Elaboration of a novel technique for purification of plasma membranes from *Xenopus laevis* oocytes

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**MATERIALS AND METHODS**

Oocyte Preparation, Injection, and Maintenance

Ovaries were surgically removed from gravid *X. laevis* frogs anesthetized with 2-aminobenzoic acid ethyl ester. Oocytes (stages V–VI) were dissected by hand, and follicular layers were removed with a collagenase treatment (17.5 mg/ml, type 1A; Sigma-Aldrich) for 1 h under mild agitation in a Ca2+-free Barth solution (in mM: 90 NaCl, 3 KCl, 0.82 MgSO4, and 5 HEPES, pH 7.6). Oocytes were allowed to rest overnight at 18°C in normal Barth solution [same as above with 0.4 mM CaCl2 and 0.33 mM Ca(NO3)2] supplemented with horse serum (5%), sodium pyruvate (2.5 mM), and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml kanamycin). On the next day, oocytes were injected with cRNA solutions in water and further incubated for 2 [aquaporin (AQP)2] and transient receptor potential vanilloid (TRPV)5 or 5 [Na+-glucose cotransporter (SGLT)1] days before proceeding to membrane purification. cRNA preparations were diluted to 1 µg/µl [except wild-type AQP2 (wt-AQP2), which was 0.025 µg/µl] and injected at 46 nl/oocyte with a microinjection apparatus (Drummond Scientific, Broomall, PA).

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Vectors and cRNA

All constructs used in this study were elaborated with the pT7Ts vector. For AQP2, the wild-type protein (wt-AQP2) construct was kindly provided by Peter M. T. Deen (Nijmegen, The Netherlands). The two point mutations, D150E and G196D, were inserted with site-directed mutagenesis, and validity of constructs was confirmed through sequencing. For SGLT1, a fully functional myc-tagged version of the protein was used (1), along with two nonfunctional SGLT1 mutants, C351A and C361A (10). Vectors for TRPV5, wild type and mutants (N518C and F531C), were kindly provided by L. Parent (University of Montreal) (8). For preparation of cRNAs, all vectors were linearized and cRNAs were synthesized with the mMessage machine T7 kit (Ambion, Austin, TX).

Preparation of Total and Plasma Membrane Fractions of Oocytes

Total membranes. Five oocytes were rinsed in Barth solution and homogenized in 1 ml of the same solution supplemented with protease inhibitor cocktail (Sigma-Aldrich) with 10 strokes of a Potter-Elvehjem tissue grinder (Wheaton). Homogenates were centrifuged at 10,000 × g for 20 min at 4°C to discard cell debris, and the supernatant was centrifuged at 16,000 × g for 20 min at 4°C to pellet down total membranes. Pellets were resuspended in 10 μl of Barth solution (2 μl solution/oocyte) and frozen until use.

Plasma membranes. Forty oocytes (except in Fig. 1, where 10–120 oocytes were treated) were rinsed in MES-buffered saline solution (MBSS; 80 mM NaCl, 20 mM MES pH 6.0) and incubated for 10 min at room temperature in the same solution with 0.005% subtilisin A (Sigma-Aldrich) under very mild agitation to partially digest the vitelline membranes. From this moment on, oocytes are somewhat sticky although resistant enough to tolerate subsequent treatments. The following polymerizing steps were performed at 4°C under mild agitation. Polymerization was performed by two sequential 60-min incubations in MBSS, first with 1% lodo and then with 0.1% polyacrylic acid (Sigma-Aldrich). Between each step, oocytes were thoroughly rinsed in MBSS. The oocytes were then homogenized in an Eppendorf tube with 0.5 ml of cold HbA (in mM: 5 MgCl₂, 5 NaH₂PO₄, 1 EDTA, 80 sucrose, and 20 Tris pH 7.4). This homogenization was performed by hand with a P200 pipettor until no particles were linearized and cRNAs were synthesized with the mMessage machine T7 kit (Ambion, Austin, TX).

Western Blots

Western blots were performed as described previously (1), using total membranes and purified plasma membranes isolated from X. laevis oocytes. Samples loaded represent either 1 oocyte (total membranes) or 40 oocytes (purified plasma membranes), except for Fig. 1, where the number of oocytes varied from 10 to 120. For TRPV5 and SGLT1, total membrane fractions were solubilized in 1% Triton X-100 to eliminate a major contaminating band of equivalent molecular mass that alters the normal migration of both proteins analyzed. Interestingly, this contaminant is not found in purified plasma membrane. Samples were run on either a 7.5% [TRPV5 and SGLT1] or a 12% (AQP2) gel and transferred onto nitrocellulose membranes. The efficiency of the overall procedure was monitored by Ponceau red staining. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) + Tween 20 (TBS-T, 0.1%) and probed with specific antibodies. For AQ2, we used α-AQP2 at 1/100 (N-20 from Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase (HRP)-linked chicken anti-goat at 1/5,000 (Santa Cruz Biotechnology). For SGLT1, we used α-myc at 1/300 (9E10 clone from Santa Cruz Biotechnology) followed by HRP-linked goat anti-mouse at 1/3,000 (Jackson Immunoresearch). For TRPV5, we used α-CAT21-A at 1/100 (Jackson Immunoresearch) followed by HRP-linked donkey anti-rabbit at 1/10,000 (Jackson Immunoresearch, PA). For protein disulfide isomerase (PDI), we used α-PDI at 1/200 (Santa Cruz Biotechnology) followed by HRP-linked donkey anti-rabbit at 1/10,000 (Santa Cruz Biotechnology). All incubations were performed in TBS-T with milk. Membranes were rinsed adequately between every step with TBS-T and revealed with enhanced chemiluminescence detection (Phototope-HRP, New England Biolabs, Pickering, ON, Canada).

Volume Measurements

Functionality of AQ2 was assessed by water flux measurements in noninjected and AQ2-injected oocytes. Briefly, the oocytes were placed in a 0.07-ml bath on the stage of an inverted low-power microscope equipped with a camera and a recording system for analysis of the oocyte cross section. The oocytes were challenged with a hypotonic solution (−20 mosmol/kgH₂O), and the swelling induced was monitored. The variations in volume were used to determine water permeability values (Pₜ) which are given in micrometers per second. For a more detailed description of this setup and procedure, see Duquette et al. (9) and Charron et al. (4).

RESULTS AND DISCUSSION

Technical Considerations

The need for an improved technique for the purification of plasma membrane of X. laevis oocytes came from the fact that all available techniques report various levels of contaminants originating from different intracellular stores (11, 13, 21), except that of Kamsteeg and Deen (14), which results in a high level of purification without any evidence of contaminants. Unfortunately, this technique requires a certain level of technical skill and turns out to be inappropriate when >40 oocytes are required for a given experiment.

The present technique was elaborated out of preexisting techniques and essentially permits rapid and efficient purification of highly purified plasma membranes from X. laevis oocytes. The overall technique is accessible, does not require the manual removal of the vitelline membranes, and is accomplished in ~3 h. The first step consists of the partial enzymatic digestion of the vitelline membrane with the use of subtilisin A. The purpose of this procedure is to make the vitelline membrane more permeable to the polymerizing agents applied thereafter. In contrast to techniques in which the vitelline membrane is physically removed with the use of microforceps, the partial digestion of this layer does not make the oocytes fragile and sticky, which greatly facilitates subsequent manipulations. In addition, the manual removal of this membrane is cumbersome and thus greatly limits the number of oocytes that can be treated by a single person. This treatment has been proposed to facilitate patch-clamp studies on oocytes (5). The enzymatic permeabilization of the vitelline membrane enables an efficient polymerization of the plasma membrane to the vitelline layer.
The use of polymerization as a strategy for the purification of oocyte plasma membranes was proposed by Chaney and Jacobson (2) and subsequently modified by Kamsteeg and Deen (14). This technique presents rapid and easy purification of plasma membranes without the use of more fastidious techniques such as density-gradient centrifugation (13). Polymerized plasma membranes from the latter technique, although adequate, generate small membrane fragments that are difficult to separate from the remainder of the homogenate. On the other hand, when a similar purification is performed on oocytes in which the plasma membranes have been polymerized to the vitelline layer, large leaflets are generated that are easier to precipitate and also allow for their direct visualization through the successive centrifugation steps. The final pellet consists of highly purified plasma membranes attached to the vitelline fraction.

Limitation of Technique

In a first set of experiments, we aimed at defining the limitations of this technique, delineating the number of oocytes that could be adequately processed in a given sample and also the ability to detect variations in expression levels in plasma membrane of the oocyte. For these purposes, we have chosen to express the AQP2 water channel since its detection in oocytes through Western blots is found to be quite sensitive. In Fig. 1, plasma membranes were purified from oocytes expressing AQP2 (1 ng cRNA per oocyte) in batches of 10–120 oocytes. As seen in Fig. 1, the specific signal found at 29 kDa gradually increases with the increasing number of oocytes used in the purification procedure. Although quite faint, AQP2 can be visualized even with as few as 10 oocytes, thus validating the technique from very low to high sample sizes. This flexibility in sample size makes it convenient to adapt for varying expression levels and Western blot sensitivity. Second, we also wanted to demonstrate the ability to visualize variations in expression levels of a given protein at the plasma membrane. In Fig. 2, plasma membranes were purified with the same sample size (40 oocytes per sample) expressing increasing levels of AQP2 (0–1 ng per oocyte) and visualized through Western blot. As seen in Fig. 2A, specific signals increasing in intensity are found in correlation with increasing activity levels for AQP2 (Fig. 2A) corresponding to injected cRNA. Identification of possible contamination from intracellular stores, as determined by evaluation of PDI in the same blots, failed to show any specific contaminants (data not shown).

Validation of Technique

In subsequent experiments, total membranes and purified plasma membrane fractions were tested for the presence of heterologously expressed proteins through Western blot analysis. For the sake of demonstrating the efficacy of the technique, it was applied to proteins normally targeted to the plasma membrane and to mutant forms known to be mistargeted or nonfunctional. Again, we have chosen AQP2 as a model to test this technique since the wild-type form of the protein (wt-AQP2) is strongly expressed at the plasma membrane while most mutant forms are known to be completely retained in intracellular stores. Also, we took advantage of one AQP2 mutant (D150E) that demonstrates predominant endoplasmic reticulum (ER) retention with low expression at the plasma membrane, thus displaying partial targeting. In Fig. 3A, we present water permeabilities of control and AQP2-injected oocytes. As shown in Fig. 3A, oocytes injected with 1 ng of wt-AQP2 cRNA display a net increase in water permeability (93 ± 22 µm/s) while G196D-AQP2-injected oocytes (10 ng)
display water permeabilities similar to controls (3.6 ± 1.5 and 4.4 ± 1.4 μm/s, respectively). The oocytes injected with D150E-AQP2 (10 ng) exhibit intermediary water permeability (24.8 ± 6.5 μm/s), consistent with its partial targeting at the plasma membrane. Figure 3B presents Western blot detection of AQP2 injected oocytes. Oocytes were injected with wild-type (wt-AQP2, 1 ng) or mutant (D150E and G196D, 10 ng) AQP2 and tested for both activity and Western blot detection. Functionality was assessed by water permeability analysis (A) for controls (Ctl; \( P_f = 3.6 \pm 1.5 \) μm/s), wild-type (\( P_f = 93 \pm 22 \) μm/s), D150E (\( P_f = 25 \pm 6.5 \) μm/s), and G196D (\( P_f = 4.4 \pm 1.4 \) μm/s) AQP2s. B: Western blot performed on total membranes (5 oocytes) and purified plasma membranes (40 oocytes) from same samples. In total membranes, mature AQP2 is shown at 29 kDa while high-mannose form is seen at 31 kDa for both mutants. In purified plasma membrane, only the mature form of the protein is found. Note the strong signal for wild type compared with the fainter D150E. G196D is not detected in this purified fraction. Bottom: protein disulfide isomerase (PDI), an endoplasmic reticulum (ER) marker, was used to show the absence of this major contaminant in the purified membrane fraction.

**Fig. 4.** Western blot of total membranes and purified plasma membranes from \( \text{Na}^+ \)-glucose cotransporter (SGLT1)-injected oocytes. Total membranes (5 oocytes) and purified plasma membranes (40 oocytes) from oocytes injected with 46 ng of wild-type or mutant (C356A and C361A) SGLT1, along with controls, were tested in Western blot. As shown in total membranes, products are generated for all SGLT1 variants, although the mature protein (69 kDa) is only found in the wild type. The 2 mutants only display low-molecular-mass products. In purified plasma membranes, only the mature SGLT1 protein is detected in the wild type while immature forms are never found. Bottom: detection of PDI (ER marker) indicates the absence of contamination in purified membrane fractions.

**Fig. 5.** Western blot of total membranes and purified plasma membranes from transient receptor potential vanilloid (TRPV)5-injected oocytes. Total membranes (5 oocytes) and purified plasma membranes (40 oocytes) from oocytes injected with 46 ng of wild-type or mutant (N518C and F531C) TRPV5, along with controls, were tested by Western blot. As shown in total membranes, products are generated for all TRPV5 variants, showing essentially low-molecular-mass forms with mature glycosylated forms. In purified plasma membrane fractions, only wild-type proteins are detected, with glycosylated forms being predominant this time. Bottom: PDI detection indicates the absence of contamination in purified membrane fractions.
D150E-AQP2 (29 kDa) is found in the purified plasma membrane fraction, as for wt-AQP2. Once again, PDI was used as marker for intracellular contaminants. Even in overexposed blots, no trace of PDI could be seen in purified plasma membrane fractions.

To further substantiate the efficacy of this technique, we have performed similar studies on two other proteins: the Na\(^+\)/glucose cotransporter SGLT1 and the TRPV5 Ca\(^{2+}\)-selective channel, testing both wild-type proteins and mutants for which a lack of function has been established. SGLT1 has been extensively studied in X. laevis oocytes. Functional studies as well as immunofluorescence labeling have shown adequate targeting to the plasma membrane (1). In Western blots, both mature (glycosylated) and immature forms of the protein are found (18). In Fig. 4, we compare Western blots for SGLT1 of total membrane and purified plasma membranes for both wild type and mutants (C351A and C361A). Both of these mutants have been previously reported to be nonfunctional in oocytes, but even though mistargeting was suspected their actual processing has not been determined (10). The Western blot of total membranes confirms that both mutant SGLT1 proteins are being synthesized, although the mature glycosylated form is lacking, which is found with the wild-type form of the protein (69- and 61-kDa forms, also see Ref. 18). In purified plasma membranes, only the mature form of the wild-type protein is found. Immature forms of the protein, be it of the wild type or the mutated proteins, are not evidenced. It thus seems that both mutants of SGLT1 are sequestered in intracellular stores, most probably the ER. In Fig. 4, bottom, evaluation of PDI indicates that no intracellular contaminants are found in the purified plasma membrane fractions.

The TRPV5 is also well expressed in X. laevis oocytes (3, 8). The protein is adequately targeted at the plasma membrane and exhibits normal functionality. In Western blots from oocytes, TRPV5 is expressed as a doublet that represents glycosylation variants (79 and 89 kDa; also see Ref. 3). We have expressed in oocytes the wild-type form as well as two nonfunctional mutants (N518C and F531C) of TRPV5 (8) and assessed their plasma membrane targeting with Western blots. As shown in Fig. 5, in total membrane samples all three forms of TRPV5 are well expressed, essentially as unglycosylated forms, although glycosylated variants are present for all three proteins. On the other hand, in purified plasma membranes only the wild-type protein is detected, with the mature glycosylated form being predominant. In contrast, the dominant form of the wild-type TRPV5 in the total membrane preparation resides in the lower-molecular-mass form (79 kDa). It may thus be assumed that the lack of function of the two TRPV5 mutants originates from entrapment of the proteins in intracellular stores. Once again, as shown in Fig. 5, bottom, evaluation of PDI shows the absence of intracellular contaminants in the purified plasma membrane fractions.

In conclusion, we have elaborated a technique that permits rapid and efficient purification of plasma membranes from X. laevis oocytes that combines partial digestion of the vitelline layer with polymerization of the plasma membrane. High-level purification is thus achieved without fastidious technical steps, such as manual vitelline removal or centrifugation on density gradients. This improved technique provides a new means of investigation for the study of plasma membrane protein targeting, a key feature of both physiological and pathological interest.

GRANTS

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