein complex in the regulation of adherens junction formation [4]. In the present study, we examined the association of the polycystin multiprotein complex with plasma membrane lipid microdomains.

Lipid rafts are membrane microdomains, enriched in sphingolipids and cholesterol that are thought to organize complexes of membrane proteins, particularly signalling molecules [5,6]. The organization of such lipid rafts is frequently orchestrated by specialized lipid-binding proteins such as the caveolins and flotillins (reggie proteins) and therefore these proteins are widely considered markers of lipid rafts. Among other significant functions, caveolins were found to bind cholesterol, as well as to regulate cholesterol trafficking, balance and cellular distribution [7,8]. Flotillin-1 and -2 were identified as membrane components of lipid rafts and implicated in signal transduction pathway events [9,10]. In some studies, flotillins were found to be associated with caveolae and caveolins [11], whereas in others, they were expressed independently from caveolins [12]. Similar to caveolins, flotillins were found to co-localize and associate with

cholesterol in biochemical [13], electron microscopy [14] and *in vitro* binding studies (Z. Surviladze, unpublished work).

Recent investigations determined a possible association between lipid rafts and cellular events critical in the establishment of cell–cell adhesion, such as actin reorganization and filopodia formation [10,15]. Interestingly, lipid rafts are also involved in the recruitment of a wide range of ion channels, including Ca^{2+} permeable channels [16,17]. Based on these observations, we speculated that polycystin-1, which, together with polycystin-2, forms a Ca^{2+} permeable channel [18], and is involved in the establishment of cell adhesion and polarity, might be associated with lipid rafts.

MATERIALS AND METHODS

Reagents

All reagents, including Brij 96 (Fluka, Gillingham, Dorset, U.K.), were obtained from Sigma–Aldrich unless otherwise specified. Lubrol was purchased from Calbiochem (EMD Biosciences, San Diego, CA, U.S.A.). The cDNA encoding the monomeric form of PFO (perfringolysin O) [19] was cloned by Dr A. E. Johnson and colleagues (Texas A&M University, College Station, TX, U.S.A.). This PFO^{C459A, G324V, G325V, A215C} mutant is a non-oligomerizing form of cytolytic toxin, which recognizes plasma membrane cholesterol, but does not form pores in the cell membrane. The monomeric form of PFO was purified and labelled in the laboratory of Dr B. Wilson (University of New Mexico HSC, Albuquerque, NM, U.S.A.).

Abbreviations used: ADPKD, autosomal dominant polycystic kidney disease; CC, chelerythrine chloride; CRAC, cholesterol recognition/interaction amino acid; GFP, green fluorescent protein; EGFP, enhanced GFP; FBS, foetal bovine serum; HEK-293 cells, human embryonic kidney 293 cells; HRP, horseradish peroxidase; mAb, monoclonal antibody; MBP, myelin basic protein; M β CD, methyl- β -cyclodextrin; MDCK cells, Madin–Darby canine kidney cells; PFO, perfringolysin O; PKC, protein kinase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.

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Cells and cell culture

All tissue culture reagents were obtained from Gibco BRL (Invitrogen, Carlsbad, CA, U.S.A.). Human primary kidney epithelial cells were derived from previously healthy individuals via the NDRI (National Disease Research Interchange, Philadelphia, PA, U.S.A.) isolated and cultured as described in [20,21]. Primary cystic epithelia were isolated and cultured as described. Samples from patient 3 are known to express hyperphosphorylated polycystin-1 [4]. MDCK (Madin–Darby canine kidney) cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing FBS (foetal bovine serum), L-glutamine and penicillin–streptomycin solution [22]. HEK-293 (human embryonic kidney 293 cells; A.T.C.C., Manassas, VA, U.S.A.) were used for GFP–flotillin transfections (where GFP stands for green fluorescent protein) and were grown in Eagle's minimum essential medium with Earle's balanced salt solution containing FBS, non-essential amino acids, L-glutamine and penicillin–streptomycin solution.

Antibodies

A polyclonal antibody raised against polycystin-1, NM005, was raised and purified as described in [4]. Mouse mAb (monoclonal antibodies) raised against human E-cadherin, β -catenin and flotillin-2 were obtained from Transduction Laboratories (BD Biosciences, Rockville, MD, U.S.A.). Mouse mAb raised against actin clone C4 was from ICN Biomedicals (Costa Mesa, CA, U.S.A.), and rabbit polyclonal antibody raised against polycystin-2 was from Zymed Laboratories (South San Francisco, CA, U.S.A.). A rabbit polyclonal antibody raised against caveolin-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Biotinylated anti-human IgG was from Vector Laboratories (Burlingame, CA, U.S.A.). HRP (horseradish peroxidase)-labelled secondary antibodies and streptavidin–horseradish peroxidase conjugate were obtained from Amersham Biosciences (GE Healthcare, Piscataway, NJ, U.S.A.). Texas Red-conjugated streptavidin, rhodamine-conjugated donkey anti-rabbit, FITC-conjugated donkey anti-mouse and CyTM5-conjugated donkey anti-mouse secondary antibodies were from Jackson Immunoresearch Laboratories (Westgrove, PA, U.S.A.).

Sucrose-density-gradient centrifugation

Sucrose-density-gradient centrifugation was performed as described in [23]. Primary human kidney cells were grown to confluence in 100 mm culture dishes for the analysis of endogenous protein complexes. HEK-293 cells were used to analyse the fractionation of the C-terminus of polycystin-1. Different non-ionic detergents [0.1, 0.01 or 0.05 % (v/v) Triton X-100 or 1 % (v/v) Lubrol or 1 % (v/v) Brij 96] prepared in Tris buffer (20 mM Tris, pH 8, 150 mM NaCl and 5 mM EDTA) were used for cell lysis. The cell lysate was passed three to five times through a 22G syringe needle and mixed 1:1 with 80 % sucrose in Tris buffer. The cell lysate sample was layered on 80 % sucrose and overlaid with a step gradient prepared from (top to bottom) 10, 15, 20, 25, 30 and 35 % sucrose solutions in Tris buffer. Centrifugation was performed at 135 000 g_{av} for 16–19 h at 4 °C; 400 μ l fractions were collected beginning at the top of the sucrose gradient.

Plasmids and transient transfections

Flotillin-2 (reggie-1)–EGFP (where EGFP stands for enhanced GFP) encoding full-length rat reggie-1 fused in frame at the C-terminus to EGFP was as described in [24]. The sIg₇-PKD1MN6 construct, kindly provided by Dr R. Maser (University of Kansas, Lawrence, KS, U.S.A.), encodes 1–165 amino acids of poly-

cystin-1 C-terminus fused to transmembrane region of CD7, followed by CH2 and CH3 domains of human IgG1. The construct represents a subclone derived from a clone originally isolated by Dr G. Walz (Renal Division, University Hospital Freiburg, Freiburg, Germany) [25].

Flotillin-2 (reggie-1)–EGFP was transfected into MDCK cells and the C-terminus of polycystin-1 was transfected into HEK-293 cells, using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were analysed 24 h post-transfection.

Immunofluorescence microscopy

Cells grown on coverslips or on 0.4 μ m pore filter supports (Falcon-BD, Franklin Lakes, NJ, U.S.A.) were permeabilized with 0.1 % Triton X-100 or 0.025 % (w/v) saponin and processed for immunofluorescence [4]. Fluorescence staining of total cholesterol in cultured cells was performed using filipin. Filipin was applied after cell fixation, at a concentration of 0.05 mg/ml in PBS, for 2 h at room temperature (24–25 °C). For the detection of plasma membrane cholesterol, live cells were incubated with monomeric (non-pore-forming), biotinylated PFO for 5 min at room temperature prior to fixation and subsequently fixed and detected with Texas Red-conjugated streptavidin secondary antibody (Jackson Immunoresearch Laboratories). Samples were imaged on Zeiss LSM510 and Zeiss LSM510-META confocal imaging systems in the University of New Mexico CRTC Fluorescence Microscopy Facility.

Immunoprecipitations

SDS/PAGE and immunoprecipitations with anti-polycystin NM005 were performed as described in [4]. The sIg fusion with the C-terminus of polycystin-1 was precipitated with Protein A–Sepharose as detailed by Walz and co-workers [25]. The immunoprecipitates probed for flotillin-2 were resolved under non-reducing conditions, i.e. without boiling and without 2-mercaptoethanol. Using this procedure, IgG remains as a heavy chain/light chain complex with molecular mass above 100 kDa. This method prevents interference of IgG heavy chain during the immunoblot detection of flotillin-2 and allows a clear visualization of the flotillin-2 band (molecular mass 42 kDa). Immunoprecipitation of proteins from sucrose density gradient fractions was performed by collecting the 'light' (pooled fractions 1–5) and 'heavy' (fractions 6–10) fractions separately, diluting with PBS (3-fold) and then adding the appropriate antibody for immunoprecipitation. For immunoblot analyses, the proteins were separated on 4–15 % gradient Criterion precast gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.). High molecular mass calibration marker ranging from 10 to 250 kDa molecular mass (Bio-Rad Laboratories) was used as a standard.

M β CD (methyl- β -cyclodextrin) treatment

Cell lysates were subjected to immunoprecipitation with an antibody raised against polycystin-1. To remove cholesterol from the protein complex, immunoprecipitates were incubated with 10 mM M β CD/PBS solution for 1 h at room temperature. Control samples were incubated with PBS alone. As a further control for cholesterol specificity, one immunoprecipitate was incubated for 1 h at room temperature in the presence of 10 mM M β CD saturated with cholesterol (8:1, mol/mol). The cyclodextrin–cholesterol complex was prepared as described in [26]. Briefly, cholesterol in a chloroform solution was dried under nitrogen in a glass culture tube. An appropriate volume of sterile-filtered M β CD in PBS was added to the tube, and the resulting suspension

was vortex-mixed and bath sonicated. The complex was then incubated overnight at 37 °C.

In vitro kinase and phosphatase assays

Primary human kidney cell lysates were immunoprecipitated with anti-polycystin-1 antibody, and the immunoprecipitates were subjected to *in vitro* kinase assays. The kinase reaction mix (30 μ l final volume) contained kinase buffer (25 mM Hepes, pH 7.2, 2.5 mM MnCl₂, 2.5 mM MgCl₂ and 0.1 % Triton X-100) and 2 μ Ci [γ -³²P]ATP. MBP (myelin basic protein) was used as the exogenous kinase substrate. Kinase inhibitors PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine) and CC (chelerythrine chloride; Calbiochem, La Jolla, CA, U.S.A.) were added to the kinase reaction mix in concentrations 20 nM and 3 μ M respectively. To detect phosphatase activity, the *in vitro* kinase assay was performed with or without 1 mM sodium vanadate in 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 5 mM dithiothreitol and 1 mM MnCl₂.

Densitometry

Densitometric analyses were performed on a GS 800 calibrated densitometer (Bio-Rad Laboratories) using PDQUEST software.

RESULTS

The polycystin–E-cadherin– β -catenin complex associates with the lipid raft marker flotillin-2

We previously showed that polycystin-1 and -2 form a multiprotein complex with the adherens junction proteins E-cadherin and β -catenin at the plasma membrane of human primary kidney epithelial cells, and suggested that formation of this complex is critical for normal renal epithelial polarity [4]. In the present study, we examined a possible association of the polycystin multiprotein complex with lipid-enriched signalling microdomains, lipid rafts. To address this question, we first tested if polycystin-1 was associated with the lipid raft markers caveolin-1 or flotillin-2. Co-immunoprecipitation experiments did not detect an association between polycystin-1 and caveolin-1 (results not shown). However, flotillin-2 was readily co-immunoprecipitated with the polycystin-1–E-cadherin– β -catenin multiprotein complex, but not in preimmune controls, suggesting that flotillin-2 is an integral part of the complex in primary human kidney epithelial cells (Figure 1A). Co-localization studies corroborated the biochemical data. Dual immunofluorescence staining with the antibodies raised against polycystin-1 and flotillin-2 revealed that the two proteins were partially co-localized both at the plasma membrane (mostly at the cell–cell contacts) and on intracellular membranes (Figure 1B). Together, these results suggested a possible lipid raft association of the polycystins and their assembled proteins.

Caveolin-1 and flotillin-2 constitute distinct domains in primary human kidney epithelia

Caveolin-1 and flotillin-2 are two lipid raft markers that have been variously reported to co-distribute or form distinct domains in different cell types [12,27,28]. Sucrose-density-gradient fractionation, co-immunoprecipitation and immunofluorescence co-localization studies were undertaken to evaluate the interrelationship of these two raft markers in human kidney epithelia. The recovery of caveolin-1 and flotillin-2 in light density fractions of sucrose gradients markedly depended on the nature of the

Figure 1 Polycystin-1 associates with the lipid raft marker protein flotillin-2

(A) Lane 1, total cell lysates immunoblotted for flotillin-2, E-cadherin and β -catenin using specific mouse mAbs raised against each protein. Lane 2, cell lysate immunoprecipitated (IP) with a polyclonal antibody raised against polycystin-1 and immunoblotted with specific mouse mAb against flotillin-2, E-cadherin and β -catenin to reveal co-precipitated proteins. Lane 3, control sample, where cell lysate was immunoprecipitated with an antibody raised against polycystin-1 and probed only with secondary HRP-labelled anti-mouse antibody. Lane 4, control sample, where cell lysate was immunoprecipitated with preimmune serum from rabbit used to raise NM0005 and immunoblotted (WB) for flotillin-2. Both controls confirm the specificity of polycystin-1/flotillin-2 association. (B) Dual immunostaining performed with antibodies directed against polycystin-1 (red) and flotillin-2 (green). Prominent sites of co-localization are indicated with arrows. Scale bar, 10 μ m.

detergent used for cell solubilization. In experiments with 1 % Lubrol and 1 % Brij 96, more caveolin-1 and flotillin-2 were detected in the light-buoyant-density fractions of the gradient and their distributions were more similar than in experiments with Triton X-100 (Figures 2A and 3B). The low-density sucrose gradient fractions 1–5 following solubilization in Brij 96 (Figure 2A) were collected and immunoprecipitated with an antibody raised against caveolin-1. Despite the co-distribution of flotillin-2 and caveolin-1 in sucrose density gradients under some detergent extraction conditions, these two lipid raft markers did not co-immunoprecipitate, suggesting that they define distinct domains (Figure 2B). Immunofluorescence staining of human kidney epithelial cells was used to probe the native membrane organization of the two lipid raft markers in human kidney cells. No significant co-localization of caveolin-1 (red) and flotillin-2 (green) (Figure 2C) was detected. Therefore we conclude that the two lipid raft marker proteins caveolin-1 and flotillin-2 constitute distinct membrane domains in primary human kidney epithelia.

Figure 2 Caveolin-1 and flotillin-2 do not associate in human primary kidney epithelial cells

(A) Distribution of flotillin-2 and caveolin-1 on 10–80 % sucrose density gradient. Detergent: 1 % Brij 96. (B) Fractions 1–5 of the sucrose gradient from (A) were immunoprecipitated (IP) with the antibody raised against caveolin-1 and probed for flotillin-2. IgG appears as a high (>100 kDa) molecular mass band, because the sample was treated under non-reducing conditions (see the Materials and methods section). Controls probed for caveolin-1 following immunoprecipitation with the anti-caveolin antibody were positive (results not shown). Flotillin-2, normally detected just below 50 kDa was notably absent. (C) Dual immunostaining of primary human kidney epithelial cells grown on coverslips for flotillin-2 (green) and caveolin-1 (red). Scale bar, 10 μ m.

The polycystin multiprotein complex is not associated with detergent-resistant, low-buoyant-density fractions

Lipid rafts and raft-associated proteins are typically distributed in light-buoyant-density fractions of sucrose gradients following sonication or detergent extraction. Surprisingly, sucrose-density-gradient centrifugation experiments indicated that polycystin-1, as well as E-cadherin and β -catenin, was significantly co-distributed in the 'heavy', high-density fractions of sucrose density gradients (Figure 3A). The protein distributions shown were consistently observed, irrespective of the type of non-ionic detergent used for protein solubilization (Figures 3A–3D). Numerous experiments were performed using different concentrations and different types of non-ionic detergents (0.1, 0.01 or 0.05 % Triton X-100 or 1 % Lubrol or 1 % Brij 96), as well as no detergent where the cells were homogenized by sonication. In all cases, endogenous polycystin-1 from normal human kidney cells was consistently found in the high-density fractions where it was partially co-distributed with E-cadherin, β -catenin, actin and flotillin-2. E-cadherin, β -catenin and flotillin-2 were in some cases also detected in lower density, detergent-resistant fractions that are often equated with being representative of 'lipid rafts' (Figure 3C, fractions 1–5). Caveolin was principally enriched in the light-buoyant-density fractions and, upon solubilization in 1 % Lubrol, was largely distinct from flotillin-2 (Figure 3D). Actin depolymerization prior to sucrose-density-gradient centrifugation did not shift the distribution of polycystin-1 or adherens junction proteins, suggesting that the migration of these proteins in the heavy-buoyant-density fractions of the gradient is not due to their association with the actin cytoskeleton (results not shown).

The light-buoyant density of detergent-resistant membrane fractions is in part dependent on the lipid to protein mass ratio [29]. Given the large size of native polycystin-1, we comparatively evaluated the gradient fractionation of a chimaeric, trans-membrane form of the C-terminus of polycystin-1 that is known

Figure 3 Endogenous polycystin-1 and flotillin-2 have a heavy buoyant density on sucrose gradients

Cell lysates were prepared by detergent solubilization under different conditions and fractionated on 10–80 % sucrose density gradient as detailed in the Materials and methods section. The resulting distributions of endogenous polycystin-1, E-cadherin, β -catenin, flotillin-2, caveolin-1 and actin were evaluated by immunoblotting. (A–D) Cell lysates from normal human kidney cells were subjected to sucrose gradient fractionation following solubilization with (A–C) Triton X-100 (0.05, 0.1 or 1 %) or (D) Lubrol (1 %).

Figure 4 The overexpressed C-terminus of polycystin-1 associates with flotillin-2

The slg.7-PKD1MN6 construct was used to express the C-terminus of polycystin-1 (165 amino acids) as a transmembrane fusion protein with human slg in HEK-293 cells. **(A)** The Ig-containing fusion protein was immunoprecipitated (IP) directly using Protein A-Sepharose beads. The bound proteins were resolved by SDS/PAGE and immunoblotted (WB) for (lane 1) polycystin-1 using biotinylated anti-human IgG or (lane 2) flotillin-2 using a specific mouse mAb. Control experiments demonstrated that flotillin-2 was not bound to Protein A-Sepharose beads from control lysates (results not shown). **(B)** Cell lysates from HEK-293 cells overexpressing the C-terminus of polycystin-1 fused to slg were subjected to sucrose gradient fractionation following solubilization with 0.1 or 1% Triton X-100.

to be active in intracellular signalling [30–32]. The overexpressed C-terminus of polycystin-1 retained the ability to complex with flotillin-2 based on co-precipitation experiments (Figure 4A). Untransfected cell lysates and lysates from cells expressing a control chimaera with an identical extracellular and trans-membrane segment served as negative controls in the co-precipitation studies (results not shown). The polycystin-1 C-terminus chimaera was distributed both in low- and high-density fractions of the sucrose density gradient, where it partially co-distributed with β -catenin and flotillin-2 (Figure 4B). The distribution of the polycystin-1 C-terminus chimaera was modestly affected by changes in detergent concentration with peak fractions redistributing to light-buoyant-density fractions (Figure 4B). Together, these results imply that polycystin-1 and associated proteins are most likely part of atypical protein–lipid complexes that have a sizeable protein mass, and association with flotillin-2 membrane domains is in part dictated by C-terminal sequences in polycystin-1.

Associations between flotillin-2 and some components of the polycystin multiprotein complex are mediated by cholesterol

The co-immunoprecipitation and co-fractionation of the polycystin multiprotein complex with the raft marker flotillin-2 raised the question as to whether or not the complex contained lipids, particularly cholesterol molecules. To address this question, primary human kidney cell lysates were subjected to immunoprecipitation with an antibody raised against polycystin-1 and the immunoprecipitate was divided into two samples, one of which was incubated with M β CD and the other was left untreated. *In vitro* M β CD treatment was used because the treatment of living cells with cyclodextrin is often harmful (may cause cell death) and may, therefore, indirectly affect the outcome of the experiment. Our procedure allowed us to observe the direct effect of cholesterol depletion on protein–protein interactions within the complex. The results revealed that cholesterol depletion from the immunoprecipitated protein complex significantly reduced the association between polycystin-1 and flotillin-2 (Figures 5A and

5F). M β CD treatment also significantly decreased the association of polycystin-1 with E-cadherin (Figure 5B) and β -catenin (Figures 5B and 5F), but only minimally decreased its association with polycystin-2 (Figure 5B).

Two controls provide evidence for the specificity of the M β CD treatment (Figure 5C). Immunoblotting for polycystin-1 served as a control to show that depletion of cholesterol did not affect the stability of the antibody–antigen complex, as expected. Thus the amount of polycystin-1 in the immunoprecipitate was unchanged following M β CD treatment (Figure 5C, left panel). A second control performed with cholesterol-saturated M β CD confirmed that the treatment of the immunoprecipitates with M β CD specifically depleted cholesterol, but not other lipids within the protein complex. Cholesterol-saturated M β CD is often used on live cells to demonstrate cholesterol specificity [26,33]. As shown in the right panels of Figure 5(C), M β CD saturated with cholesterol failed to disrupt the association between the proteins of polycystin-1 complex. The M β CD–cholesterol complex is expected to prevent depletion of cholesterol (but not of other molecules) from the immunoprecipitates, thereby confirming the importance of cholesterol in the overall stability of the complex.

Immunoprecipitation of polycystin-1 from high-density sucrose gradient fractions revealed flotillin-2 complexed with E-cadherin and β -catenin in ‘non-raft’ fractions (Figure 5D). The association between the polycystin–flotillin-2 multiprotein complex purified on sucrose gradients was also sensitive to cholesterol depletion. We conclude that even though the large multiprotein complex formed by polycystin-1, E-cadherin, β -catenin and flotillin-2 is not associated with typical ‘lipid rafts’, it contains cholesterol molecules that mediate interactions between complexed proteins and may represent a specific membrane domain organization.

Overexpressed flotillin-2 co-localizes with cellular cholesterol at the plasma membrane

The lipid raft marker caveolin-1 is also known for its cholesterol-binding properties and its regulatory roles in cellular cholesterol balance and distribution [7,8]. Given the dependence of the polycystin–flotillin-2 complex stability on cholesterol, we analysed the distribution of cellular cholesterol relative to overexpressed flotillin-2 using two different probes. First, overexpressed flotillin-2–GFP in MDCK cells was co-localized with plasma membrane cholesterol using biotinylated, monomeric PFO as a probe. The monomeric form of PFO toxin specifically binds cholesterol and thus monitors the abundance of plasma membrane cholesterol in the absence of fixation [19]. Significantly, the overexpressed flotillin-2 was most prominent at cell–cell contact sites where it markedly co-localized with cholesterol identified both with PFO (Figures 6A–6F) and with filipin (Figures 6G–6I). In some transfected cells, there was a notable redistribution of cholesterol from an intracellular pool to the plasma membrane enriched in flotillin-2–GFP. Endogenous flotillin-2, polycystin-1 and cholesterol could also be shown to be co-localized at the plasma membrane and intracellularly (Figure 7). It should be noted that for the triple staining experiment, detergent permeabilization was required to reveal the intracellularly localized endogenous flotillin-2; therefore, cholesterol staining is not as robust as in the experiments using GFP–flotillin and staining for cholesterol prior to fixation.

Caveolin and other cholesterol-binding proteins may be identified by the presence of the CRAC (cholesterol recognition/interaction amino acid) motif [L/V-X(1-5)-Y-X(10-5)-R/K] [34,35]. Flotillin-2 contains two putative, previously unidentified CRAC motifs encompassing amino acid residues 120–127

Figure 5 Associations between select proteins in the polycystin complex are mediated by cholesterol

Cell lysates were immunoprecipitated (IP) with our antibody against polycystin-1 and the immunoprecipitates were divided into two aliquots, one of which was incubated with 10 mM M β CD for 1 h at room temperature (+ C) and the other was left untreated (– C). The immunoprecipitated proteins were recovered and probed with antibodies raised against (A) flotillin-2, (B) E-cadherin, polycystin-2 (PC-2) and β -catenin, and (C, left panel) PC-1. (C, right panel) Immunoprecipitates were treated, in parallel, with M β CD saturated with cholesterol (+ C/Ch) or M β CD only (+ C) (1 h at room temperature in both cases), as a further control. Immunoprecipitated proteins were recovered and probed with antibodies raised against β -catenin and flotillin-2. (D, E) The high-density fractions of the sucrose gradient were collected and immunoprecipitated with antibody raised against polycystin-1. Immunoprecipitates were probed with antibodies raised against E-cadherin, β -catenin and flotillin-2. (E) Immunoprecipitate was left untreated (– C) or treated with M β CD (+ C). (F) The data from three independent experiments as described for (A–C) were quantified and the mean values plotted. The amount of β -catenin and flotillin-2 in untreated precipitates was taken as 100 % (white bars). After M β CD treatment (grey bars), the amount of β -catenin decreased by 72.5 % and that of flotillin-2 by 88.8 %.

(VEQIYQDR) and 157–169 (VYDKVDYLSSLGK). The CRAC motifs are found 100 residues downstream of the fatty acid modifications and in the N-terminal half of the protein that is important for membrane association [24]. Taken together, flotillin-2 has the hallmarks of a cholesterol-binding protein and when overexpressed affects the cellular distribution of cholesterol, suggesting that similar to caveolin-1, flotillin-2 may locally concentrate cholesterol and thereby mediate the cholesterol-dependent organization of protein complexes.

The polycystin–flotillin-2 multiprotein complex constitutes a signalling microdomain

Lipid-enriched microdomains, or lipid rafts, are thought to function as ‘signalling platforms’ that selectively organize a host of signalling molecules, including among others Src-family kinases [36] and phosphatases [37]. We therefore considered if the polycystin–flotillin-2 multiprotein complex might function as a signalling microdomain. The possible association of kinase and phosphatase activities with the protein complex was assessed by *in vitro* kinase assays performed on the polycystin multiprotein complexes isolated by immunoprecipitation. A high level of *in vitro* phosphorylation of the exogenous kinase substrate MBP (Figure 8A, lane 2) was observed, suggesting that the polycystin multiprotein complex indeed includes kinases. Kinase assays performed in the presence of two different kinase inhibitors were used to identify the possible kinases. The Src kinase inhibitor

PP2 reduced the level of MBP phosphorylation by 36 %, whereas the PKC (protein kinase C) inhibitor CC had no significant effect on kinase activity (Figure 8B). Thus the polycystin protein complex includes important signalling molecules such as Src-family kinases. In this regard, it is noteworthy that flotillin-2 is subject to phosphorylation and possible regulation by Src kinases (C. Neumann-Giessen and R. Tikkanen, unpublished work).

The general phosphatase inhibitor sodium vanadate was used to assay for the presence of phosphatase activity in the complex. Inclusion of vanadate in the phosphorylation reaction revealed the activity of phosphatases in the complex and induced roughly a 50 % increase in phosphorylation of MBP and polycystin-1, relative to the phosphorylation detected in the absence of vanadate (Figures 8C and 8D). On low-percentage gels, a notable increase in electrophoretic mobility of the polycystin-1 protein was detectable when phosphatase activity was inhibited with vanadate (Figure 8C). Based on the *in vitro* phosphorylation data, we conclude that both kinases and phosphatases are part of the polycystin/flotillin-2 multiprotein complex and may regulate protein interactions or activity of the complex. In addition, polycystin-1 and flotillin-2 are most likely targets of these endogenously associated kinases and phosphatases.

In published studies, we reported that polycystin-1 is in some cases hyperphosphorylated in primary cystic human kidney epithelial cells derived from ADPKD patients [4]. Initial gradient fractionation studies performed using cell lysates from two different ADPKD primary cell lines did not reveal any striking

Figure 6 Overexpressed flotillin-2-GFP co-localizes with cellular cholesterol

Flotillin-2-GFP was overexpressed in MDCK cells. (A–F) Plasma membrane cholesterol was visualized with biotinylated PFO. Texas Red-conjugated streptavidin was used for detection. (G–I) Total free cellular cholesterol was visualized using the cholesterol-binding fluorochrome filipin. Black and white panels show individual localizations of flotillin-2-GFP and cholesterol and merge shows extent of overlap. Scale bar, 10 μ m.

changes in the sedimentation properties of polycystin-1, despite some variability in protein expression levels (Supplementary Figure 1; see <http://www.BiochemJ.org/bj/392/bj3920029add.htm>). Patient 1 is known to have a germline missense mutation in polycystin-1, while the germline mutation in patient 3 has not been definitively identified (P. Harris and A. Wandering-Ness, unpublished work). Polycystin-1 has been shown to be hyperphosphorylated in patient 3 [4]. Polycystin-1 from patient 3 remained complexed to flotillin-2, based on co-immunoprecipitation experiments, but the complex was depleted from the plasma membrane and the proteins were observed co-localized intracellularly (Supplementary Figure 1).

DISCUSSION

In the present study on primary human kidney epithelial cells, combined biochemical and immunofluorescence experiments showed that a polycystin multiprotein complex was associated with the lipid raft marker flotillin-2, cholesterol as well as

protein kinases and phosphatases. Thus we identify flotillin-2 as a new polycystin-1/polycystin-2-associated protein that probably serves to organize polycystins and the associated E-cadherin/ β -catenin proteins into a novel signalling membrane domain. We envision that regulated phosphorylation and dephosphorylation of associated proteins may influence their plasma membrane disposition.

The large, cholesterol-containing, multiprotein complex was distributed in high-density sucrose gradient fractions and proved to be distinct from low-density lipid rafts. On the basis of the known cell-surface distribution of the polycystin–E-cadherin– β -catenin complex [4], we propose that the polycystin multiprotein complex constitutes a new type of signalling membrane microdomain. Even though signalling protein complexes are frequently associated with low-density lipid rafts, some complexes may be formed outside these lipid-rich raft fractions. For example, GPI (glycosylphosphatidylinositol)-anchored protein Thy-1 complexes can be found both in low- and high-density sucrose gradient fractions [23]. The existence of ‘heavy rafts’ recently suggested in high-density fractions was explained by

Figure 7 Endogenous flotillin-2, cholesterol and polycystin-1 all co-localize at the plasma membrane and intracellularly

Triple fluorescence staining was performed in primary human kidney epithelial cells. Polycystin-1 (red) and flotillin-2 (green) were detected using specific antibodies. Cholesterol was visualized using the cholesterol-binding fluorochrome filipin (blue). Black and white panels show individual localizations of endogenous polycystin-1, flotillin-2 and cholesterol. Prominent sites of co-localization are indicated with arrows in the upper panel. Lower panel: higher magnification of the cell enclosed by a rectangle in the upper panel. Scale bar, 10 μ m.

a higher protein/lipid ratio within such microdomains [29]. Proteins may exhibit a light buoyant density because they are encased in 'lipid shells' of cholesterol and sphingolipids of varying sizes [38]. Even with such a lipid shell surrounding the polycystin-1 protein, the large size of the polycystin-1 itself (polycystin-1 contains 4304 amino acid residues with a large, 2579 residue, extracellular domain) may preclude it and any associated protein from flotation on sucrose density gradients. Accordingly, the significantly smaller chimaeric Ig-polycystin-1 C-terminus retained an association with flotillin-2 and was distributed both in high- and low-density gradient fractions. The cumulative data support the idea that polycystin-1 fractionation on density gradients is affected by the large molecular mass of the polycystin-1 and possibly the number of associated proteins, which in turn affects the protein/lipid ratio of the complex. The presence of flotillin-2 and dependence of complex integrity on cholesterol are taken as indicators for it constituting a unique non-raft, membrane domain rather than an isolated protein complex.

The considerable distribution of flotillin-2 in 'non-raft' high-density fractions strongly suggests that it plays an important role in the function and organization of proteins and protein complexes, which are not included in typical lipid rafts. Flotillins and caveolins have been suggested to have overlapping or redundant functions in some cell types [39]. However, in neurons [12], lymphocytes [27,28] and, as we show here, in kidney epithelia, caveolin-1 and flotillin-2 have quite distinctive localizations. The polycystin multiprotein complex was strictly associated with flotillin-2. Therefore membrane domains, defined by flotillin-2 and caveolin-1, appear to have unique functions in polarized human kidney epithelial cells.

It is of interest to consider the role of flotillin-2 in cholesterol recruitment and the regulation of the polycystins. As a part

of the polycystin-1 multiprotein complex, flotillin-2 may serve to regulate the level and distribution of cholesterol within the microdomain and thereby affect both the spatial distribution and activities of associated proteins. The presence of CRAC domains and increased recruitment of cholesterol to the lateral cell-cell contacts enriched in overexpressed flotillin-2-GFP are taken as indicators of the capacity of flotillin-2 to redistribute specifically and organize cholesterol. In low-density lipid rafts, glycosphingolipids, facilitated by cholesterol and in some cases raft proteins, are documented to induce local clustering and affect the recruitment of signalling molecules [40,41]. We envision that flotillin-2 and cholesterol in the polycystin multiprotein complex similarly co-ordinate the close spatial clustering of the polycystins with the E-cadherin/ β -catenin cell-cell adhesion molecules and regulatory signalling molecules. The integration of polycystin-2-related calcium channels, such as TrpC1, in caveolin membrane domains is suggested to promote close association with regulatory G-proteins and is functionally important for Ca^{2+} signalling [16,42]. Interestingly, flotillin-2 overexpression has been linked to the co-ordinate overexpression of a G-protein-coupled receptor in melanoma cells [43]. Therefore it will be of interest in future studies to examine the functional relationship between polycystin-1/polycystin-2 embedment in flotillin-2 domains and their tightly interrelated G-protein and Ca^{2+} signalling activities [25,44].

Flotillin-2 may also serve a scaffolding role and, similar to caveolins, biochemically partition the membrane into microdomains by its propensity to oligomerize and associate with other proteins [10,45,46]. Flotillins were initially identified based on their association with cell-adhesion molecules and later implicated in cellular events critical for cell adhesion, such as filopodia formation and actin reorganization [9,10,24]. However, the actin associations were labile and not maintained during

Figure 8 The polycystin-1-flotillin-2 multiprotein complex contains kinases and phosphatases

(A) Kinase and phosphatase activities associated with polycystin-1 immunoprecipitates were measured as described in the Materials and methods section. Lane 1, immunoprecipitates incubated without the exogenous kinase substrate MBP but with [γ - 32 P]ATP; lane 2, immunoprecipitates incubated with MBP and [γ - 32 P]ATP alone. (B) MBP phosphorylation was inhibited in the presence of the Src kinase inhibitor PP2, but not by the PKC inhibitor CC. Values were normalized to kinase reaction without inhibitors. (C, D) Effect of vanadate, a general phosphatase inhibitor. Inclusion of vanadate in the phosphorylation reaction resulted in altered polycystin-1 mobility (C) and increased the maximal phosphorylation of both MBP and polycystin-1 roughly by 50% above the values without vanadate (D).

detergent lysis and gradient fractionation. Thus flotillin-2 appears to scaffold a polycystin multiprotein complex required for cell-cell adhesion and may co-ordinate, together with the adherens junction proteins, the actin assemblies necessary for initiating and stabilizing cell-cell adhesion.

The flotillin-2/polycystin microdomain contained kinases and phosphatases, suggesting that the domains also serve to integrate signalling. Polycystin-1 contains numerous sites in its C-terminus that are targets for regulatory phosphorylation by Src kinase and PKA [47,48]. Opposing activities of tyrosine kinases (Src and PKC, among others) and, as we show here, closely associated tyrosine phosphatases control the dephosphorylation of polycystin-1. Indeed in ADPKD, the hyperphosphorylation of polycystin-1 contributes to the disruption of the polycystin-1-E-cadherin complex [4], suggesting a possible loss of associated phosphatases. ADPKD cells expressing a hyperphosphorylated form of polycystin-1 retained an association with flotillin-2, but the proteins were localized intracellularly. Counteracting kinase and phosphatase activities also regulate the formation and disassembly of adherens junctions and the plasma membrane localization of E-cadherin [49–51]. Therefore the organized assembly of Src kinases and tyrosine phosphatases, in keeping with the biological functions associated with the polycystin protein complex, may have a significant impact on polycystin-1 signalling and the control of stable adherens junction-mediated adhesion by affecting the plasma membrane stability/localization of these proteins.

In summary, the work presented here identifies a large, cholesterol-containing signalling microdomain, which is distinct

from low-density, detergent-resistant membrane fractions typically used to characterize 'lipid raft' domains. The cholesterol-binding capacity and oligomerization capacity of flotillin-2 contribute to the structural integrity and association of the adherens junction proteins with the polycystins. The domain also promotes the concentration of the requisite kinases and phosphatases that are known to affect the activities of the polycystins and adhesion proteins.

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