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Reconstitution and Assay of Biogenic Membrane-Derived Phospholipid Flippase Activity in Proteoliposomes

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1. Introduction

Newly synthesized phospholipids in biogenic (self-synthesizing) membranes, such as the eukaryotic endoplasmic reticulum (ER) and bacterial cytoplasmic membrane (bCM), are initially located in the cytoplasmic leaflet of the membrane bilayer. In order to populate the exoplasmic leaflet of the bilayer to allow uniform bilayer growth, phospholipids have to be translocated (flipped) to the opposite membrane leaflet. Flipping does not occur spontaneously; in artificial bilayers, liposomal systems, and certain biomembranes, transverse movement of phospholipids, i.e., spontaneous transfer of a phospholipid from one side of the membrane to the other, occurs only very slowly—if at all. Nevertheless, it is clear from a number of studies that transbilayer movement of phospholipids occurs rapidly in the ER and bCM by a bidirectional, diffusion process facilitated by specific membrane proteins and requiring no metabolic energy. The latter observation largely rules out conventional activities of the ABC family of transporters, which are involved in energy-coupled, vectorial transport of solutes and some phospholipids. Although certain α-helical membrane-spanning peptides appear able to promote transbilayer movement of some classes of phospholipid in synthetic membranes ***(1,2)***, it is generally hypothesized that specific proteins, called flippases, are required for phospholipid flipping in the ER and bCM ***(1,3–9)***.

Biogenic membrane phospholipid flippases have yet to be identified. This chapter deals with recently described protocols to reconstitute flippase activity in proteoliposomes generated from detergent extracts of ER or bCM, as well as

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procedures to measure phospholipid flip-flop in the reconstituted vesicles ***(4–8)***. Briefly, ER or bCM preparations are solubilized in Triton X-100, and a clear detergent extract is prepared by eliminating insoluble material by ultracentrifugation. The Triton extract is supplemented with egg phosphatidylcholine and trace amounts of a labeled reporter phospholipid, before being treated with detergent adsorbing beads. Gradual removal of detergent promotes the formation of unilamellar proteoliposomes with diameters in the size-range 150–300 nm. These vesicles are then assayed for their ability to flip the labeled reporter phospholipid across the bilayer.

By varying the amount of Triton extract included in the reconstitution, it is possible to generate proteoliposomes containing, on average, a single functional flippase per vesicle. Increasing the amount of Triton extract beyond the amount required to populate each vesicle with a single flippase results, as expected, in an increase in the rate of flipping ***(7);*** decreasing the amount of Triton extract results in fewer flippase-equipped vesicles, and consequently a reduction in transport amplitude with no alteration in transport rate. The protein:phospholipid ratio of proteoliposome preparations containing an average of one functional flippase per vesicle provides an excellent basis to estimate the relative abundance of active flippases present in the crude Triton extract. Given the average size of the vesicles (determined by dynamic light scattering or other means) and based on assumptions concerning the average molecular mass of biogenic membrane proteins (approx 50 kDa is a good estimate (*see* **ref. *4***), thickness of the membrane bilayer (approx 4 nm), the cross-sectional area of a phospholipid (approx 0.7 nm^2), and the number of phospholipid molecules per vesicle (approx 550,000 phospholipid molecules per 250 nm diameter vesicle), it is possible to calculate the abundance of functional flippases. We have determined this to be approx 0.2% by weight of biogenic membrane proteins (the number is similar for both rat liver ER and *Bacillus subtilis [B. subtilis]* cytoplasmic membrane ***[4,5,7]***); estimates as high as 0.6% have been reported for rat liver ER ***(9)***. The reconstitution protocol and activity assay have been used to enrich for functional flippases and can be used to isolate, identify, and characterize the protein. In purification protocols we reconstitute at a low protein: phospholipid ratio such that not all vesicles contain a flippase. Under these conditions, an increase in the protein:phospholipid ratio for a protein fraction produces a proportional increase in the extent of transport (because more vesicles are flippase-competent at the higher protein:phospholipid ratio). Such data can be used to generate a traditional specific activity measure (transport amplitude normalized to the protein:phospholipid ratio) that can, in turn, be used to guide a purification effort (*see* **refs. *4–8*** for further details).

The assay to measure flippase activity makes use of [^{3}H]dipalmitoylphosphatidycholine ([^{3}H]DPPC) as a transport reporter, and relies on the

ability of phospholipase A_2 to hydrolyze only those phospholipids present in the outer leaflet of liposomes and proteoliposomes without compromising the intactness of the vesicle membrane ***(7)***. Thus, in protein-free liposomes or proteoliposomes lacking a flippase, only 50–60% of the [^{3}H]DPPC will be hydrolyzed, corresponding to the outer leaflet pool. However, for proteoliposomes equipped with a flippase, [^{3}H]DPPC located in the inner leaflet will be flipped out and hydrolyzed as well (phospholipids and hydrolysis products will be transported from the outer leaflet to the inner leaflet to compensate), resulting in a predicted hydrolysis of 100%. Although the extent of phospholipase A_2-mediated hydrolysis is considerable, the simultaneous presence of the hydrolysis products, lyso phospholipid and fatty acid, preserves the membrane permeability barrier. [^{3}H]DPPC resembles natural phospholipids and is commercially available, making it an attractive choice for transport reporter. The procedure we describe is easily adapted to other measures of flippase activity, for instance using fluorescent NBD-phospholipids or other phospholipid analogs. Examples of assays using other phospholipid reporters may be found in ***(4–6)***, and ***(8–9)***

2. Materials

2.1. Solubilization of Biogenic Membrane Proteins

1. Biogenic membrane preparation, e.g., rat liver endoplasmic reticulum vesicles, bacterial cytoplasmic membrane vesicles (procedures for the preparation of these membranes may be found in ***[4–9]***). The protein concentration of the membrane suspension is typically in the range 5–25 mg/mL. The membranes are typically flash-frozen in liquid nitrogen after preparation and stored at −80°C.
2. Buffer A1: 1 *M* potassium acetate, 10 m*M* HEPES pH 7.5.
3. Buffer A2: 0.5 *M* potassium acetate, 10 m*M* HEPES pH 7.5.
4. Buffer B: 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.
5. Buffer C: 2% Triton X-100 (w/v), 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.

2.2. Reconstitution of Liposomes and Proteoliposomes

1. Egg phosphatidylcholine (egg PC), 100 mg/mL in chloroform (stored at −20°C).
2. Triton X-100, 10% (w/v) solution. Triton X-100 that has been specially purified for membrane research is recommended. Store at 4°C.
3. A stock solution of 200 m*M* HEPES pH 7.5.
4. A stock solution of 2 *M* NaCl.
5. 13 × 100 mm Pyrex screw-cap tubes and caps.
6. SM-2 BioBeads, 20–50 mesh (BioRad, Hercules, CA).
7. Methanol.
8. Nitrogen or argon for drying.
9. 2 mL Dounce homogenizer.
10. [choline-methyl-^{3}H]dipalmitoylphosphatidylcholine ([^{3}H]DPPC).

2.3. Flippase Assay

1. Buffer B: 10 m*M* HEPES pH 7.5, 100 m*M* NaCl.
2. Liposomes or proteoliposomes, prepared as described in **Subheading 3.2.** containing [^{3}H]DPPC.
3. Perchloric acid, ammonium molybdate and ascorbic acid for phosphate assay.
4. Phospholipase A_2, from *Naja naja* venom (obtained as a powder from Sigma; dissolved in water at a concentration of 10,000 U/mL in water, and stored in aliquots at –20°C).
5. 100 m*M* $CaCl_2$.
6. 2 *M* NaCl.
7. 120 m*M* ethylene glycol-*bis* (β-aminoethyl ehter) *N,N,N',N'*,-tetraacetic acid (EGTA) (pH 7.0).
8. Chloroform and methanol for extraction.
9. Thin layer chromatography (TLC) solvent, freshly prepared at time of use: chloroform:methanol:28% ammonia (65:25:5, by volume).
10. Silica 60 TLC plates, activated by heating in a 100°C oven for at least 1 h.

3. Methods

3.1. Solubilization of Biogenic Membrane Proteins Including the Flippase

1. Wash membranes with high salt to remove peripheral membrane proteins; make sure membranes are fully suspended by using a Dounce homogenizer (*see* **Note 1**). Add an equal volume of Buffer A1. Mix well and incubate on ice for 30 min. Centrifuge to pellet the membranes (175,000*g*, 30 min, 4°C). Remove the supernatant and wash the pellet with ice-cold Buffer A2.
2. Extraction of integral membrane proteins (*see* **Notes 2** and **3**): Resuspend the membranes in Buffer B, by homogenizing in a total volume comparable to that of the starting membrane preparation. Add an equal volume of Buffer C. Mix well and incubate on ice for approx 30 min. The mixture should be less turbid than the starting material. Spin the sample in an ultracentrifuge at 175,000 *g* for 30 min, 4°C.
3. Carefully remove the supernatant (Triton Extract or TE) without disturbing the fluffy surface of the pellet. The TE typically contains 50–65% of the protein in the salt-washed membranes (*see* **Note 4**).

3.2. Reconstitution of Liposomes and Proteoliposomes

1. The procedure described is for the reconstitution of vesicles from a 1-mL sample containing approx 4.5 μmol egg phosphatidylcholine with or without Triton Extract (to prepare proteoliposomes or liposomes, respectively) in 10 m*M* HEPES pH 7.5, 100 m*M* NaCl, 1% (w/v) Triton X-100. We routinely scale this procedure to 2 mL samples by doubling all the reagents but processing all samples for the same length of time as for the 1-mL reconstitution. The procedure is adapted from Lévy et al. ***(9)***.
2. Wash the SM2 Bio-Beads (weigh out more than you need, as beads are invariably lost during the washing protocol) successively with methanol (twice); water (three

times); then 10 m*M* HEPES pH 7.5, 100 m*M* NaCl (once). Use wash volumes of 25 mL/*g* beads. A 1-mL reconstitution procedure requires 300 mg of wet beads: 100 and 200 mg aliquots of wet washed beads may be prepared ahead of time and stored, respectively, in capped 1.5 mL Eppendorf tubes. Make sure that the caps are tightly closed so that beads remain moist. (*see* **Note 5**).

3. Dry 32 μL 100 mg/mL egg PC (approx 4.5 μmol) in a screw-cap 13 × 100-mm glass tube under a stream of nitrogen (*see* **Note 6**). Use a Hamilton syringe specifically set aside for this purpose—avoid plastic-ware.
4. Add water, Triton X-100, HEPES buffer, and NaCl to the dried lipid such that the final volume and concentration after the addition of protein sample will be 1 mL at 1% Triton X-100, 10 m*M* HEPES, 100 m*M* NaCl. Before adding protein (Triton Extract), vortex every few minutes until the lipid is dissolved—this takes about 15 to 20 min at room temperature. The solution should be completely clear. Bubbles/froth may be cleared by centrifuging the sample briefly in a table-top clinical centrifuge (e.g., 1000*g*, 5 min), leaving the tube uncapped. (*see* **Note 7**).
5. Add the TE. Vortex gently to mix, but avoid generating froth. For liposomes, omit TE. The total volume of the sample should be 1 mL.
6. Add ~100 mg of wet washed beads. Reserve the ~200 mg aliquot of beads for the next step. Incubate at room temperature on an end-over-end mixer (*see* **Note 8)** for 3–4 h. The mixture should become slightly turbid.
7. Add the 200-mg aliquot of wet beads. Transfer the end-over-end mixer to 4°C and continue mixing for at least 12 h. This step is usually done overnight, so that the proteoliposomes are ready for the flippase activity assay the following morning.
8. Remove the turbid supernatant from the beads (rinse the beads, if desired, with a small volume of 10 m*M* HEPES pH 7.5, 100 m*M* NaCl, then add the rinse to the supernatant) and centrifuge at 265,000*g* for 45 min in a TL-100 ultracentrifuge (Beckman) to pellet the vesicles. Remove the supernatant and resuspend the vesicles in 10 m*M* HEPES pH 7.5, 100 m*M* NaCl; centrifuge again, and resuspend the pelleted vesicles in a small volume (approx 400 μL) of the same buffer. It is important that the proteoliposome suspension appears uniform by eye; rather than pipet-mixing for this purpose, we recommend using a 2-mL Dounce homogenizer.
9. Protein recovery after reconstitution is typically approx 50%, phospholipid recovery ranges between 50–70%. Measurement of protein in the reconstituted vesicles is frequently compromised by the presence of phospholipid. To circumvent this problem, the protein is precipitated using a chloroform/methanol mixture, then dissolved in SDS (*see* **Note 9**).

3.3. Flippase Assay

1. Take a small aliquot (5–10 μL) of the liposome or proteoliposome sample to measure phospholipid content (*see* **ref.** ***[11]***). Based on this measurement, resuspend an aliquot of the vesicles at a concentration of 1 m*M* (lipid phosphorus) in Buffer B (*see* **Notes 10** and **11**).

2. Setup reactions in Eppendorf tubes on ice. Each Eppendorf tube should contain: 21 μL Buffer B, 1.5 μL 100 m*M* $CaCl_2$; 1.5 μL 2 *M* NaCl; 3 μL membranes (1 m*M* lipid phosphorus).
3. Preincubate at 30°C for 1 min.
4. Start the assay by adding 3 μL phospholipase A_2 (10,000 U/mL). Vortex briefly to mix. The total reaction volume is 30 μL. Incubate at 30°C; hydrolysis is complete in approx 15 min (*see* **Note 12**).
5. At desired times, stop hydrolysis by adding 30 μL 120 m*M* EGTA and 165 μL water (note that phospholipase A_2 activity is Ca^{2+}-dependent).
6. Extract lipids: Add 250 μL chloroform and 250 μL methanol, then vortex to mix and centrifuge to get a clear separation of phases (a few minutes in a microfuge). Save the lower phase. Remove the upper phase and transfer to a fresh eppendorf tube. Add 250 μL mock lower phase, vortex, and centrifuge. Collect the lower (organic) phase and pool with the original lower phase. Wash the pooled lower phase with mock upper phase. Collect the lower phase, dry under nitrogen, and dissolve in 40 μL chloroform:methanol (1:1, v/v).
7. TLC analysis: Spot the lipid extract on an activated Silica 60 TLC plate. Develop in freshly prepared chloroform:methanol:28% ammonia (65:25:5, by volume). Air-dry the plate, and visualize the distribution of radioactivity on the plate with a TLC scanner, phosphorimager, or fluorography followed by densitometry of the spots. Rf values for lyso PC and PC in this solvent system are approx 0.1–0.2 and approx 0.3–0.4, respectively.
8. The extent of hydrolysis is calculated as [(lyso [^{3}H]PC)/(lyso [^{3}H]PC + [^{3}H] DPPC)].
9. Roughly 45–50% hydrolysis can be expected in the case of liposomes (i.e., % hydrolysis of phospholipids in the outer leaflet of the vesicles). For proteoliposomes, % hydrolysis ranges from 50–75% depending on the activity of the proteins and the ratio of protein/phospholipid in the reconstituted vesicles (*see* **Note 13**).

4. Notes

1. Resuspension after each centrifugation step should be done using a Dounce homogenizer. Repeated pipeting is not adequate to break up small membrane clumps. For example, 10 or 20 passes with the Dounce helps to minimize clumping and maximize the membrane surface available for detergent extraction when preparing the Triton extract, or for phospholipase A_2-catalyzed hydrolysis during the flippase activity assay.
2. In this protocol, we specify Triton X-100 because we know it to be compatible with the flippase activity assays we have tested. Liposomes and proteoliposomes prepared using extracts from other detergents may be compatible with many downstream applications, but some detergents cause artifactual flip-flop of phospholipids in liposomes (unpublished results).
3. After solubilization of flippase activity in 100 m*M* NaCl, 1 % (w/v) Triton X-100, it is possible to lower both salt and detergent concentration 10-fold to facilitate

chromatography without causing any apparent decrease in the amount of soluble flippase activity.

4. The Triton extract may be frozen (–20°C) for storage without loss of flippase activity.
5. SM2 BioBeads should be washed freshly for each reconstitution. It is possible to keep them in buffer at 4°C for a few days, but prolonged storage of washed beads leads to poor performance.
6. Lipid tracers (e.g., [^{3}H]DPPC) should be dried from solution along with the egg PC. These will be incorporated into both leaflets of the phospholipid bilayer.
7. It is sometimes desirable to reconstitute larger volumes of a sample. This method has been used in instances where almost all of the final reconstitution volume came from the protein sample. For example, the lipids may be dissolved in 82 μL of 10% Triton X-100, then brought to 1 mL by adding 918 μL of a protein sample in 0.2% Triton X-100, 10 m*M* HEPES, 100 m*M* NaCl.
8. Mixing end-over-end is essential to remove all the detergent. A platform rocker may be used instead of an end-over-end mixer, but the mechanics of mixing are different—we have had more consistent success with end-over-end mixing.
9. To determine the protein/phospholipid ratio of the proteoliposomes, the protein is precipitated from 100 μL of proteoliposomes by adding 375 μL chloroform/methanol 1:2 (v/v) in a 1.5-mL Eppendorf tube. After centrifugation in a microcentrifuge for 10 min, the supernatant is transferred to a glass test tube. The pellet is washed with another 300–500 μL chloroform/methanol 1:2 (v/v), and the supernatants are pooled, dried, and may be used for a lipid phosphorus determination (*see* **Note 10** and ***[11]***). To quantify protein, the pellet is dried and dissolved overnight at room temperature in 100 μL 1% sodium dodecyl sulfate (SDS). The redissolved protein can be assayed by the micro-BCA method (Pierce Chemical Co.), which is tolerant of 0.1 % SDS and <1 % Triton X-100. We have also successfully used a different assay procedure (***12***) that does not require prior precipitation of protein.
10. Accurate determination of lipid phosphate before phospholipase A_2 treatment is crucial to the performance of the assay (*see* **Note 2**). Phosphate determination by the method of Rouser et al. ***(7)*** takes about 90 min, from start to finish. Briefly, phospholipids are extracted with a chloroform:methanol mixture, the extract is dried, hydrolyzed with perchloric acid to release inorganic phosphate from the glycerophospholipid molecules, and the released phosphate is assayed in a color reaction using an ascorbic acid-ammonium molybdate mixture.
11. The ratio of phospholipase A_2 to the number of vesicles is important. The proportions given here result in an average of at least 1 phospholipase molecule/vesicle. A lower ratio does not allow for hydrolysis of all vesicles, because the phospholipase A_2 tends to associate with vesicles and "hops" only infrequently between them.
12. For kinetic studies of flipping activity, the incubation time can be varied. Phospholipids in the outer leaflet of the vesicle are hydrolyzed within one minute ($t_{1/2}$ approx 0.1 min ***[7]***), and the entire accessible DPPC pool in flippase-containing proteoliposomes is hydrolyzed within 15 min.
13. We found that vesicles maintain their integrity during phospholipase treatment. This was tested by monitoring the leakage of fluorescent FITC-Dextran (4300 Da).

The Dextran was trapped in the vesicles at a self-quenching concentration (the dextran was added at a sufficiently high concentration prior to the addition of SM2 Bio-Beads for reconstitution, resulting in a proportion of the dextran being trapped within the lumen of the vesicles). The dilution of the FITC-dextran accompanying release from the vesicles could be monitored by an increase in fluorescence. Treatment of proteoliposomes with phospholipase A_2 for 20 min at 30°C resulted in a low level of leakge (5%, compared with the signal obtained when the vesicles were disrupted with detergent) (*see* **ref. 7**).

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