

The human polycystin-2 protein represents an integral membrane protein with six membrane-spanning domains and intracellular N- and C-termini

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PKD2 is one of the two genes mutated in ADPKD (autosomal-dominant polycystic kidney disease). The protein product of *PKD2*, polycystin-2, functions as a non-selective cation channel in the endoplasmic reticulum and possibly at the plasma membrane. Hydrophobicity plots and its assignment to the TRP (transient receptor potential) family of cation channels suggest that polycystin-2 contains six transmembrane domains and that both the N- and C-termini extend into the cytoplasm. However, no experimental evidence for this model has so far been provided. To determine the orientation of the different loops of polycystin-2, we truncated polycystin-2 within the predicted loops 1–5 and tagged the constructs at the C-terminus with an HA (haemagglutinin) epitope. After transient expression and selective membrane permeabilization, immunofluorescence staining for the HA epitope revealed that loops 1, 3 and 5 extend into the lumen

of the endoplasmic reticulum or the extracellular space, whereas loops 2 and 4 extend into the cytoplasm. This approach also confirmed the cytoplasmic orientation of the N- and C-termini of polycystin-2. In accordance with the immunofluorescence data, protease protection assays from microsomal preparations yielded protected fragments when polycystin-2 was truncated in loops 1, 3 and 5, whereas no protected fragments could be detected when polycystin-2 was truncated in loops 2 and 4. The results of the present study therefore provide the first experimental evidence for the topological orientation of polycystin-2.

Key words: membrane topology, polycystin-2, transient receptor potential family.

INTRODUCTION

At a prevalence of at least 1:1000, ADPKD [autosomal-dominant PKD (polycystic kidney disease)] is one of the most common inherited human diseases [1]. The main symptom of ADPKD is cyst formation within the kidney parenchyma, but additional symptoms, such as liver and pancreatic cysts, heart valve defects and aneurysms, are common. In total, 85% of the patients carry mutations in the *PKD1* gene, and the remaining 15% suffer from mutations in the *PKD2* gene [2–5]. The protein encoded by *PKD1*, polycystin-1, is a transmembrane protein encompassing 4302 amino acids [6–8], 11 transmembrane domains, an extracellular N-terminus and a cytoplasmic C-terminus [9]. Polycystin-2, the protein product of the *PKD2* gene, is an integral membrane protein of 968 amino acids with a predicted molecular mass of 110 kDa [10,11]. It functions as a non-selective channel for mono- and bi-valent cations, and its activity is regulated by Ca²⁺ ions [12], probably through an EF-hand domain within the C-terminal domain [13,14]. The subcellular location of polycystin-2 is discussed controversially. Beside its location in the ER (endoplasmic reticulum), attributed to a 34-amino-acid cluster in the C-terminus [10], polycystin-2 may also be present at the plasma membrane [15]. It is generally accepted, however, that polycystin-2 is integrated in the plasma membrane of the primary cilium [16,17]. This pathway is regulated by a trafficking motif located within the first 15 amino acids of the protein [18].

The close homology of polycystin-2 with the TRP (transient receptor potential) family of cation channels suggests that polycystin-2 consists of six transmembrane domains with both the

N- and C-terminus extending into the cytoplasm. In the absence of crystallography data for this membrane protein and in order to gain structural information which might help to better understand its function, we determined the topology of polycystin-2 by molecular and biochemical approaches.

MATERIALS AND METHODS

Expression plasmids and transfections

The full-length human *PKD2* cDNA (a gift from Dr Stefan Somlo, Yale University, New Haven, CT, U.S.A.) was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen). Various expression constructs were generated which coded for polycystin-2 proteins of 460, 493, 547, 577, 582 and 625 amino acids in length respectively. In most cases an HA (haemagglutinin) epitope was fused to the C-terminus of the proteins (Table 1).

COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum). They were transiently transfected at a confluency of 70–80% with 15 μg of DNA per 60-mm dish using the DEAE-dextran protocol [19].

Microsomal preparations

All manipulations were carried out at 4°C. Five 100-mm dishes with COS-7 cells were transiently transfected with the respective expression plasmids. Cells were washed three times 48 h later

Abbreviations used: ADPKD, autosomal-dominant polycystic kidney disease; Cy3, indocarbocyanine; ER, endoplasmic reticulum; HA, haemagglutinin; HRP, horseradish peroxidase; PKD, polycystic kidney disease; PNGase F, peptide N-glycosidase F; TRP, transient receptor potential.

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Table 1 Sequence of polycystin-2 constructs

The last ten amino acids of polycystin-2 are shown on the left of each sequence (the numbers in parentheses indicate the position of the most C-terminal amino acid of the human polycystin-2 protein). The HA epitope is underlined, and the italicized residues result from the cloning process.

Construct	Sequence
Polycystin-2 (fl)-HA	...GGNGSSNVHV(968) <u>YYPYDVPDYA</u> LE
Polycystin-2 (loop 5)-HA	...YLVFGTQVDD(625) <u>LSYPYDVPDYA</u> ID
Polycystin-2 (loop 4)-HA	...LFKFINFNRT (582) <u>LSYPYDVPDYA</u> ID
Polycystin-2 (1-577)	...FVWIKLKF(577)
Polycystin-2 (1-577)-HA	...FVWIKLKF(577) <u>YLSYPYDVPDYA</u> ID
Polycystin-2 (loop 3)-HA	...FLEDQNTFPN(547) <u>LSYPYDVPDYA</u> ID
Polycystin-2 (loop 2)-HA	...IFYVVVEIL(493) <u>DL</u> <u>SY</u> <u>PY</u> <u>DV</u> <u>PD</u> <u>YA</u> <u>ID</u>
Polycystin-2 (loop 1)-HA	...VIPSWQFQPL(460) <u>SY</u> <u>PY</u> <u>DV</u> <u>PD</u> <u>YA</u> <u>ID</u>

with 1 × PBS, scraped off of the dishes and centrifuged for 15 min at 200 g at 4°C. The cell pellet was resuspended in buffer A [10 mM Hepes/KOH (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 5 mM sodium EDTA, 5 mM EGTA and 250 mM sucrose], incubated for 15 min and then homogenized with 20 strokes in a Dounce homogenizer. Nuclei and intact cells were removed by centrifugation for 30 min at 1000 g. The supernatant was cleaned once more by centrifugation for 15 min at 1000 g before membrane vesicles were collected by centrifugation for 30 min at 55 000 rev./min (Beckman Coulter TLA-55). This microsomal fraction was finally resuspended in buffer B [10 mM Hepes/KOH (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 5 mM sodium EDTA, 5 mM EGTA, 250 mM sucrose and 100 mM NaCl].

Protease protection assay

The microsomal preparation (in buffer B) was divided into two halves and centrifuged again at 55 000 rev./min (Beckman Coulter TLA-55) for 30 min. Subsequently, one pellet was resuspended in buffer B containing 1% Triton X-100 and the other pellet in buffer B only. The resuspended vesicles were incubated for 1 h at 4°C in the presence or absence of 1 mg/ml proteinase K [except in the case of polycystin-2 (loop 3)-HA when 4 mg/ml proteinase K was used]. A control reaction in buffer B without Triton X-100 was performed in parallel. Reactions were stopped by incubating in 5 mM PMSF for 20 min at room temperature (20°C), and analysed by immunoprecipitation, as described below.

PNGase F (peptide N-glycosidase F) assay

Proteinase K-treated and untreated microsomal preparations of transiently transfected COS-7 cells synthesizing polycystin-2 (loop 1)-HA were denatured for 10 min at 100°C in 1 × denaturing buffer (New England Biolabs) as recommended in the manufacturer's instructions. Samples were then supplemented with (final concentration) 1% Nonidet P40 and 1 × G7 buffer (New England Biolabs) and incubated with 8.3 units/μl of PNGase F (New England Biolabs) for 90 min at 37°C; control samples were incubated without PNGase F. Finally, the deglycosylated proteins were subjected to immunoprecipitation as described below.

Immunoprecipitation

For immunoprecipitations, non-permeabilized samples from the protease protection assays were first permeabilized for 30 min

on ice by adding 1% Triton X-100 (final concentration). The reactions were performed in 600 μl of buffer B containing 20 μl of Protein A-Sepharose beads (GE Healthcare) coupled with the monoclonal anti-(HA epitope) antibody 12CA5 (500 μl of hybridoma supernatant per 20 μl of beads). Immunoprecipitations were performed for 4 h at 4°C on a rotating wheel, then the beads were briefly spun down, washed four times in buffer B and resuspended in an equal volume of 2 × sample buffer. Supernatants obtained after the immunoprecipitation were kept for later Western blot analysis.

Western blot analysis

Total cell lysates were prepared 48 h after the transient transfection of COS-7 cells. The cells were washed three times with 1 × PBS, scraped off the Petri dishes and centrifuged for 15 minutes at 200 g and 4°C. Afterwards, the cell pellet was lysed in 1 × PBS, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 0.5 mM PMSF, and the protein concentration was determined using the Bradford assay. Total protein or immunoprecipitated protein (30 μg) were subjected to denaturing PAGE before being transferred on to a PVDF membrane (Millipore). The membrane was incubated with one of the following primary antibodies: the rat monoclonal anti-(HA epitope) antibody 3F10 (Roche Diagnostics; diluted 1:4000), a rabbit polyclonal anti-calreticulin antibody (Stressgen; diluted 1:2000) and a rabbit polyclonal anti-actin antibody (Sigma; diluted 1:2500). The following secondary antibodies were used: HRP (horseradish peroxidase)-conjugated anti-(rabbit IgG) (Sigma; diluted 1:20 000) and HRP-conjugated anti-rat IgG (Dianova; diluted 1:20 000).

Immunofluorescence

For immunofluorescence labelling, cells were fixed for 1 h at room temperature with Bouin's solution [0.1 M sodium phosphate (pH 7.4), 15% saturated picric acid and 2% formaldehyde], washed three times with 1 × PBS, and permeabilized for 15 min with 0.25% Triton X-100 at room temperature or with 5 μg/ml digitonin at 4°C. Fixation of non-permeabilized cells was performed for 1 h at room temperature with 1 × PBS and 4% paraformaldehyde, then cells were washed three times in 1 × PBS, and blocked with 1 × PBS/2% BSA for 1 h at room temperature. The fixed cells were incubated with the primary antibodies (diluted in 1 × PBS/2% BSA) for 2 h at room temperature. After the cells were washed once with high-salt 1 × PBS (total concentration of 500 mM NaCl) and twice with 1 × PBS, they were incubated with the secondary antibodies (diluted in 1 × PBS/2% BSA) for 1 h at room temperature. The cells were washed again with high-salt and regular 1 × PBS before the nuclei were stained for 1 min at room temperature with 10 μg/ml Hoechst 33258. Finally the cells were washed three times with 1 × PBS and mounted in 1 × PBS and 40% glycerol. Images were acquired using a Zeiss LSM 710 confocal laser-scanning microscope and processed with Adobe Photoshop. The following primary antibodies were used: a mouse monoclonal anti-(acetylated tubulin) antibody (Sigma; diluted 1:200); a rabbit polyclonal antiserum raised against the peptide N-CSIPQDLRDEIKE-C (amino acids 331–343 of human polycystin-2) (diluted 1:100), the mouse monoclonal anti-(HA epitope) antibody 12CA5 (hybridoma supernatant; diluted 1:30), a polyclonal anti-(HA epitope) antibody (Sigma; diluted 1:100), the mouse monoclonal anti-CD8 antibody OKT8 (hybridoma supernatant; diluted 1:30), a mouse monoclonal anti-calnexin antibody (BD Biosciences; diluted 1:30), and the rabbit polyclonal

10	20	30	40	50	60	70		
MVNSSRVQPQ	QPGDAK	KRPPA	PRAPDPGR	LM	AGCAAVGASL	AAPGGLCEQR	GLEIEMQRIR	QAAARDPPAG
80	90	100	110	120	130	140		
AAASPSPLS	SCSRQAWSRD	NGPFEAEEEE	EEVEGEEGGM	VVEMDVEWRP	GSRRSAASSA	VSSVGARSRG		
150	160	170	180	190	200	210		
LGGYHGAGHP	SGRRRRREDQ	GPPCPSVGG	GDPLHRHLPL	EGQPPRVAWA	ERLVRGLRGL	WGTRLMEESS		
220	230	240	250	260	270	280		
TNREKYLKSV	LRELVTYLLF	LIVLCILTYG	MMSSNVYYT	RMMSQLFLDT	PVSKTEKTNF	KTLSSMEDFW		
290	300	310	320	330	340	350		
KFTEGSLLDG	LYWKMQPSNQ	TEADNRSFIF	YENLLLGVPR	IRQLRVRNGS	CSIPQDLRDE	IKECYDVYSV		
360	370	380	390	400	410	420		
SSEDRAPFGP	RNGTAWIYTS	EKDLNGSSHW	GIIATYSGAG	YYLDLSRTRE	ETAAQVASLK	KNVWLDGRTR		
430	440	450	460	470	480	490		
ATFIDFSVYN	ANINLFCVVR	LLVEFPATGG	VIPSWQFQPL	KLIRYVTTFD	FFLAACEIIF	CFFIFYVVE		
500	510	520	530	540	550	560		
EILEIRIHKL	HYFRSFWNCL	DVVIVVLSV	AIGINIYRTS	NVEVLLQFLE	DQNTFPNFEH	LAYWQIQFNN		
570	580	590	600	610	620	630		
IAAVTVFFVW	IKLKFKINFN	RTMSQLSTTM	SRCAKDLFGF	AIMFFIIFLA	YAQLAYLVFG	TQVDDFSTFQ		
640	650	660	670	680	690	700		
ECIFTQFRII	LGDINFAEIE	EANRVLGPIY	FTTFVFFMFF	ILLNMFLAII	NDTYSEVKSD	LAQQKAEMEL		
710	720	730	740	750	760	770		
SDLIRKGYHK	ALVKLKLKKN	TVDDISESLR	QGGGKLNFE	LRQDLKKGKH	TDAEIEAIFT	KYDQDGDQEL		
780	790	800	810	820	830	840		
TEHEHQMRD	DLEKEREDLD	LDHSSLPRPM	SSRSFPRSLD	DSEEDDEDSD	GHSSRRRSGI	SSGVSYEEFQ		
850	860	870	880	890	900	910		
VLVRRVDRME	HSIGSIVSKI	DAVIVKLEIM	ERAKLKRREV	LGRLLDGVAE	DERLGRDSEI	HREQMERLVR		
920	930	940	950	960				
EELERWESDD	AASQISHGLG	TPVGLNGQPR	PRSSRPSSSQ	STEGMEGAGG	NGSSNVHV			

Figure 1 Putative transmembrane domains in the human polycystin-2 protein (GenBank® accession number NP_000288.1)

The transmembrane domains suggested by a previous publication [11] are indicated by bars above the sequence and those predicted by Swiss-Prot (accession number Q13563) are indicated by bars below the sequence. The ciliary-targeting motif (amino acids 1–15) and the retention signal for the ER (amino acids 787–820) are boxed.

anti-polycystin-2 antiserum YCB9 directed against amino acids 103–203 of human polycystin-2 (diluted 1:4000). The following secondary antibodies were used: FITC-conjugated anti-mouse IgG (ICN Biomedicals; diluted 1:200), Cy3 (indocarbocyanine)-conjugated anti-(mouse IgG) (Dianova; diluted 1:300) and Cy3-conjugated anti-(rabbit IgG) (Dianova; diluted 1:400).

RESULTS

Determining the topology of polycystin-2 by immunofluorescence

According to two hydrophobicity plot-based structural predictions ([11] and Swiss-Prot database accession number Q13563), the polycystin-2 protein contains six transmembrane domains, with the N-terminus and the C-terminus extending into the cytoplasm (Figure 1). Although both models show minor differences with respect to the position of transmembrane domains 1, 2, 3, 5 and 6, they diverge markedly on the first and last amino acid positions of the fourth transmembrane helix (Figure 1). To elucidate the topology of polycystin-2 experimentally and to better characterize the position of its transmembrane domains and loops, we examined full-length and C-terminal truncation

mutants of polycystin-2 tagged at the C-terminus with an HA epitope (Figure 2). Upon transient transfection into COS-7 cells, the cellular location (plasma membrane or ER) of the HA-epitope-tagged proteins was determined with an anti-(HA epitope) antibody in the presence of selective permeabilization agents such as digitonin and Triton X-100. Digitonin allows the specific permeabilization of the plasma membrane, whereas Triton X-100 permeabilizes all cellular membranes [20]. The specificity of the selective permeabilization treatments was verified by immunofluorescence using an antibody against a cytoplasmic protein (acetylated tubulin) and an antibody against a protein located in the lumen of the ER (calnexin).

To first verify whether the N- and C-termini of polycystin-2 extend into the cytoplasm as postulated by structural predictions, COS-7 cells were transiently transfected with a construct coding for the full-length polycystin-2 protein tagged with a C-terminal HA epitope (Figure 2). At 48 h after transfection, cells were fixed and incubated either with a monoclonal anti-(HA epitope) antibody or with a polyclonal antibody directed against amino acids 103–203 of polycystin-2 (Figure 3). In either case, the full-length protein was already detectable in digitonin-permeabilized cells (Figure 3) which confirms the predicted cytoplasmic

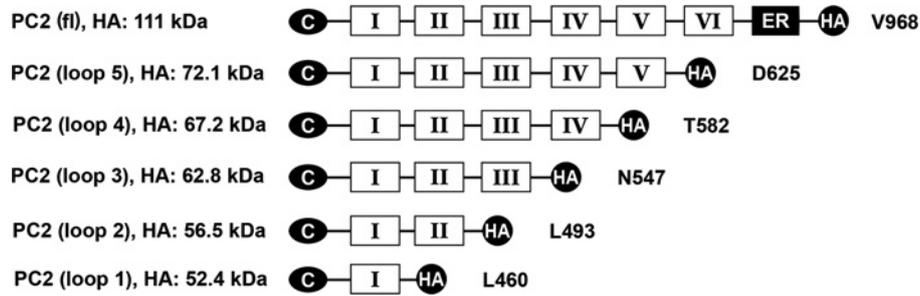


Figure 2 Constructs used for the topology study

PC2 (fl)–HA encodes the full-length polycystin-2 protein, and the constructs named PC2 (loop 1–5)–HA represent truncation mutants of human polycystin-2. The last amino acid of each construct is shown on the right-hand side, and the predicted molecular mass is shown on the left-hand side. The oval represents the N-terminal ciliary-targeting motif (C), open rectangles represent the putative transmembrane domains (numbered I–VI), the full rectangle represents the retention signal for the ER and the circle represents the HA epitope (HA) which was attached to the very C-terminus of each construct. Not drawn to scale.

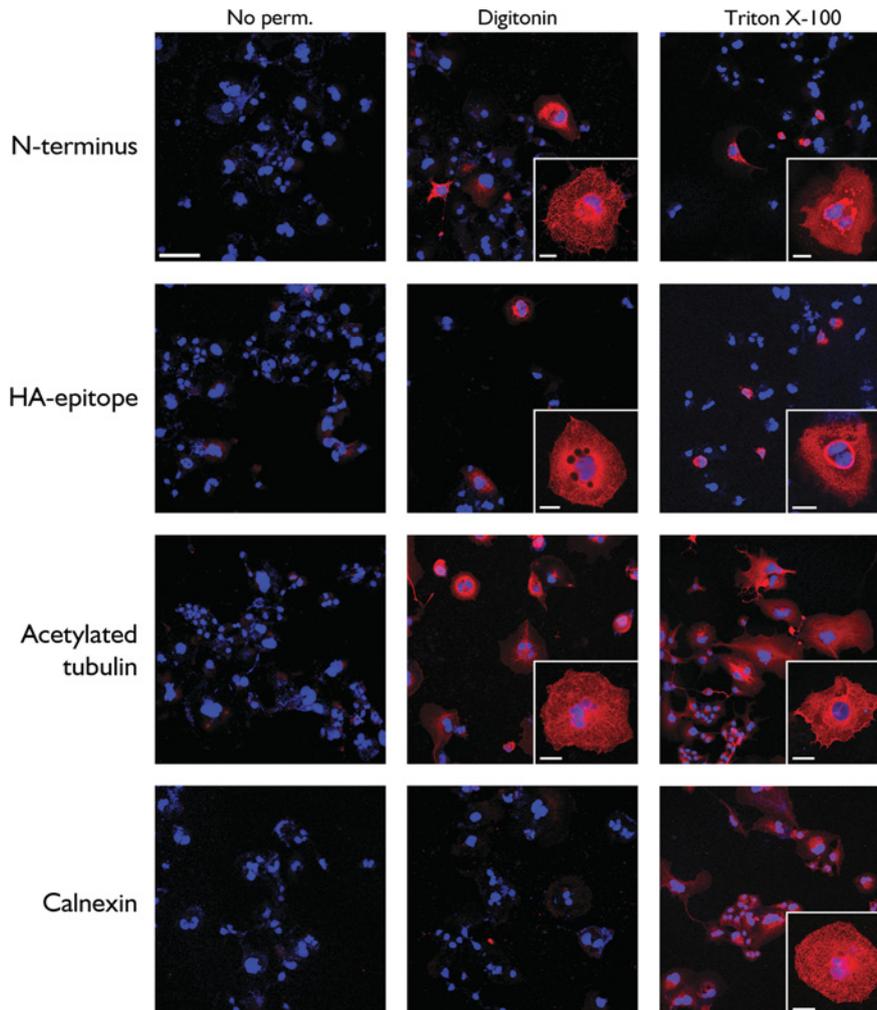


Figure 3 The N- and C-termini of polycystin-2 extend into the cytoplasm

COS-7 cells were transiently transfected with an expression plasmid encoding full-length polycystin-2 tagged with an HA epitope at the C-terminus. Non-permeabilized (No perm.), digitonin-treated and Triton X-100-treated cells were stained with an antibody directed against amino acids 103–203 (N-terminus) of polycystin-2 and with an antibody against the HA epitope. Both the N- and the C-termini were already visible after digitonin treatment, thus demonstrating their cytoplasmic orientation. Control stainings against tubulin and calnexin confirmed that the plasma membrane was intact in non-permeabilized cells and that the membrane of the ER was still intact in the presence of digitonin. Nuclei were counterstained with the DNA-binding dye Hoechst 33258. Scale bars are 100 μm in overviews and 25 μm in insets.

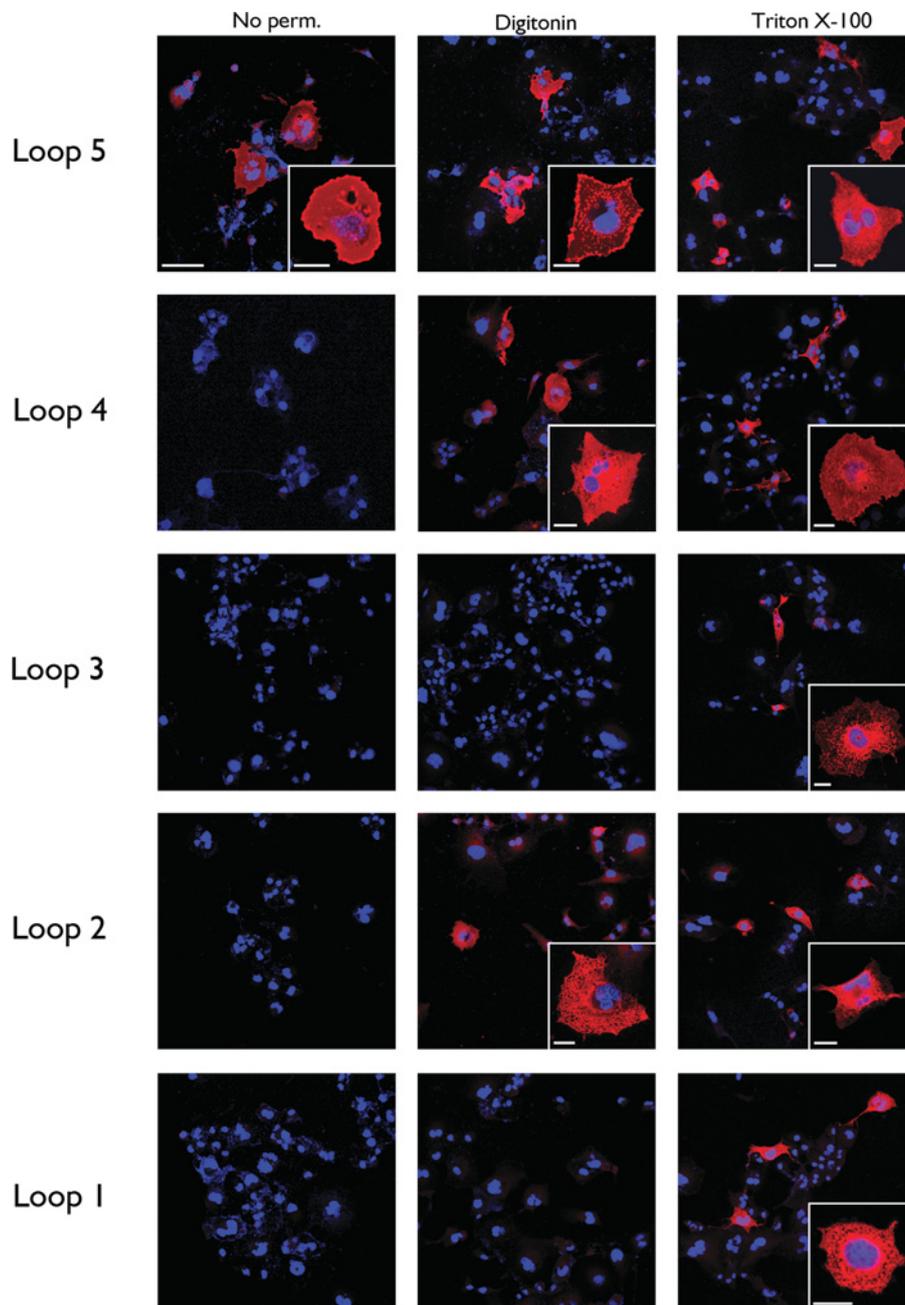


Figure 4 Topology determination of human polycystin-2 by selective permeabilization

Non-permeabilized (No perm.), digitonin-treated and Triton X-100-treated COS-7 cells transiently producing polycystin-2 (loop 1–5)–HA were stained with an anti-(HA epitope) antibody. Constructs truncated in loops 4 and 5 were transported to the plasma membrane, whereas those truncated in loops 1, 2 and 3 were retained intracellularly. Loop 5 extends into the extracellular space, loops 4 and 2 extend into the cytoplasm, and loops 3 and 1 extend into the lumen of the ER. Nuclei were counterstained with the DNA-binding dye Hoechst 33258. Scale bars are 100 μm in overviews and 25 μm in insets.

location of the N- and C-termini of full-length polycystin-2. Our experimental approach was validated by control staining for acetylated tubulin and calnexin. A tubulin signal was detected in digitonin- and Triton X-100-permeabilized cells, but not in non-permeabilized cells (Figure 3), whereas calnexin was first visible after permeabilizing the cells with Triton X-100 (Figure 3).

We next focused on elucidating the orientation of the five putative loops of polycystin-2 using the same indirect immunofluorescence approach. For that purpose, progressive C-terminal deletions of polycystin-2 were generated which ended

within the predicted loops and which were tagged with an HA epitope at the C-terminus (Figure 2). All five truncated proteins were detected by Western blot analysis (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/433/bj4330285add.htm>). Since these proteins lack the C-terminal retention signal for the ER they were expected to reach the plasma membrane, and (depending on the orientation of the respective loop) should be detectable in either non-permeabilized or digitonin-treated cells. Polycystin-2 mutant proteins truncated in loops 4 and 5 indeed reached the plasma membrane (Figure 4),

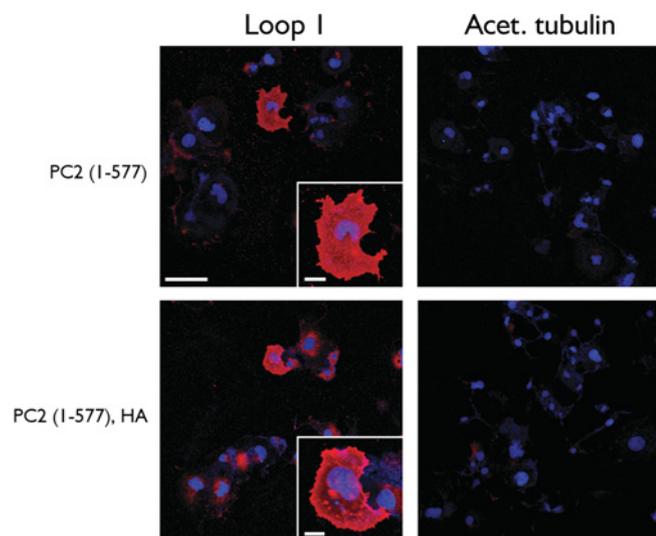


Figure 5 Immunofluorescence staining of non-tagged and HA-epitope-tagged human polycystin-2 truncated after amino acid 577

Non-permeabilized COS-7 cells transiently producing a 577-amino-acid long polycystin-2 protein with and without a C-terminal HA epitope were immunostained with an antibody directed against amino acids 331–343 of polycystin-2 (loop 1). Regardless of the presence of the HA epitope, both proteins were detectable at the plasma membrane of non-permeabilized cells, which shows that loop 1 extends into the extracellular space and that the presence of the HA epitope does not affect the topology of polycystin-2. An absent signal after staining for acetylated tubulin (Acet. tubulin) demonstrated the integrity of the plasma membrane. Nuclei were counterstained with the DNA-binding dye Hoechst 33258. Scale bars are 100 μm in overviews and 25 μm in insets.

which was confirmed by double-immunofluorescence staining for the plasma membrane marker CD8 (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/433/bj4330285add.htm>). In contrast, polycystin-2 proteins truncated in loops 1, 2 and 3 did not reach the plasma membrane and were retained intracellularly, which was evident by a cytoplasmic staining pattern upon either digitonin or Triton X-100 treatment (Figure 4) similar to that observed for the full-length protein (Figure 3). This intracellular location was confirmed by the absence of co-localization with the plasma membrane marker CD8 (Supplementary Figure S2 and results not shown). These observations argue for the presence of a plasma membrane export signal between amino acids 547 and 582 (H. Hoffmeister, K. Babinger, S. Gürster, A. Cedzich, C. Meese, K. Schadendorf, L. Osten, U. de Vries, A. Rasclé and R. Witzgall, unpublished work).

In spite of the different locations of the truncated polycystin-2 proteins, selective permeabilization allowed us to determine the orientation of the respective loops by immunofluorescence. In COS-7 cells synthesizing the mutant truncated in loop 5, an immunofluorescence signal was already detected without permeabilization (Figure 4), which argued that this loop extends into the extracellular space. Both the loop 4 mutant (which reaches the plasma membrane) and the loop 2 mutant (which is retained intracellularly) were first detected after digitonin treatment (Figure 4). This result argued for a cytoplasmic orientation of both loops. Permeabilization of COS-7 cells with Triton X-100 finally allowed the detection of the polycystin-2 mutant proteins truncated in loop 1 and loop 3 respectively (Figure 4), which demonstrates that both loops extend into the lumen of the ER. The orientation of loop 1 was also verified using an antibody directed against amino acids 331–341 (loop 1) (Figure 5). A polycystin-2 mutant protein located in the plasma membrane was already detected with this antibody in non-permeabilized cells (Figure 5),

a) Protected fragment of PC2 (loop 1), HA: 28.9 kDa

	*	I		
TNREKYLKSV	<u>LRELV</u> TYLLF	LIVLCILTYG	MSSNVVYYYT	RMMSQLFLDT
PVSKTEKTNF	KTLSSMEDFW	KFTEGSLDDG	LYWKMQPSNO	TEADNRSFIF
YENLLGVPR	IRQLRVRNGS	CSIPQDLRDE	IKECYDVYSV	SSEDRAPFGP
RNGTAWIYTS	EKDLNGSSHW	GIIATYSGAG	YYLDLSRTRE	ETAAQVASK
KNVWLDGRTR	ATFIDFSVYN	ANINLFCVVR	LLVEFPATGG	VIPSWQFQPL
SYPYDVPDYA	ID			

b) Protected fragment of PC2 (loop 3), HA: 6.6 to 8.0 kDa

II	*	*	III	
<u>FIF</u> YVVVEEI	LEIRIHKLHY	FRSEWNCLDV	VIVVLSVVAI	GINIYRTSNV
EVLQLEQ	NTFPNLSYPY	DVPDYAID		

c) Protected fragment of PC2 (loop 5), HA: 4.7 to 7.8 kDa

IV	*		*	V
<u>VTVFFVWIKL</u>	FKFINFNRTM	SQLSTTMSRC	AKDLFGFAIM	<u>FFIIFLAYAQ</u>
<u>LAYLVFGTQV</u>	DDLSPYDVP	DY AID		

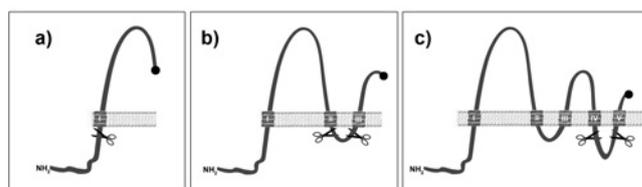


Figure 6 Predicted proteinase K cleavage sites and resulting protected fragments of HA-epitope-tagged polycystin-2 (loop 1), (loop 3) and (loop 5)

The amino acid sequences N- and C-terminal to transmembrane domains 1, 3 and 5 are shown. Bars above the sequence indicate the transmembrane domains predicted in the original description of polycystin-2 [11], whereas the underlined amino acids indicate the transmembrane helices given in the Swiss-Prot database (accession number Q13563). Asterisks in the putative loops mark the amino acids predicted as accessible to proteinase K within the cytoplasm (schematically shown in the diagrams). Molecular masses of the corresponding HA-epitope-tagged proteolytic fragments are listed next to the respective construct.

thus arguing that loop 1 extends into the extracellular space. A similar pattern was observed with this antibody upon synthesis of the same polycystin-2 fragment lacking the C-terminal HA epitope (Figure 5), which makes it unlikely that the HA epitope influences the overall topology of the C-terminal deletion mutants. Our immunofluorescence data are therefore compatible with the model: (i) that polycystin-2 contains six transmembrane domains, (ii) that its N- and C-termini extend into the cytoplasm, (iii) that its loops 1, 3 and 5 extend into the lumen of the ER and the extracellular space respectively, and (iv) that its loops 2 and 4 extend into the cytoplasm.

Determining the topology of polycystin-2 using protease protection assays

To verify the immunofluorescence data, protease protection assays were performed on microsomal preparations of transiently transfected COS-7 cells producing the truncated polycystin-2 mutant proteins. Microsomes are membrane vesicles with cytoplasmic-side-out orientation [21]. Protease treatment of non-permeabilized microsomes will therefore exclusively digest protein domains which extend into the cytosol, whereas protein domains exposed to the microsomal lumen (lumen of the ER and extracellular space) are protected. Assuming that proteinase K cleaves polycystin-2 after amino acid 221 (within the N-terminus, immediately preceding the first residue of the first transmembrane domain), amino acids 492 and 503 (within putative loop 2)

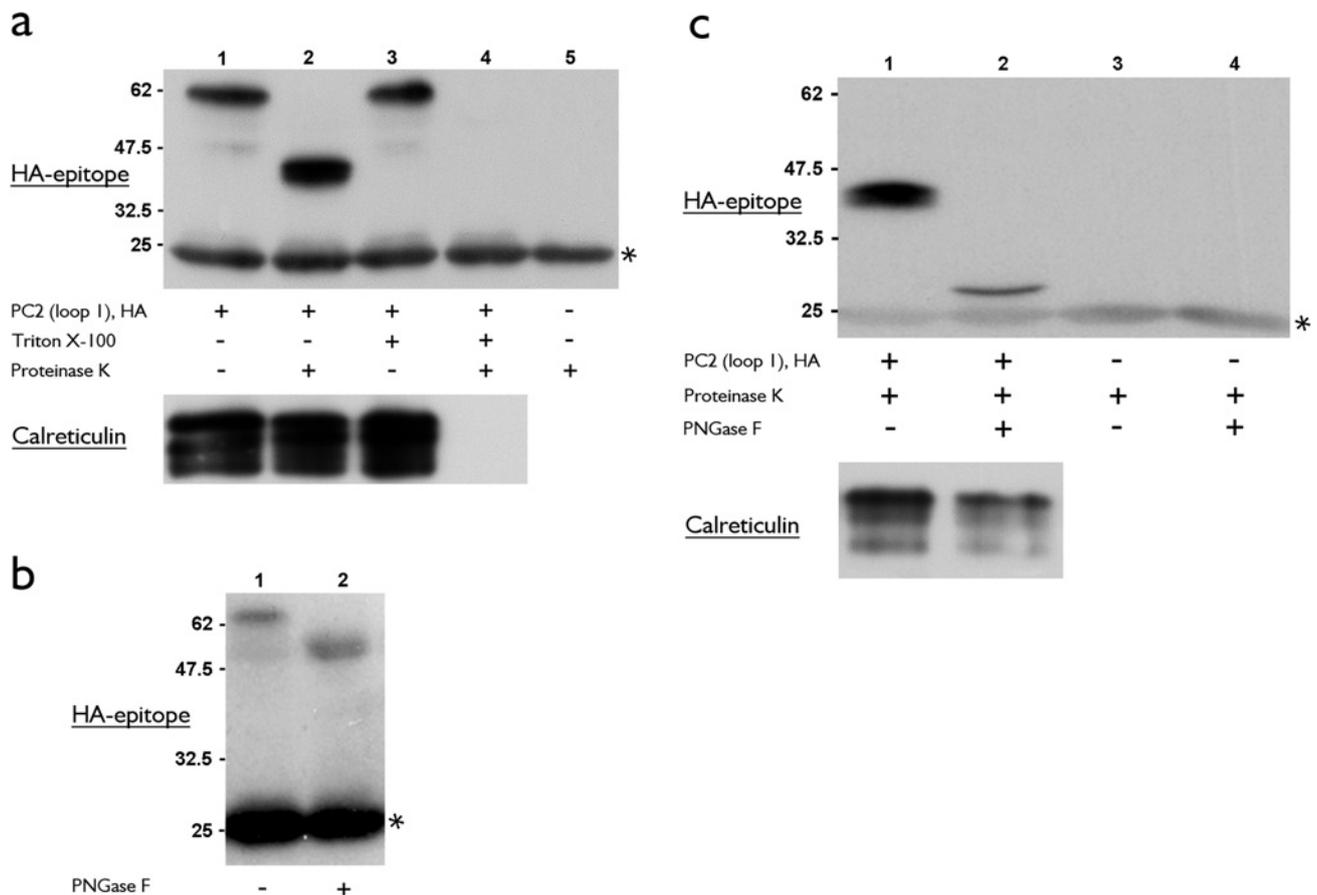


Figure 7 Orientation of loop 1 of human polycystin-2

COS-7 cells were transiently transfected with an expression plasmid encoding polycystin-2 (loop 1)-HA. Microsomes were prepared and digested 48 h later with 1 mg/ml proteinase K in the absence and presence of Triton X-100. **(a)** After immunoprecipitation with the murine anti-(HA epitope) antibody 12CA5, the precipitated proteins were subjected to Western blot analysis with the rat anti-(HA epitope) antibody 3F10. A protected fragment was only detected in non-permeabilized microsomes, although at a molecular mass nearly twice as high as predicted (lane 2). A Western blot of the supernatant fraction with an anti-calreticulin antibody (lower panel) demonstrates the integrity of the microsomal preparations. **(b)** Permeabilized microsomes were incubated in the absence or presence of PNGase F, immunoprecipitated with the 12CA5 antibody and subjected to Western blot analysis with the 3F10 antibody. The shift to a lower molecular mass after PNGase F treatment showed that this deletion mutant of polycystin-2 was N-glycosylated. **(c)** Non-permeabilized microsomes were first incubated with proteinase K and subsequently with PNGase F as indicated. After immunoprecipitation with the 12CA5 antibody and Western blot analysis with the 3F10 antibody, a shift to a lower molecular mass was seen in the presence of PNGase F. Asterisks mark the light chain of the antibody. The molecular mass in kDa is indicated on the left-hand side.

and amino acids 571 and 598 (within putative loop 4), one would expect protected fragments with molecular masses of ~28.9 kDa, 6.6–8.0 kDa and 4.7–7.8 kDa respectively, upon proteinase K digestion in the absence of detergent for the loop 1, loop 3 and loop 5 constructs (Figure 6). Protease protection assays were performed with microsomes in the absence and presence of 1% Triton X-100 and in the absence and presence of proteinase K. Protected fragments were immunoprecipitated with the murine monoclonal anti-(HA epitope) antibody 12CA5 and detected by Western blot analysis with the rat monoclonal anti-(HA epitope) antibody 3F10. The integrity of the microsomal preparations was controlled by Western blot analysis of the post-immunoprecipitation supernatants using an antibody directed against the luminal ER protein calreticulin. As predicted, calreticulin was only degraded by proteinase K when Triton X-100 was added to the microsomal fractions (Figure 7a, lane 4). Proteinase K cleavage of non-permeabilized vesicles from cells producing polycystin-2 (loop 1)-HA yielded a protected fragment (Figure 7a, lane 2) and therefore corroborated the immunofluorescence data that loop 1 extended into the lumen of the ER. However, the observed molecular mass of the protected

fragment (~40 kDa) exceeded the expected size markedly. Since full-length polycystin-2 is known to be N-glycosylated [10,22,23], possibly at five predicted glycosylation sites within loop 1, we investigated whether the higher molecular mass of the protected fragment was due to glycosylation. Microsomal preparations of cells synthesizing polycystin-2 (loop 1)-HA were incubated with the enzyme PNGase F which cleaves all N-glycosidic bonds. Upon PNGase F treatment, the polycystin-2 (loop 1)-HA protein migrated with a markedly higher mobility (Figure 7b, lane 2), thus confirming that loop 1 is glycosylated. PNGase F treatment of proteinase K-digested polycystin-2 (loop 1)-HA generated a protected fragment of ~28 kDa (Figure 7c, lane 2), which again is close to the predicted molecular mass of 28.9 kDa (Figure 6). These data thus confirm that loop 1 of polycystin-2 is N-glycosylated and that it extends into the lumen of the ER. By inference, the higher apparent molecular mass of polycystin-2 (loop 2)-HA and (loop 3)-HA proteins (Supplementary Figure S1, lanes 1–3) is probably due to glycosylation within loop 1.

Analogous protease protection assays were performed from microsomal preparations of COS-7 cells producing polycystin-2 (loop 3)-HA and polycystin-2 (loop 5)-HA, and revealed

protected fragments of the expected size (Figure 6) of ~ 8.0 kDa (Figure 8a, lanes 3 and 4) and ~ 5 kDa (Figure 8b, lane 2) respectively. This confirmed that loops 3 and 5 also extend into the microsomal lumen, in agreement with the immunofluorescence data. In the case of polycystin-2 (loop 3)-HA, a 4-fold higher concentration of proteinase K was necessary to achieve complete digestion. The resulting protected fragment was slightly smaller than expected (Figure 8a, lane 4), which could not be explained by the penetration of proteinase K into damaged microsomes because calreticulin levels remained unaffected (Figure 8a, lower panel, compare lanes 3 and 4). The fact that protected fragments of the expected size were detected demonstrates that the predicted cleavage sites (Figure 6) between amino acids 492 and 503 and 571 and 598 respectively, were accessible to proteinase K. This in turn suggests a cytoplasmic orientation of loops 2 and 4. Indeed no protected fragment were detected with microsomes of COS-7 cells synthesizing polycystin-2 (loop 2)-HA and (loop 4)-HA (results not shown). Taken together, these protease protection assays confirm our immunofluorescence data and strongly support a topology model for polycystin-2 in which loops 1, 3 and 5 extend into the lumen of the ER (or the extracellular space), whereas loops 2 and 4 extend into the cytosol (Figure 9). Furthermore, our immunofluorescence data confirm the cytoplasmic orientation of the N- and C-termini of polycystin-2 (Figure 9).

DISCUSSION

PKD2 is one of the two genes mutated in patients with ADPKD. Despite extensive efforts, it remains unclear how mutations lead to PKD. *PKD2* encodes the non-selective cation channel polycystin-2, a member of the TRP family of cation channels. Since structural information provides clues on the function of polycystin-2 and will help to better characterize disease-associated mutations, in the present study we elucidate the topology of this calcium channel.

Topology studies of integral membrane proteins are ideally carried out with the full-length protein, e.g. by inserting specific immunological epitopes or N-glycosylation sites into the protein. The location of the inserted domains is subsequently determined by the access of the epitope to the respective antibody in non-permeabilized and permeabilized cells, and by the access of the novel glycosylation sites to the glycosylation machinery which is exclusively located in the lumen of the ER and Golgi apparatus. Our initial attempts to elucidate the topology of polycystin-2 by using the latter approach failed because polycystin-2 became increasingly unstable the more of the endogenous N-glycosylation sites were removed, a typical phenomenon attributed to the loss of structural integrity of the protein [24]. In light of the very limited structural data for polycystin-2 we chose the strategy to generate progressively shorter polycystin-2 proteins with an HA epitope at their very C-terminus. One of the possible drawbacks of our approach could be an altered topology of the truncated proteins. This objection cannot be held against our topology study because the cytoplasmic orientation of the N-terminus was demonstrated both by immunofluorescence in the context of the full-length protein, and by the identification of a protected fragment for polycystin-2 (loop 1)-HA in the proteinase K protection assay. The cytoplasmic location of the N-terminus confirms published data obtained with the pathogenic polycystin-2 mutant protein R742X [10,25]. Finally the observation that loop 1 was detected in the lumen of the ER or extending into the extracellular space in polycystin-2 constructs of different lengths and independently of the presence of a C-terminal HA epitope indicates that neither the C-terminal deletions nor the HA epitope altered the overall topology of polycystin-2.

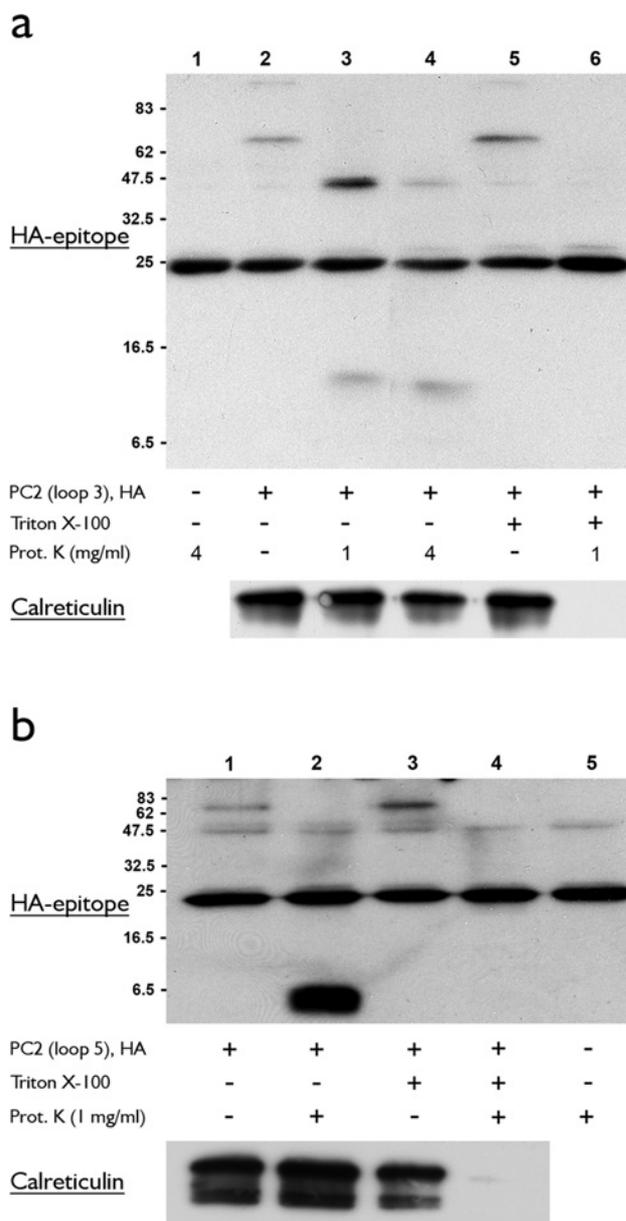


Figure 8 Orientation of loops 3 and 5 of human polycystin-2

COS-7 cells were transiently transfected with an expression plasmid encoding polycystin-2 (loop 3)-HA and polycystin-2 (loop 5)-HA. Microsomes were prepared and digested 48 h later with proteinase K (Prot. K) in the absence and presence of Triton X-100. **(a)** Protease protection assay for polycystin-2 (loop 3)-HA. The reactions were immunoprecipitated with the murine anti-(HA epitope) antibody 12CA5 and subjected to Western blot analysis with the rat anti-(HA epitope) antibody 3F10. An excess of proteinase K was necessary to obtain complete digestion which resulted in a protected fragment in the predicted size range (lanes 3 and 4). This argues that loop 3 is present in the lumen of the microsomes. **(b)** Protease protection assay for polycystin-2 (loop 5)-HA. Similar to the finding in **(a)**, the observation of a protected fragment of the expected molecular mass argues that loop domain 5 extends into the lumen of the microsomes (lane 2). The molecular mass in kDa is indicated on the left-hand side.

Our model that the N-terminus, the C-terminus, and loops 2 and 4 of polycystin-2 extend into the cytoplasm, whereas loops 1, 3 and 5 are located in the lumen of the ER, is in agreement with two hydrophobicity plot-based structural predictions of polycystin-2 ([11] and Swiss-Prot database accession number Q13563). As a matter of fact our experimental data solve a discrepancy between both predictions regarding the exact position of transmembrane

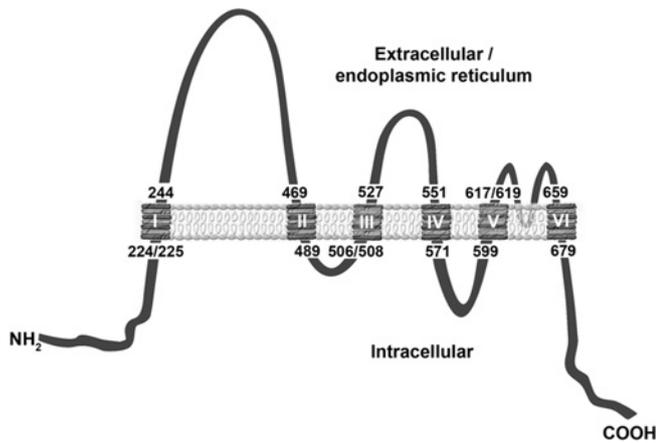


Figure 9 Proposed membrane topology of human polycystin-2

On the basis of the data obtained in the present study we propose a model in which all six predicted transmembrane segments traverse the membrane. The N- and C-termini, as well as loops 2 and 4, extend into the cytoplasm, whereas loops 1, 3 and 5 extend into the lumen of the ER or the extracellular space. The numbers indicate the beginning and the end of each transmembrane segment based on structural predictions ([11], and Swiss-Prot database accession number Q13563) and the results of the present study.

domain 4 (Figure 1). We show that the constructs truncated at amino acid positions 573–577 were accessible to the anti-(HA epitope) antibody upon digitonin treatment (results not shown), which indicates that this part of loop 4 extends into the cytoplasm and cannot be part of the transmembrane domain 4 as suggested [11]. Our experimental data rather support the Swiss-Prot database model which proposes that transmembrane domain 4 ends at amino acid residue 571.

Our data also confirm the predicted cytoplasmic location of the C-terminus of full-length polycystin-2. A cytoplasmic orientation of the C-terminus is in agreement with the fact that this portion of polycystin-2 interacts with many cytosolic proteins [26]. A similar argument holds true for the cytoplasmic orientation of the N-terminus of polycystin-2 which interacts with cytoplasmic proteins such as α -actinin and KIF3B (kinesin family member 3B) [27,28]. The following additional findings support the orientation of the N- and C-terminus in the cytoplasm: (i) phosphorylation sites have been identified in the N-terminus [29] and C-terminus [22,25] of polycystin-2, (ii) a ciliary trafficking motif has been identified in the N-terminus [18], and (iii) a retention signal for the ER has been identified in the C-terminus of polycystin-2 [10]. The proposed orientation of loop domain 5 also fits with previous data. Addition of an antibody directed against loop 5 of murine polycystin-2 abrogates the flow-induced calcium response of embryonic kidney cells, which is attributed to a functional plasma membrane complex of polycystin-1 and polycystin-2 [30].

In summary, the present study provides the first experimental evidence for the topology of polycystin-2. It constitutes another step towards a better characterization of the function of polycystin-2 and will improve our understanding of mutations in *PKD2*.

AUTHOR CONTRIBUTION

Helen Hoffmeister, Anna-Rachel Gallagher, Anne Rasclé and Ralph Witzgall designed the experiments; Helen Hoffmeister and Anna-Rachel Gallagher performed the experiments; Helen Hoffmeister, Anna-Rachel Gallagher, Anne Rasclé and Ralph Witzgall analysed the experiments; and Helen Hoffmeister, Anne Rasclé and Ralph Witzgall wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

The human polycystin-2 protein represents an integral membrane protein with six membrane-spanning domains and intracellular N- and C-termini

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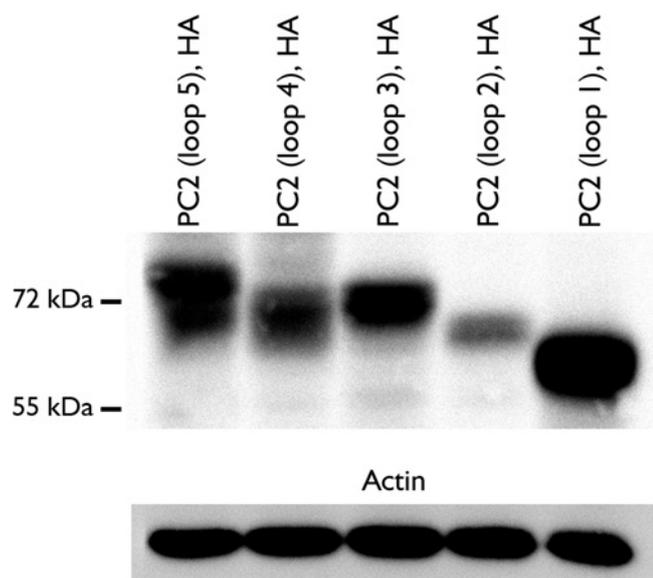


Figure S1 Western blot detection of polycystin-2 (loop 1–5)–HA constructs

COS-7 cells were transiently transfected with expression constructs encoding polycystin-2 (loop 1–5)–HA. Total cell lysates were analysed by Western blot with the rat monoclonal anti-(HA epitope) antibody 3F10 and with a polyclonal antibody directed against actin to control for equal loading. Chemoluminescence was quantified with the FUSION-FX7 camera system (Vilber Lourmat) and yielded relative expression levels of 100%, 115%, 86%, 43% and 242% for polycystin-2 (loop 5), (loop 4), (loop 3), (loop 2) and (loop 1) respectively. Of note, the polycystin-2 (loop 1–3)–HA constructs migrated at higher molecular masses than predicted. The molecular mass in kDa is indicated on the left-hand side. PC2, polycystin-2.

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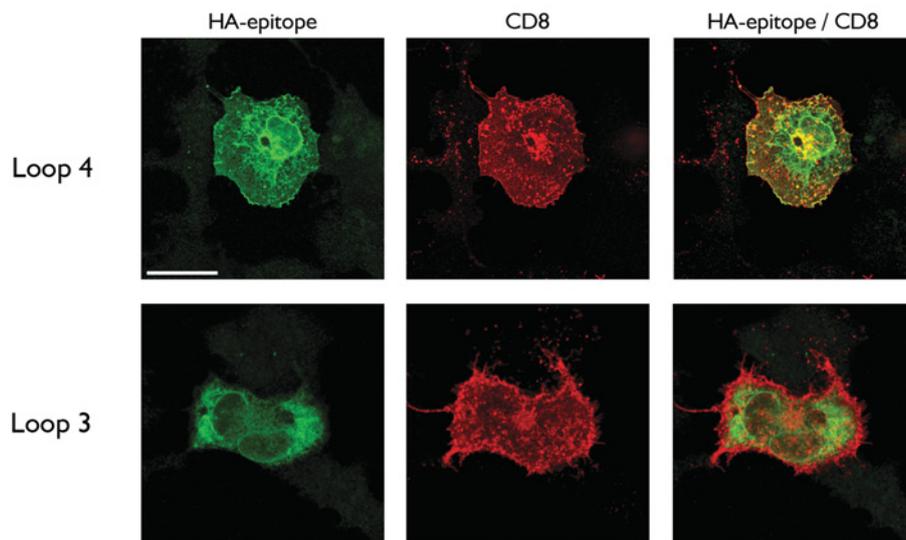


Figure S2 Double-immunofluorescence staining for polycystin-2 (loop 3)-HA, polycystin-2 (loop 4)-HA and the plasma membrane marker CD8

COS-7 cells were transiently co-transfected with expression plasmids encoding polycystin-2 (loop 4)-HA and CD8, and with expression plasmids encoding polycystin-2 (loop 3)-HA and CD8. Cells (48 h later) were fixed, permeabilized with Triton X-100 and stained with an anti-(HA epitope) antibody (green) and the anti-CD8 antibody OKT8 (red). Polycystin-2 (loop 4)-HA co-localized with CD8 at the plasma membrane, but polycystin-2 (loop 3)-HA did not. Scale bar, 50 μ m.

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