A modified mammalian tandem affinity purification procedure to prepare functional polycystin-2 channel

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Abstract The tandem affinity purification (TAP) procedure was initially developed as a tool for rapid purification of native protein complexes expressed at their natural levels in yeast cells. This purification procedure was also applied to study interactions between soluble proteins in mammalian cells. In order to apply this procedure to mammalian membrane proteins, we created a modified TAP tag expression vector and fused with the PKD2 gene, encoding a membrane cation channel protein, polycystin-2, mutated in 15% of autosomal dominant polycystic kidney disease. We generated epithelial Madin–Darby canine kidney cell line stably expressing TAP-tagged polycystin-2, improved the subsequent steps for membrane protein release and stability, and succeeded in purifying this protein. Using patch clamp electrophysiology, we detected specific polycystin-2 channel activities when the purified protein was reconstituted into a lipid bilayer system. Thus, this modified TAP procedure provides a powerful alternative to functionally characterize membrane proteins, such as ion channels, transporters and receptors, using cell-free system derived from mammalian cells.

Keywords: Tandem affinity purification; Mammalian cell; Planar lipid bilayer; Polycystin-2; Electrophysiology

1. Introduction

Classical biochemical purification methods rely heavily on the biological properties of a given protein, such as size, shape, charge, hydrophobicity and solubility, and are generally time-, labor- and cost-consuming. More importantly, as each protein is unique, purification protocols for individual proteins are often designed on an empirical basis [1]. The complexity of the mammalian genome and lack of a generic protein purification protocol hamper the progress in elucidating protein functions. Thus, there is critical demand for developing widely applicable and efficient purification procedures for isolating proteins from mammalian cells.

Among a variety of protein purification techniques, epitope-tagged affinity purification appears to be the most powerful and a gentle discriminatory separation technique for protein retrieval. In particular, well-characterized small affinity tags such as FLAG, hexahistidine, hemagglutinin, maltose and glutathione *S*-transferase have been extensively utilized in affinity purification of recombinant proteins. In the last decade, a tandem affinity purification (TAP) method was developed [2,3], which utilized two consecutive affinity purification steps [3]. Nowadays, there are many affinity purification tags available, several of which have low affinity for their cognate ligands such that low protein yield is obtained [1]. Rigaut et al. [2] compared several tag combinations aiming at high recovery rates without affecting protein functions and developed what has later become a standard TAP tag. This TAP tag consists of two immunoglobulin (IgG)-binding domains of *Staphylococcus aureus*, protein A and a calmodulin-binding peptide (CBP), separated by a cleavage site for the tobacco etch virus (TEV) protease. The TAP-tagged protein is expressed in host cells at close to physiological concentrations to form a complex with endogenous components. Extracts prepared from cells expressing TAP-tagged proteins are subjected to two successive purification steps. The first step involves binding of the protein A tag to an IgG column and eluting bound materials by incubation with TEV protease. This elution step significantly reduces the contaminating proteins bound non-specifically to the column. The eluates that contain the target proteins are subjected to the second-step purification, consisting of incubation with calmodulin-coated beads to remove TEV protease and contaminants. Highly purified target proteins are eluted by removing free calcium ions using calcium chelator EGTA. Therefore, these two steps of affinity purification can produce highly pure protein of interest together with associated partners under standard conditions [2–5].

This TAP approach was more recently introduced by some researchers into various types of host cells, such as bacteria, *Drosophila*, plant and mammalian cells and used to identify novel protein–protein interactions [6–12]. In this report, we attempted to purify a membrane-associated channel protein, polycystin-2, for in vitro electrophysiological studies. Due to low abundance of endogenous polycystin-2 protein in many cells, it is uneasy to study its biophysical properties and interaction with protein partners. Heterologous expression of

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Abbreviations: TAP, tandem affinity purification; MDCK, Madin–Darby canine kidney; IgG, immunoglobulin; CBP, calmodulin-binding peptide; TEV, tobacco etch virus; CMV, cytomegalovirus; TBS, Tris-buffered saline

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polycystin-2 in mammalian cells can significantly augment its abundance. However, overexpressed polycystin-2 targets mostly to membranes of intracellular organelles such as that of endoplasmic reticulum [13,14], which makes it difficult for electrophysiological studies. Currently, most of polycystin-2 channel studies use in vitro translated proteins or purified proteins from insect cells [15–17]. Here, we performed two consecutive affinity purifications of the polycystin-2 protein from cultured mammalian cells. To develop a generic protein purification protocol that is applicable to the identification of membrane proteins in mammalian cells, we incorporated a TAP tag to the N-terminus of human polycystin-2. We stably expressed polycystin-2 in mammalian cells at desirable levels by liposome-based gene transfer and purified the protein from total cell lysates. The combination of two different high-affinity tags greatly reduced the number of non-specific proteins in the mixture. We demonstrated the presence of polycystin-2 protein in a final elution buffer by Western blotting, which validates our modified TAP purification approach. We reconstituted purified polycystin-2 into planar lipid bilayer to determine whether it exhibits channel function. Indeed, specific cation channel activities were observed using a lipid bilayer patch clamp system. The modified TAP approach should also apply to other proteins and provide an efficient way to purify them to homogeneity from complex mixture found in mammalian cell lysates.

2. Materials and methods

2.1. Plasmid construction

The N-terminal TAP-tagged polycystin-2 was constructed in three steps based on the vector pEGFPC2 (Clontech, Palo Alto, CA). First, the TAP tag was amplified from the plasmid pBS1761 (courtesy gift from Dr. R.M. Zhao, University of Toronto, Canada) with a pair of primers (5′-CTGCAGGCTAGCACCGGTATGGCAGGCCTTGCG CAACA-3′ and 5′-AAGCTTGAGCTCGAGATCAG CTTATCGT-CATCATCAAG-3) and subcloned into the vector pGEM-Teasy (Promega, Madison, MI). Second, the resulting fragment was used to substitute the EGFP sequence located between *Age*I and *Hin*dIII in pEGFPC2 to generate pTAPC2 vector. Finally, the entire open reading frame of the human PKD2 gene was inserted into the *Xho*I–*Kpn*I site of pTAPC2 to generate pTAPC2-PKD2. The plasmid construct was confirmed by sequencing.

2.2. Transfection and selection of stable cell line

Mouse fibroblast NIH 3T3 and Madin–Darby canine kidney (MDCK) type II cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, penicillin-streptomycin, and 10% fetal bovine serum. Cells were plated onto a 6-well cluster and cultured to 90% confluence. Transfection was performed using Lipofectamine 2000 (Invitrogen, Toronto, ON) according to the manufacturer's recommendation. For generation of pooled stable cell line, 800 μg/μl hygromycin (Invitrogen) was added to DMEM to select viable clones one (recovery) day following transfection. The selection process was maintained for eight weeks with daily medium change.

2.3. Purification of TAP-tagged polycystin-2 protein

In order to efficiently release polycystin-2 membrane proteins from cultured cells, four different lysis buffers were tried and compared before making batch purification. They include two mild lysis buffers with non-ionic detergent NP40 (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Nonidet P40) and Triton X-100 (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100), one strong RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS) and the commercial CellLytic™-M reagent (Cat #: C2978, Sigma–Aldrich Canada, Oakville, ON).

All the following purification steps were conducted at 4 °C. MDCK monolayers grown at 150 mm dishes were lysed in 1.2 ml of ice-cold Sigma CellLytic™-M reagent supplemented with protease inhibitor cocktail (Sigma–Aldrich Canada) and then scraped off. The resulting homogenate was cleared by centrifugation at $15\,000\times g$ for 20 min. Batch purification of the IgG binding domain-tagged protein was performed using 300 μl (5 dishes) of protein A Sepharose 4 Fast Flow resin (Sigma–Aldrich Canada). Lysates were incubated with the resin overnight, washed three times with 10 ml of Tris-buffered saline (TBS) plus 0.1% Tween 20, and then three times with 10 ml of TEV buffer (50 mM Tris, pH 8.0, 1 mM EDTA). The beads were cut off from the tagged proteins using 300 units (500 μl) of TEV protease (Invitrogen) overnight. Supernatants from the TEV reaction containing the cleaved TAP tagged polycystin-2 were collected. The beads were washed twice with a 2 ml calmodulin binding buffer (50 mM Tris, pH 8.0, 75 mM NaCl, and 2 mM $CaCl_2$). The supernatants were combined and batch-purified by binding 200 μl of calmodulin affinity gel resin (Sigma–Aldrich Canada) for 4 h, washed five times with 5 ml of TBS, and eluted with a calmodulin elution buffer (50 mM Tris, pH 8.0, 25 mM EGTA). To validate drug-resistant colonies and fusion protein production, immunofluorescent staining and Western blotting were conducted as described previously [14] and silver staining was performed as per the manufacturer's protocol (Bio-Rad, Mississauga, ON).

2.4. Preparations of planar lipid bilayer and proteoliposome containing polycystin-2

The methods of preparation were based on those described previously [15,17]. Briefly, planar lipid bilayers were formed with a mixture of synthetic phospholipids (Avanti Polar Lipids, Birmingham, AL) in *n*-decane. The lipid mixture (25 mg/ml) was composed of phosphatidylethanolamine and phosphatidylserine in a 7:3 ratio. To prepare proteoliposome containing polycystin-2, the protein was dialyzed with phospholipids for lipid bilayer membrane assays. In brief, the same lipid mixture as above was sonicated with a solution containing 150 mM NaCl, 0.1 mM EDTA, 25 mM Na^+-cholate and 20 mM HEPES, pH 7.2. Polycystin-2 was added to a Na^+-cholate (0.55 mM) solution and the phospholipid mixture (25 mg/ml). The mixture was dialyzed for three days in 150 mM NaCl, 0.1 mM EDTA, and 20 mM HEPES, pH 7.2, with daily buffer change to eliminate residual detergent.

2.5. Electrophysiological measurements

Lipid membrane was formed across a 0.2 mm hole in a Delrin cup inserted in a special acrylic chamber (Harvard Apparatus, Montreal, QC) by painting with a glass stick containing either the lipid mixture or proteoliposome. Two solution-filled compartments were formed and separated by the membrane and are designated *cis* (or intracellular) and *trans* (or extracellular). The *cis* compartment contained 150 mM KCl, 15 μM Ca^{2+} (by 1 mM EGTA and 1.01 mM $CaCl_2$), pH 7.4 (adjusted by MOPS-KOH). The *trans* chamber contained either the identical solution as in *cis* (to form a symmetrical condition) or 15 mM KCl (asymmetrical). Polycystin-2 protein was added to the *cis* chamber in proximity of the membrane formed by the lipid mixture. Alternatively, polycystin-2-containing proteoliposome was directly painted to form the membrane with proteins expectedly inserted. The *cis* compartment was clamped to a range of voltages using a Gapfree protocol generated by Clampex 8 (Axon Instruments, Union City, CA) and the *trans* compartment was held at ground (0 mV). Currents and voltages were recorded at 200 μs per sample, Bessel filtered at 1K or 3K Hz with the amplifier PC-ONE (Dagan Corporation, Minneapolis, MN), the AD/DA converter Digidata 1320A and the software Clampex 8 (Axon Instruments).

2.6. Statistics and data analysis

Data were analyzed using Clampfit 9.2 (Axon Instruments). Single-channel current amplitudes were obtained from Gaussian fits to All-Point Histograms. For the analysis of mean open time (MOT) and mean close time (MCT), recordings with single openings filtered (Gaussian) at 200 Hz were used. Analyzed data were fitted and plotted using Sigmaplot 8 (Jandel Scientific, San Rafael, CA) and expressed in the form of means ± S.E. (*N*). The Goldman–Hodgkin–Katz (GHK) current equation [18] was used to evaluate the main unitary conductance.

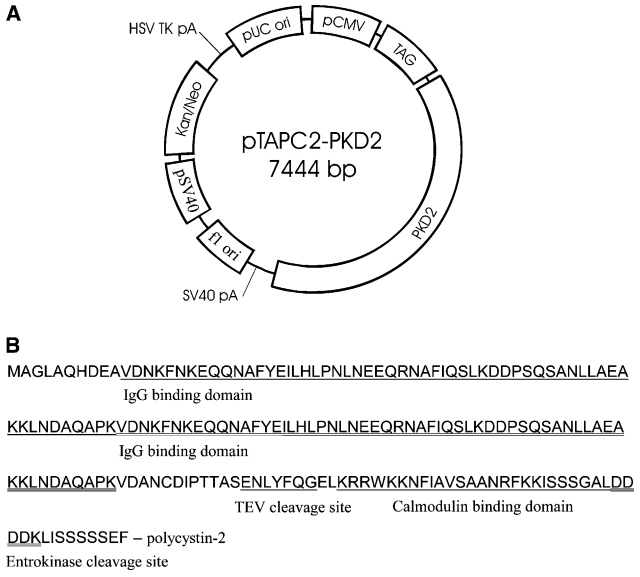

Fig. 1. Mammalian expression plasmid pTAPC2-PKD2 construct. (A) Schematic diagram of the plasmid pTAPC2-PKD2. (B) Structure and sequence of the N-terminal tandem tags. The TAP tag consists of two tandem protein A IgG binding domains (58 a.a. each), a TEV cleavage sequence (7 a.a., cleavage site between Q and G), and calmodulin binding peptide sequence (26 a.a). There is also an entrokinase recognition site (5 a.a.) [2]. They were placed downstream of CMV promoter, followed by the in-frame human PKD2 gene, and terminated with SV40 polyadenylation (pA) signal. Neo/Kan, neomycin or kanamycin resistance gene with SV40 promoter and herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal. Ori, origin of replication.

3. Results and discussion

3.1. Generation of stable mammalian cell line expressing TAP-tagged polycystin-2

The TAP method has been proven to be a powerful generic protein purification method for functional proteomics studies in various host cells. To develop a comparable methodology for protein purification from cultured mammalian cells, we constructed a TAP vector based on the mammalian expression vector pEGFPC2 (Clontech). As shown in Fig. 1, the TAP cassette contains two copies of the IgG binding domain of protein A of *S. aureus* and a CBP with a cleavage site for TEV protease. Both affinity tags have been selected for high efficient recovery of proteins present at low concentrations. Protein A binds tightly to an IgG matrix, which requires the use of TEV protease to elute materials under native conditions. The eluate was then incubated with calmodulin-coated beads for further purification. There is also an entrokinase recognition site which allows to remove the whole TAP tag if necessary. The full-length human PKD2 gene was inserted in-frame into downstream of the TAP sequence. The total gene cassette was placed under the control of the cytomegalovirus (CMV) promoter to drive its constitutive expression. There is a Neo gene for G418 drug resistance marker for selection in mammalian cells.

To assess whether these tandem affinity tags are in a correct fusion format and suitable for mammalian cell expression, we transiently transfected pTAPC2-PKD2 into two extensively studied cell lines, mouse fibroblast NIH 3T3 and canine epithelial MDCK cells. Indeed, a specific band of about 140 kDa was visualized by Western blotting probed with the anti-polycystin-2 [14] or anti-rabbit IgG antibody (Fig. 2A) from the total lysates of both cell lines. Immunofluorescent staining showed complete image overlaps with the same pair of antibodies (Fig. 2B), indicative of the feasibility of this purification strategy in mammalian cells. Subsequently, we chose MDCK cells for generation of a stable cell line for two reasons. First, MDCK cells are derived from kidney with high growing speeds, which allows us to collect large quantities of cell lysates. Second, MDCK cells are able to form a tight epithelium to tolerate high selection pressure over a long period of time and to efficiently express exogenous proteins. To relieve ourselves from the work such as drug-resistant colony pickup and individual characterization and to reduce the cost, we performed eight-week selection in a 6-well cluster with daily medium change. The surviving colonies in the cluster exhibited various expression levels of the TAP-tagged polycystin-2 protein (Fig. 2C). This pool of cells stably expressing TAP-polycystin-2 was used for subsequent protein retrieval and purification. The same strategy was also successfully applied to purification of polycystin-L, a homologous (50% identical) membrane protein of polycystin-2 (data not shown), suggesting its applicability to other proteins.

3.2. Purification of polycystin-2 protein

Polycystin-2 is a membrane protein and associates with the actin cytoskeleton [14,19,20]. We attempted to search for the most suitable lysis buffer which can release membrane protein efficiently without disrupting its structural integrity. Four different lysis buffers were compared. Two commonly used mild lysis buffers with non-ionic detergent NP40 or Triton X-100, one strong RIPA lysis buffer with ionic and non-ionic detergent, and one commercial lysis buffer CellLytic from Sigma were compared. Obviously, the RIPA or CellLytic buffers gave more complete protein release from MDCK cells than NP40 or Triton X-100 buffers, as judged by signal intensity of Western blotting in the pellet after protein extraction (Fig. 3). Between RIPA and CellLytic, it is clear that the latter produced higher amounts of soluble protein present in the supernatant, perhaps due to its stabilization effect on proteins. Therefore, the CellLytic buffer was used in our subsequent protein release.

Protein extracts were prepared from MDCK cells and incubated with IgG-sepharose 4 Fast Flow beads. The IgG beads were recovered as described above and a portion of the eluates was analyzed on a SDS–PAGE gel. As shown in Fig. 4, IgG beads significantly enriched the TAP-tagged polycystin-2 protein, when compared with the unfractionated cell lystates (lane 1 vs. lane 2). Eluates obtained after TEV cleavage were further incubated with calmodulin beads. After washing the beads five times with the TBS buffer, proteins bound to the CBP beads were eluted in the presence of EGTA (25 mM). The second-step purification with the CBP further increased purity of the tagged polycystin-2 protein. A single 120 kDa protein band was visualized by Western blotting probed with the anti-polycystin-2 antibody in the eluates from TEV removal and CBP beads (Fig. 4). This smaller band is due to the removal of two IgG binding domains from the TAP-tagged polycystin-2. Thus, polycystin-2 protein was successfully purified from cultured

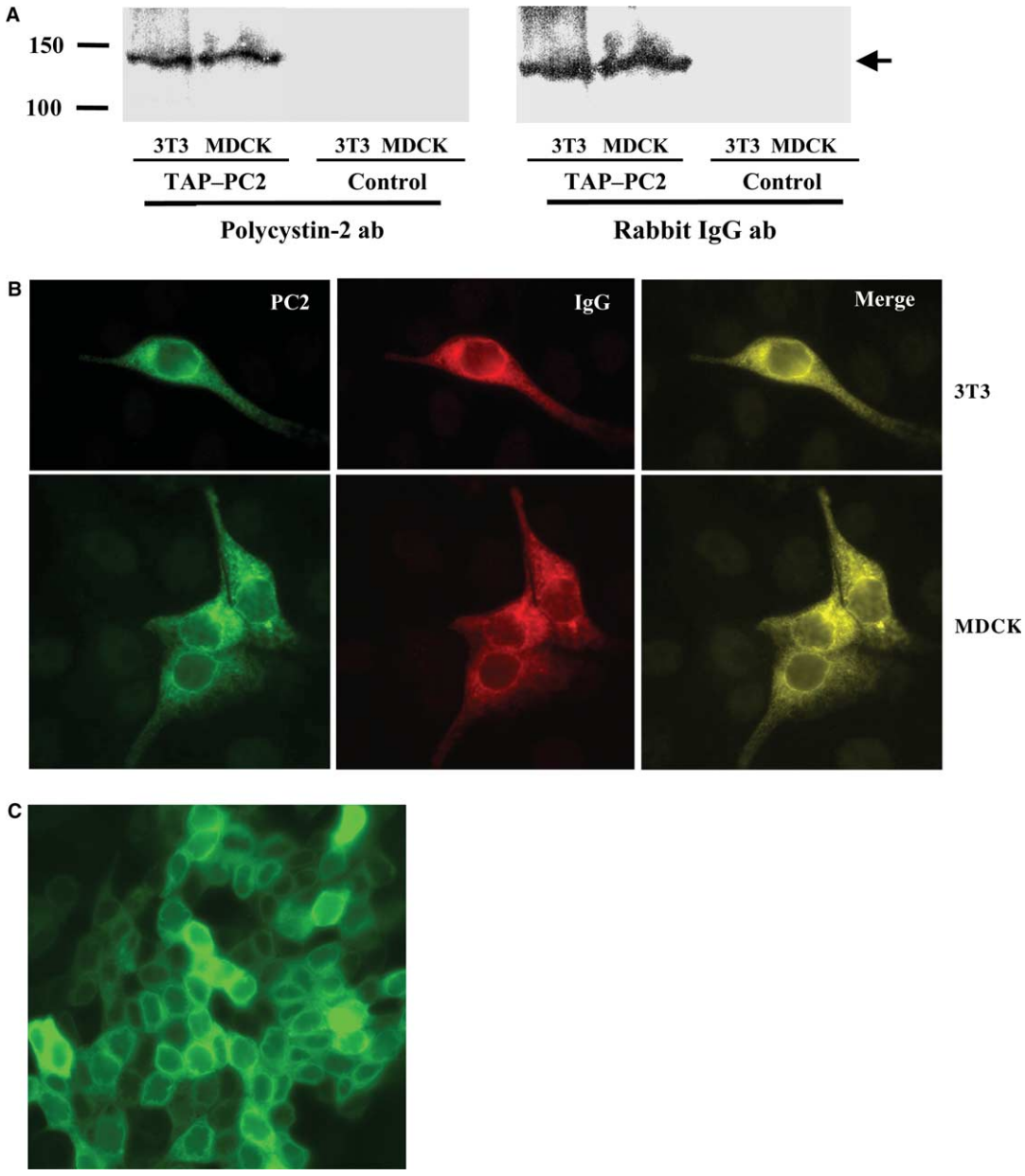

Fig. 2. TAP-tagged polycystin-2 overexpressed in NIH 3T3 and MDCK cells. (A) Western blot analysis of TAP-tagged polycystin-2 expression in transiently transfected NIH 3T3 and MDCK cells and their controls. Total cell lysates were subjected to 8% SDS–PAGE, transferred to nitrocellulose membrane, and blotted with the anti-polycystin-2 antibody. The same membrane was then stripped off and re-probed with the anti-rabbit IgG antibody. Both control cells without transfection have no band with the same antibodies. Numbers at left are molecular masses (kDa). (B) Transiently transfected NIH 3T3 and MDCK cells were co-stained with the anti-polycystin-2 and anti-rabbit IgG antibodies. One 3T3 and three MDCK cells overexpressing TAP polycystin-2 showed complete image overlay (Merge). The surrounding non-overexpressing 3T3 cells serve as control and display no signal for both antibodies, while the rest of MDCK cells display faint signals for the anti-polycystin-2 antibody, likely representing the presence of endogenous polycystin-2. (C) MDCK stable cell line which survived in 800 μg/ml G418 for eight weeks was stained with the anti-polycystin-2 antibody.

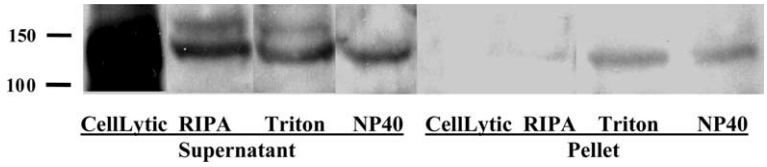

Fig. 3. Release of polycystin-2 protein from MDCK cells using different detergents. Four different lysis buffers, CellLytic, RIPA, NP40 and Triton, containing one or more detergents were used for protein extraction. Precleared supernatant and its respective pellet (150 μg each) from total cell lysates were run on 8% SDS–PAGE and blotted with the anti-polycystin-2 antibody. Molecular mass (in kDa) is indicated.

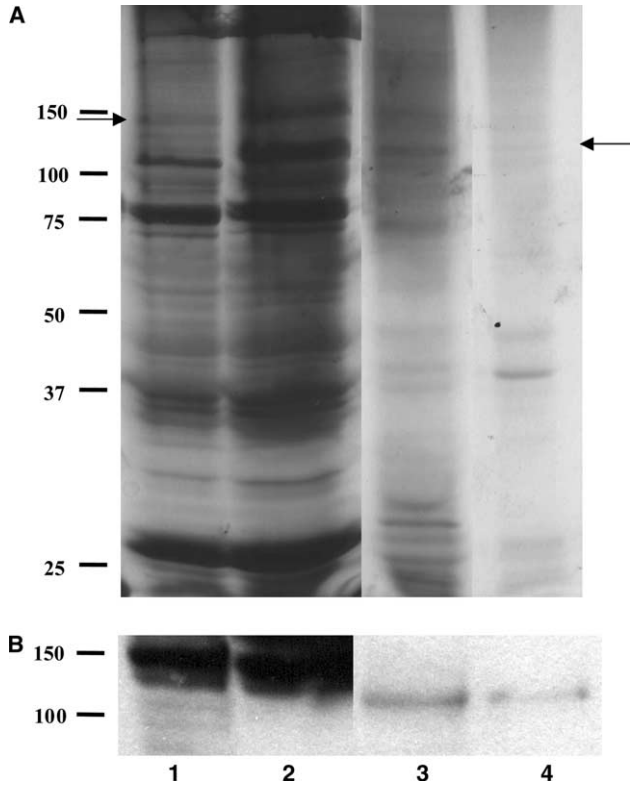

Fig. 4. Tandem affinity purification of polycystin-2 channel protein from MDCK cells. A portion of proteins in each stage of purification was separated using 8% SDS–PAGE and visualized by silver staining (A) and Western blotting probed with the anti-polycystin-2 antibody (B). Lane 1, total cell lysate prior to fractionation; lane 2, enriched protein complexes after incubation with protein A beads; lane 3, protein eluates after incubation with TEV protease; lane 4, proteins eluted from the calmodulin beads in the presence of 25 mM EGTA. Samples were mixed with 4× sample buffer, denatured at 80 °C for 5 min, then 20 μl (lanes 1 and 2) or 40 μl (lanes 3 and 4) was loaded. Arrows indicate position of the fused polycystin-2 protein. Numbers at left are molecular masses (kDa).

mammalian cells using this two-step tandem purification procedure.

3.3. Characterization of polycystin-2 channel reconstituted in lipid bilayer

To determine whether purified polycystin-2 protein preserves its cation channel activity, we reconstituted it into planar lipid bilayers and performed electrophysiological measurements. Experiments were conducted under symmetrical (150 mM KCl in both the *cis* and *trans* chambers) and asymmetrical (150 mM KCl in the *cis* chamber and 15 mM KCl in the *trans* chamber) solution conditions. Spontaneous channel activity was observed at both positive and negative membrane potentials under asymmetrical and symmetrical conditions (Fig. 5), demonstrating that purified polycystin-2 protein by our modified TAP protocol possesses the essential characteristics normally present under physiological conditions, e.g., those of endogenous or overexpressed polycystin-2 in mammalian cells. The main unitary conductance averaged 122 ± 19 pS ($N = 6$) under the symmetrical condition, which is similar to the previously reported value of 134 pS using in vitro translated polycystin-2 [16]. Under the asymmetrical condition, our data were fitted with the GHK equation, which

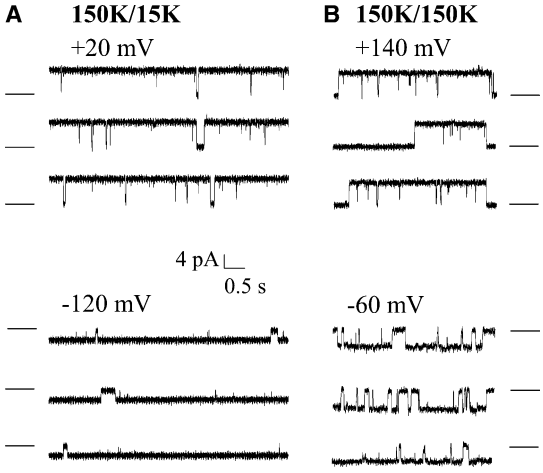

Fig. 5. Polycytin-2 channel activities in reconstituted planar lipid bilayer system. Functional assessment of the TAP-purified polycystin-2 was conducted in a lipid bilayer system. Single channel activity was obtained under asymmetrical (A, 150 K/15 K) and symmetrical (B, 150 K/150 K) conditions. (A) Shown are representative traces of single-channel currents recorded at +20 and −120 mV. The *cis* compartment contained 150 mM KCl and 15 μM $CaCl_2$ and the *trans* compartment contained 15 mM KCl and 15 μM $CaCl_2$. (B) representative traces at +140 and −60 mV. Both compartments contained 150 mM KCl and 15 μM $CaCl_2$. Signals were filtered (Gaussian) at 200 Hz in Clampfit 9 (Axon Instruments). The close state is indicated by horizontal lines.

yielded a main unitary conductance of 242 ± 31 pS, which is larger than the previously reported value of 177 pS [16], possibly due in part to the different voltage ranges used (−140 to +20 mV in the current experiments vs. −70 to +70 mV in [16]). The MOT values were rather independent of the membrane potential but were variable, ranging from few milliseconds to hundreds of milliseconds. MOT averaged 125 ± 33 ms ($N = 19$) and 128 ± 58 ms ($N = 11$), from data recorded at voltages ranging from −120 to +120 mV, under symmetrical and asymmetrical conditions, respectively. The MCT values were relatively less variable and averaged 40 ± 10 ms ($N = 25$) and 33 ± 7 ms ($N = 18$), respectively. Thus, there was no significant difference in the MOT and MCT values between the symmetrical and asymmetrical conditions.

4. Conclusion

The data presented in this study demonstrated the feasibility of purification of a membrane protein, polycystin-2 channel, from cultured mammalian cells. Our modified TAP system indeed yielded proteins capable of correct assembly in reconstituted planar lipid bilayer to form functional cation channels. This provides an alternative and efficient avenue to study the function of membrane proteins, such as ion channels, transporters and receptors, as they are often expressed at low abundance and/or sometimes in intracellular membranes (difficult for electrophysiological studies). So far, in vitro translation and protein purification from bacterium, yeast and insect are common approaches for preparing membrane proteins but they may produce proteins that are different from their physiological forms in mammalian cells, e.g., due to post-translational modifications. Although a reconstituted lipid bilayer is an artificial system, it forms a pure system for studying ion channels, which can be advantageous over a living cell. For

example, hetero-complexing of polycystin-2 with other channels, such as TRPC1 [21], is a puzzling problem for discerning channel signals in living cells. Thus, our modified TAP method is not only applicable to identification and analysis of novel protein–protein interactions and protein complexes [8,9] but also, together with lipid bilayer electrophysiology, provides an adequate approach to study the function of an ion channel and its functional modulation by a protein partner.

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