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Fluorescence-Based Measurements of Phosphatidylserine/Phosphatidylinositol 4-Phosphate Exchange Between Membranes

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Abstract

Several members of the evolutionarily conserved oxysterol-binding protein (OSBP)-related proteins(ORP)/OSBP homologs (Osh) family have recently been found to represent a novel lipid transfer protein (LTP) group in yeast and human cells. They transfer phosphatidylserine (PS) from the endoplasmic reticulum (ER) to the plasma membrane (PM) *via* PS/phosphatidylinositol 4-phosphate (PI(4)P) exchange cycles. This finding allows a better understanding of how PS, which is critical for signaling processes, is distributed throughout the cell and the investigation of the link between this process and phosphoinositide (PIP) metabolism. The development of new fluorescence-based protocols has been instrumental in the discovery and characterization of this new cellular mechanism *in vitro* at the molecular level. This paper describes the production and the use of two fluorescently labelled lipid sensors, NBD-$C2_{Lact}$ and NBD-PH_{FAPP}, to measure the ability of a protein to extract PS or PI(4)P and to transfer these lipids between artificial membranes. First, the protocol describes how to produce, label, and obtain high-purity samples of these two constructs. Secondly, this paper explains how to use these sensors with a fluorescence microplate reader to determine whether a protein can extract PS or PI(4)P from liposomes, using Osh6p as a case study. Finally, this protocol shows how to accurately measure the kinetics of PS/PI(4)P exchange between liposomes of defined lipid composition and to determine lipid transfer rates by fluorescence resonance energy transfer (FRET) using a standard fluorometer.

Introduction

The precise distribution of lipids between different membranes and within the membranes of eukaryotic cells[1,2] has profound biological implications. Decrypting how LTPs function is an important issue in cell biology[3,4,5,6],

and *in vitro* approaches are of great value in addressing this issue[7,8,9,10,11]. Here, an *in vitro*, fluorescence-based strategy is presented that has been instrumental in establishing that several ORP/Osh proteins effect PS/PI(4)P exchange between cell membranes[12] and thereby constitute a new class of LTPs. PS is an anionic glycerophospholipid that represents 2-10% of total membrane lipids in eukaryotic cells[13,14,16]. It is distributed along a gradient between the ER and the PM, where it represents 5-7% and up to 30% of glycerophospholipids, respectively[17,18,19]. Moreover, PS is essentially concentrated in the cytosolic leaflet of the PM. This build-up and the uneven partition of PS in the PM are critical for cellular signaling processes[19]. Owing to the negative charge of PS molecules, the cytosolic leaflet of the PM is much more anionic than the cytosolic leaflet of other organelles[1,2,19,20]. This enables the recruitment, *via* electrostatic forces, of signaling proteins such as myristoylated alanine-rich C-kinase substrate (MARCKS)[21], sarcoma (Src)[22], Kirsten-rat sarcoma viral oncogene (K-Ras)[23], and Ras-related C3 botulinum toxin substrate 1 (Rac1)[24] that contain a stretch of positively charged amino acids and a lipidic tail.

PS is also recognized by conventional protein kinase C in a stereoselective manner *via* a C2 domain[25]. However, PS is synthesized in the ER[26], indicating that it must be exported to the PM before it can play its role. It was not known how this was accomplished[19] until the finding that, in yeast, Osh6p and Osh7p transfer PS from the ER to the PM[27]. These LTPs belong to an evolutionarily conserved family in eukaryotes whose founding member is OSBP and that contains proteins (ORPs in human, Osh proteins in yeast) integrating an OSBP-related domain (ORD) with a pocket to host a lipid molecule. Osh6p and Osh7p consist only of an ORD whose structural features are adapted to specifically bind PS and transfer it between membranes. Nevertheless, how these proteins directionally transferred PS from the ER to the PM was unclear. Osh6p and Osh7p can trap PI(4)P as an alternative lipid ligand[12]. In yeast, PI(4)P is synthesized from phosphatidylinositol (PI) in the Golgi and the PM by PI 4-kinases, Pik1p and Stt4p, respectively. In contrast, there is no PI(4)P in the ER membrane, as this lipid is hydrolyzed to PI by the Sac1p phosphatase. Hence, a PI(4)P gradient exists at both the ER/Golgi and ER/PM interfaces. Osh6p and Osh7p transfer PS from the ER to the PM *via* PS/PI(4)P exchange cycles using the PI(4)P gradient that exists between these two membranes[12].

Within one cycle, Osh6p extracts PS from the ER, exchanges PS for PI(4)P at the PM and transfers PI(4)P back to the ER to extract another PS molecule. Osh6p/Osh7p interact with Ist2p[28], one of the few proteins that connect and bring the ER membrane and the PM into close proximity with each other to create ER-PM contact sites in yeast[29,30,31]. In addition, the association of Osh6p with negatively charged membranes becomes weak as soon as the protein extracts one of its lipid ligands due to a conformational change that modifies its electrostatic features[32]. This aids Osh6p by shortening its membrane dwell time, thereby maintaining the efficiency of its lipid transfer activity. Combined with the binding to Ist2p, this mechanism could allow Osh6p/7p to both quickly and accurately execute lipid exchange at the ER/PM interface. In human cells, ORP5 and ORP8 proteins execute PS/PI(4)P exchange at ER-PM contact sites *via* distinct mechanisms[33]. They have a central ORD, akin to Osh6p, but are directly anchored to the ER *via* a C-terminal transmembrane segment[33] and dock into the PM *via* an N-terminal Pleckstrin homology (PH) domain that recognizes PI(4)P and PI(4,5)P_2[33,34,35]. ORP5/8 use PI(4)P to transfer PS, and it has been shown that ORP5/8 additionally regulate

PM PI(4,5)P_2 levels and presumably modulate signaling pathways. In turn, a decrease in PI(4)P and PI(4,5)P_2 levels lowers ORP5/ORP8 activity as these proteins associate with the PM in a PIP-dependent fashion. Abnormally high PS synthesis, which leads to Lenz-Majewski syndrome, impacts PI(4)P levels through ORP5/8[36]. When the activity of both proteins is blocked, PS becomes less abundant at the PM, lowering the oncogenic capability of signaling proteins[37].

Conversely, ORP5 overexpression seems to promote cancer cell invasion and metastatic processes[38]. Thus, alterations to ORP5/8 activity can severely modify cellular behavior through changes in lipid homeostasis. Further, ORP5 and ORP8 occupy ER-mitochondria contact sites and preserve some mitochondrial functions, possibly by supplying PS[39]. Additionally, ORP5 localizes to ER-lipid droplet contact sites to deliver PS to lipid droplets by PS/PI(4)P exchange[40]. The strategy described herein to measure (i) PS and PI(4)P extraction from liposomes and (ii) PS and PI(4)P transport between liposomes has been devised to establish and analyze the PS/PI(4)P exchange activity of Osh6p/Osh7p[12,32] and used by other groups to analyze the activity of ORP5/ORP8[35] and other LTPs[10,41]. It is based on the use of a fluorescence plate reader, a standard L-format spectrofluorometer, and two fluorescent sensors, NBD-$C2_{Lact}$ and NBD-PH_{FAPP}, that can detect PS and PI(4)P, respectively.

NBD-$C2_{Lact}$ corresponds to the C2 domain of the glycoprotein, lactadherin, that was reengineered to include a unique solvent-exposed cysteine near the presumed PS binding site; a polarity-sensitive NBD (7-nitrobenz-2-oxa-1,3-diazol) fluorophore is covalently linked to this residue (**Figure 1A**)[12]. To be more precise, the C2 domain of lactadherin (Bos taurus, UniProt: Q95114,residues 270-427) was cloned into a pGEX-4T3 vector to be expressed in fusion with glutathione S-transferase (GST) in *Escherichia coli*. The $C2_{Lact}$ sequence was then mutated to substitute two solvent-accessible cysteine residues (C270, C427) with alanine residues and to introduce a cysteine residue into a region near the putative PS-binding site (H352C mutation) that can be subsequently labeled with N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene diamine (IANBD) [12]. A cleavage site for thrombin is present between the GST protein and the N-terminus of the C2 domain. A major advantage is that this domain selectively recognizes PS in a Ca^{2+}-independent manner contrary to other known C2 domains or Annexin A5[42]. NBD-PH_{FAPP} is derived from the PH domain of the human four-phosphate-adaptor protein 1 (FAPP1), which was reengineered to include a single solvent-exposed cysteine that can be labeled with an NBD group near the PI(4)P binding site (**Figure 1A**)[43]. The nucleotide sequence of the PH domain of the human FAPP protein (UniProt: Q9HB20, segment [1-100]) has been cloned into a pGEX-4T3 vector to be expressed in tandem with a GST tag. The PH_{FAPP} sequence has been modified to insert a unique cysteine residue within the membrane-binding interface of the protein[43]. Moreover, a nine-residue linker has been introduced between the thrombin cleavage site and the N-terminus of the PH domain to ensure accessibility to the protease.

To measure PS extraction from liposomes, NBD-$C2_{Lact}$ is mixed with liposomes made of phosphatidylcholine (PC) containing trace amounts of PS. Owing to its affinity for PS, this construct binds to the liposomes, and the NBD fluorophore experiences a change in polarity as it comes into contact with the hydrophobic environment of the membrane, which elicits a blue-shift and an increase in fluorescence.

If PS is extracted almost completely by a stoichiometric amount of LTP, the probe does not associate with liposomes, and the NBD signal is lower (**Figure 1B**)[32]. This difference in signal is used to determine whether an LTP (*e.g.,* Osh6p) extracts PS. A similar strategy is used with NBD-PH_{FAPP} to measure PI(4)P extraction (**Figure 1B**), as described previously[12,32]. Two FRET-based assays were designed to (i) measure PS transport from L_A to L_B liposomes, which mimic the ER membrane and the PM, respectively, and (ii) PI(4)P transport in the reverse direction. These assays are performed under the same conditions (*i.e.,* same buffer, temperature, and lipid concentration) to measure PS/PI(4)P exchange**.** To measure PS transport, NBD-$C2_{Lact}$ is mixed with L_A liposomes composed of PC and doped with 5 mol% PS and 2 mol% of a fluorescent rhodamine-labelled phosphatidylethanolamine (Rhod-PE)- and L_B liposomes incorporating 5 mol% PI(4)P.

At time zero, FRET with Rhod-PE quenches the NBD fluorescence. If PS is transported from L_A to L_B liposomes (*e.g.,* upon injecting Osh6p), a fast dequenching occurs due to the translocation of NBD-$C2_{Lact}$ molecules from L_A to L_B liposomes (**Figure 1C**). Given the amount of accessible PS, NBD-$C2_{Lact}$ remains essentially in a membrane-bound state over the course of the experiment[12]. Thus, the intensity of the NBD signal directly correlates with the distribution of NBD-$C2_{Lact}$ between L_A and L_B liposomes and can be easily normalized to determine how much PS is transferred. To measure the transfer of PI(4)P in the opposite direction, NBD-PH_{FAPP} is mixed with L_A and L_B liposomes; given that it only binds to L_B liposomes that contain PI(4)P, but not Rhod-PE, its fluorescence is high. If PI(4)P is transferred to L_A liposomes, it translocates to these liposomes, and the signal decreases due to FRET with Rhod-PE (**Figure 1C**). The signal is normalized to determine how much PI(4)P is transferred[43].

Protocol

1. Purification of NBD-$C2_{Lact}$

NOTE: Although this protocol details the use of a cell disruptor to break bacteria, it can be modified to use other lysis strategies (*e.g.,* a French press). At the beginning of the purification, it is mandatory to use buffer that is freshly degassed, filtered, and supplemented with 2 mM dithiothreitol (DTT) to prevent the oxidation of cysteine. However, for the protein labelling step, it is crucial to completely remove DTT. Many steps must be carried out on ice or in a cold room to avoid any protein degradation. Samples of 30 µL volume must be collected at different steps of the protocol to perform an analysis by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% acrylamide gel to check the progress of the purification. Mix enough denaturing Laemmli sample buffer with each aliquot, and heat the mixture at 95 °C. Freeze and store the tubes at -20 °C until analysis.

1. Expression of GST-$C2_{Lact}$ in *Escherichia coli*
    1. Mix 20 µL of BL21 Gold competent cells with 18 µL of sterilized water. Then, mix 2 µL of pGEX-$C2_{Lact}$ plasmid (at ~65 ng/µL) with the bacteria, and transform them by electroporation. Resuspend the bacteria with 150 µL of autoclaved Lennox Lysogeny-Broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl in deionized water, glucose-free). Let the bacteria grow at 37 °C for 1 h in a 2 mL snap-cap microcentrifuge tube.
    2. Inoculate 25 mL of LB medium, supplemented with 50 µg/mL ampicillin, with 150 µL of bacterial

suspension in a 125 mL sterile Erlenmeyer flask. Place the flask in an orbital shaker at 37 °C, and let the bacteria grow overnight with agitation at 185 rpm.

3. Fill two sterile 2 L Erlenmeyer flasks with 500 mL of LB medium supplemented with 50 µg/mL ampicillin, and add 5 mL of preculture suspension. Let the bacteria grow at 37 °C with agitation at 220 rpm.

4. Periodically measure the optical density (OD) of the suspension at a wavelength (λ) of 600 nm. When the OD reaches a value of ~0.6-0.7, add 500 µL of a stock solution of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) to each flask to initiate the expression of GST-$C2_{Lact}$. Shake the flasks at 185 rpm for 4 h at 37 °C.

5. Transfer the contents of each flask to a polypropylene centrifuge bottle. Centrifuge the two bottles for 30 min at 4600 × *g* at 4 °C to pellet the bacteria. Discard the supernatant, and resuspend each pellet in 50 mL of cold phosphate-buffered saline.

6. Transfer the bacterial suspension contained in each bottle to a 50 mL conical centrifuge tube. Centrifuge the two tubes for 30 min at 2300 × *g* at 4 °C. Remove the supernatant, and store the tubes, each of which contains a bacterial pellet, at -20 °C.

2. Purification of $C2_{Lact}$

1. On ice, fill two 50 mL conical centrifugal tubes with 50 mL of buffer containing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl (hereafter called TN buffer), previously filtered and degassed by membrane vacuum filtration.

2. To prepare the lysis buffer in each tube, dissolve a tablet of ethylenediamine tetraacetic acid-free protease inhibitor cocktail in the TN buffer by mild sonication or vortexing. Add other antiproteases, (10 µM bestatin, 1 µg/mL pepstatin A, and 10 µM phosphoramidon). Importantly, supplement the buffer with 2 mM DTT.

3. Fill the two tubes that contain the bacterial pellets prepared in step 1.1.6, with lysis buffer to obtain a final volume of 30 mL in each tube, and slowly defrost the pellets on ice for 10 min. Crush each pellet with a stainless steel spatula, and resuspend them by vortexing the tubes and/or by pipetting the suspension back and forth with a pipette controller and a 25 mL pipette until a homogeneous suspension is obtained.

4. Perform lysis using a precooled cell disruptor (see the **Table of Materials**) by loading 30 mL of the sample inside the reservoir and running a breaking cycle in continuous mode with a pressure of 1.6 bar. Collect the lysate in the same tube, keep the tube on ice, and immediately add 250 µL from a stock solution of 200 mM phenylmethylsulfonyl fluoride (PMSF) prepared in isopropanol.

5. Lyse the other sample following the same procedure. Use the remainder of the lysis buffer to wash the cell disruptor, and collect the wash to adjust the volume of each lysate (~30 mL) to a final volume of 50 mL.

6. Supplement each lysate with 5 mM $MgCl_2$,and add 20 µg/mL of DNAse I to fragment the DNA and thus reduce the viscosity of the sample. Incubate on ice for 30 min. Collect a sample for gel analysis.

7. Transfer each 50 mL lysate to a prechilled polycarbonate ultracentrifuge tube (two in total, see

the **Table of Materials**). Centrifuge at 186,000 × *g* at 4 °C for at least 1 h using an ultracentrifuge.

8. In parallel to the centrifugation step, dispense 1.4 mL of a slurry containing glutathione coupled to 4% agarose beads into two 50 mL conical centrifugal tubes, add 20 mL of TN buffer supplemented with 1 mM DTT (TND buffer) to each tube, centrifuge at 1200 × *g* for 5 min, and discard the supernatant. Repeat this washing step twice.

9. After the centrifugation of the bacterial lysate, remove a 30 µL sample from the supernatant, and transfer the supernatant from each ultracentrifuge tube to a corresponding 50 mL conical centrifuge tube that contains clean beads. For gel analysis, resuspend the debris pellet in one of the ultracentrifuge tubes with 50 mL of TND buffer, and collect a 30 µL sample.

10. Place the tubes on a rotator for 3-4 h at 4 °C to obtain a homogeneous bead suspension. Pool the bead suspensions in an empty 25 mL chromatography column. Let the beads decant, and remove the buffer and unbound proteins by gravity flow. Take a sample from the eluate for analysis.

11. Resuspend the beads with 20 mL of TND buffer, and collect the eluate by gravity flow. Repeat this step twice to completely wash the beads. Pool the collected eluates, and retain a 30 µL sample for further analysis.

    NOTE: After a short decantation, a volume of ~2 mL of bead suspension, to which GST-$C2_{Lact}$ is attached, sediments at the bottom of the column.

12. Add 1 mL of bead suspension to two 2 mL snap-cap microcentrifuge tubes. Fill each tube with TND buffer to a final volume of 1.970 mL. Take a 30 µL sample from one tube for further analysis (B1 sample). Add 10 µL of 10 mM $CaCl_2$ solution and 25 µL from a stock solution of human thrombin protease solution at 0.02 U/µL.

13. Place the two tubes on a rotator at 4 °C overnight to allow thrombin to cleave off the GST tag from the $C2_{Lact}$ domain. On the next day, in each tube, mix 10 µL of 200 mM PMSF solution with the bead suspension to inhibit the thrombin action.

14. Centrifuge the tubes at 700 × *g* for 5 min, and collect the supernatant, which contains soluble $C2_{Lact}$ domain, from each tube, without taking the beads. Pool the supernatants into a 2 mL snap-cap microcentrifuge tube (E1 eluate) that is kept on ice.

15. Add 1 mL of TND buffer to each tube to resuspend the beads, and wash them; repeat step 1.2.14. Perform this step thrice more to recover a maximum amount of protein. Each time, pool the collected supernatants into a new 2 mL tube (E2, E3, E4, and E5 eluates), and take an aliquot for further analysis. At the end of washing steps, take an aliquot from the bead suspension (aliquot B2).

16. Analyze the 30 µL samples that were collected at the different steps of the purification protocol by SDS-PAGE separation on a 15% acrylamide gel.

17. Remove potential contaminating beads by pooling all the supernatants (*i.e.*, ~10 mL) collected during steps 1.2.14 and 1.2.15 into a 10 mL chromatography column. Collect the eluate by gravity flow, and retain the beads at the bottom of the column.

18. Concentrate the $C2_{Lact}$ sample using a centrifugal filter unit with a molecular weight cut-off (MWCO) of 3 kDa and a centrifugation speed of 2300 × *g*. Stop the concentration procedure when the volume of the protein sample is ~1 mL.

3. Preparation and purification of NBD-$C2_{Lact}$

1. Equilibrate a desalting column (see the **Table of Materials**) with TN buffer. Load the column with 1 mL of concentrated $C2_{Lact}$ sample. Allow the sample to enter the gel bed completely, add 1.5 mL of freshly degassed DTT-free TN buffer to the column, and collect the eluate by gravity flow into a 2 mL snap-cap microcentrifuge tube.

2. Dilute 50 µL of eluate in a final volume of 300 µL TN buffer, and record an absorbance spectrum from 230 to 450 nm using pure TN buffer as a blank. Determine the $C2_{Lact}$ concentration based on the absorbance measured at 280 nm, considering an extinction coefficient ε equal to 44,920 $M^{-1}.cm^{-1}$.

3. To label the $C2_{Lact}$ construct with a NBD fluorophore, mix the protein with a ten-fold molar excess of *N,N′*-dimethyl-*N*-(iodoacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide).

1. Dissolve 1 mg of IANBD in anhydrous dimethylformamide (DMF), keeping in mind that the final volume of DMF used for labeling the $C2_{Lact}$ construct must not exceed 5% (v/v) of the protein sample volume.

2. To determine the volume of DMF (V_{DMF}) to dissolve IANBD, first calculate the required amount of IANBD (m, expressed in mg) to label the protein by using formula **1**.

$m = 10{,}000 \times C \times V \times MW_{IANBD}$ (**1**)

where C is the concentration of $C2_{Lact}$ measured in step 1.3.2, V is the volume of the $C2_{Lact}$ sample, and MW_{IANBD} is the molecular weight of the fluorophore (420 g/mol).

3. Calculate V_{DMF} by using formulae **2** (with m_0=1) and **3**.

$V_{DMF} = (m_0/m) \times V_{IANBD}$ (**2**)

$V_{IANBD} = 0.05 \times V$ (**3**)

Where m_0 is the quantity of IANBD powder in mg, and V_{IANBD} is the volume of IANBD solution to be added to the $C2_{Lact}$ sample.

4. Add volume V_{IANBD} of the freshly-prepared IANBD solution to the $C2_{Lact}$ sample, and shake the reaction mixture at 800-900 rpm for 30 min at 25 °C using a thermomixer protected from light. Let the reaction proceed for 90 min on ice. In the meantime, clean a centrifugal filter unit (MWCO= 3 kDa) with 10 mL of TN buffer.

4. Add L-cysteine (in 10-fold molar excess to IANBD) to the reaction mixture to inactivate free IANBD.

5. Add 15 mL of TN buffer to the NBD-$C2_{Lact}$ solution, and transfer the NBD-$C2_{Lact}$ solution to the centrifugal filter unit. Concentrate the sample to 2 mL to separate most of the free NBD from the protein by centrifugation at 2300 × *g*. Repeat this washing step two times. Centrifuge the sample in a 2 mL snap-cap centrifuge tube for 10 min at 19,000 × *g* at 4 °C to pellet potential aggregates, and collect the supernatant.

6. Dilute 50 µL of eluate in a final volume of 300 µL of TN buffer. Record the absorbance spectrum from 230 to 650 nm using the eluate collected during the concentration procedure as a blank. Determine the NBD-$C2_{Lact}$ concentration using the maximal absorbance at λ=280 and 495 nm and extinction coefficients ε= 44,920 $M^{-1}.cm^{-1}$ (protein) and 25,000 $M^{-1}.cm^{-1}$ (NBD fluorophore).

   NOTE: If the two concentration values are alike, this indicates that the $C2_{Lact}$ construct is labeled at a 1:1 ratio with the NBD group.

7. If the NBD-$C2_{Lact}$ concentration estimated from the measurement of NBD absorbance exceeds the concentration estimated from the absorbance of tryptophan (Trp) residues, repeat step 1.3.5 to further remove free NBD.

8. Add glycerol to the sample to obtain a final concentration of 10% (v/v) to cryo-protect the NBD-$C2_{Lact}$ construct during flash-freezing. Measure the final protein concentration.

9. Prepare 50 µL aliquots of protein in 0.5 mL snap-cap microcentrifuge tubes. Flash-freeze the tubes in liquid nitrogen, and store them at -80 °C.

2. Purification of NBD-PH_{FAPP}

NOTE: The procedure to produce and label PH_{FAPP} is identical to that of NBD-$C2_{Lact}$ until the transfer of NBD-$C2_{Lact}$ solution to a centrifugal filter unit in step 1.3.4. From this step onwards, follow the protocol that is described below.

1. After the concentration step, keep 2 mL of NBD-PH_{FAPP} at 4 °C in the dark for not more than 1 day before performing size-exclusion chromatography. Prior to size-exclusion chromatography, verify that there is no orange deposit (aggregation during concentration) at the bottom of the tube. If this is the case, centrifuge the sample at 540,000 × *g* for 10 min at 4 °C, and purify the supernatant by size-exclusion chromatography.

   NOTE: Size-exclusion chromatography is performed on a column packed with crosslinked dextran-acrylamide copolymer (see the **Table of Materials**), previously equilibrated with TND buffer, using a fast protein liquid chromatography system (see the **Table of Materials**). The column must be protected from light. A flow rate of 1 mL/min was used, and the elution of the NBD-PH_{FAPP} construct was followed by recording the absorbance at λ=280 (protein) and 480 nm (NBD) at the column exit.

   1. Inject the NBD-PH_{FAPP} sample loaded in a 2 mL injection loop onto the column, and immediately collect 2.5 mL fractions of eluate.

2. Analyze all the fractions that correspond to the main peak detected at 280 and 480 nm on a 15% SDS-PAGE gel. Mix a 25 µL sample of each fraction with 15 µL of Laemmli sample buffer prior to heating and loading onto the gel.

   NOTE: A main peak, which is simultaneously detected at λ= 280 and 480 nm, appears once a volume of ~150 mL buffer is passed through the column.

3. Pool the fractions that exclusively contain NBD-PH_{FAPP} protein (~12.2 kDa), and add glycerol at a final concentration of 10% (v/v). Concentrate the sample using a centrifugal filter unit with a MWCO of 3 kDa to a final volume of 1 mL using a centrifugation speed of 2300 × *g*.

4. Prepare aliquots, and record an absorbance spectrum as described for NBD-$C2_{Lact}$. Use an extinction coefficient

ε= 29,450 $M^{-1}.cm^{-1}$ to determine the concentration of the protein based on the absorbance measured at λ=280 nm.

3. Preparation of liposomes for PS and PI(4)P extraction or transfer assays

NOTE: Perform all the steps at room temperature unless otherwise specified. Handle organic solvents, rotavapor, and liquid nitrogen with caution.

1. Prepare fresh, filtered, and degassed 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-potassium hydroxide (KOH), pH 7.4, 120 mM potassium acetate (HK) buffer.
2. For each type of liposome, take precise amounts of different lipids from stock solutions, and mix them in a 25 mL pear-shaped glass flask (**Table 1**). Add pure chloroform to adjust the volume of each mixture to 1 mL. Label each flask with the liposome name. Wrap the flasks containing lipid mixture doped with Rhod-PE with aluminum foil.
3. Place the flask on a rotary evaporator. Dry the lipids under vacuum and at 25 °C for at least 30 min at a rotation speed of 500 rpm. For lipid films that contain PI(4)P, prewarm the flask at 32-34 °C for 5 min, under gentle rotation, to properly mix PI(4)P with the other lipids before creating vacuum in the flask to remove the solvent, which will leave behind a film of dry lipids on the flask wall.
4. Disconnect the flask from the evaporator, and place it in a vacuum chamber for 45 min to remove any remaining traces of solvent. Fill the flask with 2 mL of HK buffer, and add a few 4 mm-diameter glass beads to the solution. Gently vortex the flask for 2 min to resuspend the lipids and prepare multilamellar lipid vesicles (MLVs) with a lipid concentration of 4 mM. Prepare 0.5 mL aliquots of MLVs in 1.5 mL screw-cap microcentrifuge tubes.
5. Freeze-thaw the tubes 5x (using liquid nitrogen and a water bath at 37 °C, respectively). Extrude the liposomes or store them at -20 °C.
6. Use a mini extruder to prepare the liposomes (*i.e.,* large unilamellar vesicles) from the MLVs according to the manufacturer's guidelines. Use a polycarbonate filter with uniform cylindrical pores of 200 nm diameter.
7. To prepare each type of liposome, extrude at least 250 µL of the corresponding suspension of MLVs. Store the extruded liposomes at 4 °C and in the dark if they contain Rhod-PE. Use the liposomes within 2 days.

		Lipid composition (mol/mol)	Lipid			
	Liposome name		DOPC (25 mg/mL)	POPS (10 mg/mL)	16:0 Liss Rhod-PE (1 mg/mL)	C16:0/ C16:0-PI(4)P (1 mg/mL)
Extraction assays	Liposome 2 mol% PS	PC/PS 98/2	247 µL	12.5 µL		

	Liposome 2 mol% PI(4)P	PC/PI(4)P 98/2	247 µL			153 µL
	PC liposome	PC 100	252 µL			
Transport assays	L_A	PC/PS/Rhod-PE 93/5/2	234 µL	31.4 µL	200 µL	
	L_A without PS	PC/Rhod-PE 98/2	247 µL		200 µL	
	L_B	PC/PI(4)P 95/5	237 µL			383 µL
	L_B without PI(4)P	PC 100	252 µL			
	L_{A-Eq}	PC/PS/PI(4)P/ Rhod-PE 93/2.5/2.5/2	234 µL	15.7 µL	200 µL	191 µL
	L_{B-Eq}	PC/PS/PI(4)P 95/2.5/2.5	239 µL	15.7 µL		191 µL

Table 1: Volumes of lipid stock solutions to be mixed for liposome preparation. Abbreviations: PS= phosphatidylserine; PC = phosphatidylcholine; PI(4)P = phosphatidylinositol 4-phosphate; Rhod-PE = rhodamine-labelled phosphatidylethanolamine; DOPC = dioleoylphosphatidylcholine; POPS= 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; 16:0 Liss Rhod-PE = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl).

4. Measurement of PS or PI(4)P extraction

NOTE: Measurements must be conducted using a black 96-well plate and a fluorescence plate reader equipped with monochromators: one for fluorescence excitation and one for emission, with a variable bandwidth.

1. Prepare fresh, filtered, and degassed HK buffer supplemented with 1 mM $MgCl_2$ (HKM buffer). Prepare pure PC liposomes and PC liposomes doped with 2 mol % PS or 2 mol% PI(4)P (4 mM final lipid concentration, see **Table 1**).

   NOTE: Keep the tubes filled with the suspension of extruded liposomes at room temperature throughout the experiment, and keep the proteins on ice. Additionally, protect the lipid sensors from light.

2. For the PS extraction assay, in one well, mix liposomes containing 2 mol% PS (80 µM final lipid concentration, 0.8 µM accessible PS concentration) with NBD-$C2_{Lact}$ (250 nM final concentration) in a final volume of 100 µL. Fill a second well with the same amount of liposome (80 µM,

2 mol% PS) and NBD-C2$_{Lact}$ (250 nM) mixed with 3 µM LTP (Osh6p as a positive control or a protein of interest).
NOTE: An incubation time of 5 min is sufficient for Osh6p to achieve lipid extraction.

3. Fill a third well with NBD-C2$_{Lact}$ (250 nM) mixed with pure PC liposomes (80 µM). Fill a fourth well only with pure PC liposomes (80 µM). Repeat steps 4.2-4.3 to prepare three additional series of four wells.

4. For each well, record an NBD spectrum from 505 to 650 nm (bandwidth 5 nm) upon excitation at 490 nm (bandwidth 5 nm) at 25 °C. For each series, subtract the spectrum recorded with only liposomes from the other spectra.
NOTE: F and F_{max} correspond to the intensity at 536 nm measured with PS-containing liposomes in the presence or absence of LTP, respectively, whereas F_0 is the intensity at the same wavelength with pure PC liposomes. For each series, the percentage of accessible PS that is extracted by the protein is given by using the following formula.
$100 \times (1-((F-F_0)/(F_{max}-F_0)))$ (**4**)

5. For the PI(4)P extraction assay, prepare liposomes doped with 2 mol% PI(4)P, and carry out measurements with the NBD-PH$_{FAPP}$ probe. Perform control experiments and determine the extraction percentage in the same way as described above.
NOTE: Liposome and protein concentration are identical to those used in the PS extraction assay.

5. Real-time measurement of PS transport

NOTE: A standard fluorimeter (90° format) equipped with a temperature-controlled cell holder and a magnetic stirrer is used to record lipid transfer kinetics. To accurately acquire data, it is key to permanently maintain the sample at the same temperature (set between 25 and 37 °C depending on the origin of the protein (*e.g.*, yeast or human)) and to constantly stir it. The protocol described below is for the measurement of lipid transport in a 600 µL sample contained in a cylindrical quartz cell.

1. Prepare freshly degassed and filtered HKM buffer. Keep the tubes containing extruded liposomes at room temperature. Wrap tubes containing liposomes with Rhod-PE in aluminum foil, and/or store them in an opaque box to prevent any photobleaching.

2. Adjust the excitation and emission monochromators at λ = 460 nm (with a short bandwidth (1-3 nm)) and at λ = 530 nm (with a large bandwidth (≥ 10 nm)), respectively. Set the acquisition time at 25 min with a time resolution ≤ 1 s.

3. In the quartz cuvette, dilute 30 µL of the L_A liposome suspension and a volume of NBD-C2$_{Lact}$ stock solution in prewarmed HKM buffer to prepare a 570 µL sample that contains 200 µM total lipids and 250 nM NBD-C2$_{Lact}$. Add a small magnetic stirring bar, and position the cuvette in the fluorometer holder.

4. Once the sample is thermally equilibrated (after 3-5 min), trigger the measurement. After 1 min, add 30 µL of L_B liposome suspension (final concentration of 200 µM total lipids) to the sample. After 3 min, inject LTP into the sample so that the final concentration of the LTP is 200 nM, and acquire the signal for the remaining 21 min.

5. Carry out a parallel experiment to normalize the NBD signal. Mix 30 µL of $L_{A\text{-}Eq}$ liposome suspension with 250 nM NBD-C2$_{Lact}$ in HKM buffer (final volume of 570 µL). After 1 min, inject 30 µL of $L_{B\text{-}Eq}$ liposome suspension.

NOTE: The lipid composition of L_{A-Eq} and L_{B-Eq} liposomes are similar to that of L_A and L_B liposomes used in the transfer assay, except that each of them contains 2.5 mol% PS and 2.5 mol% PI(4)P. As a result, the NBD signal that is measured, referred to as F_{Eq}, corresponds to the signal that should be measured if PS was fully equilibrated between L_A and L_B liposomes by a transfer process.

6. Convert the kinetic curves measured with an LTP of interest to determine the amount of PS (in µM) transferred from L_A to L_B liposomes over time. Normalize each data point (F) of the curve by using the following formula.

   $F_{Norm} = (F-F_0)/(F_{Eq}-F_0)$ (**5**)

   in which F_0 corresponds to the NBD signal just before the addition of an LTP, and F_{Eq} is the signal measured in step 5.5.

   NOTE: The amount of PS (in µM) transferred from L_A to L_B liposomes corresponds to 2.5 × F_{Norm}, considering that the equilibrium corresponds to a situation where one half of accessible PS molecules, contained in the outer leaflet of the L_A liposomes, (*i.e.*, corresponding to 5 mol % of 0.5 × 200 µM total lipids) has been transferred into L_B liposomes.

6. Real-time measurement of PI(4)P transport

1. Set the fluorimeter (excitation and emission wavelength, bandwidth, acquisition time, time resolution) as done for the PS transfer assay. Likewise, use the same buffer, cuvette, and liposomes to perform the experiments under constant stirring at the same temperature.

2. In the cuvette, mix 30 µL of L_B liposome suspension and NBD-PH_{FAPP} with prewarmed HKM buffer to obtain a final volume of 570 µL (200 µM total lipids, 250 nM NBD-PH_{FAPP}). Once the thermal equilibration of the sample is reached, start the measurement, and after 1 min, inject 30 µL of L_A liposome suspension. After 3 min, inject the LTP of interest (final concentration of 200 nM), and record the signal.

3. Perform a second experiment to normalize the NBD signal. Mix 30 µL of L_{B-Eq} liposome suspension with 250 nM NBD-PH_{FAPP} in 570 µL of HKM buffer. After 1 min, inject 30 µL of L_{A-Eq} liposome suspension.

   NOTE: Here, the NBD signal, referred to as F_{Eq}, corresponds to the one which should be measured if PI(4)P was fully equilibrated between L_A and L_B liposomes.

4. Convert the kinetic curves to determine the amount of PI(4)P (in µM) transferred from L_B to L_A liposomes over time. Each data point (F) is normalized by using formula **5** in which F_0 corresponds to the NBD signal prior to the addition of an LTP, and F_{Eq} is the signal measured in step 6.3.

   NOTE: The amount of PI(4)P (in µM) transferred from L_B to L_A liposomes corresponds to 2.5 × F_{Norm}, considering that the equilibrium corresponds to a situation where one half of the PI(4)P contained in the outer leaflet of the L_B liposomes (*i.e.,* 0.5 × 5 µM) has been transferred in L_A liposomes.

7. Analysis of kinetics curves

1. Quantify the extent to which an LTP is efficient by determining the speed at which this LTP transfers lipids from one liposome population to the other one in the first few seconds following its injection to the cuvette.

1. Perform a linear regression of the first data points of the transfer kinetics to obtain a slope. Divide the slope value by the LTP concentration in the reaction mixture to determine the number of lipid molecules transferred per protein per time unit (min or s).

Representative Results

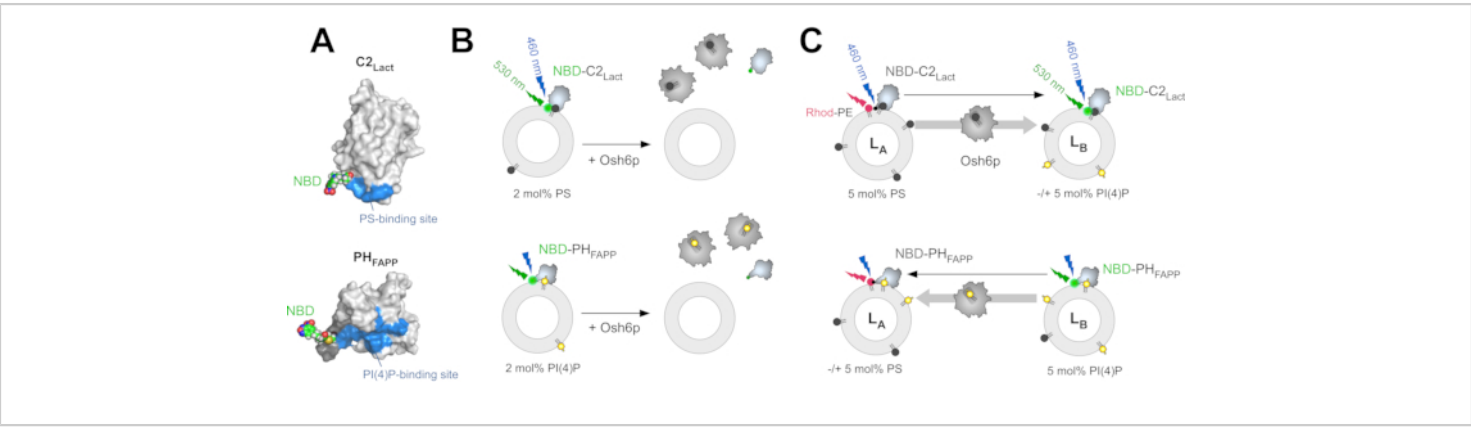

Figure 1: Description of the fluorescent lipid sensors and *in vitro* assays. (**A**) Three-dimensional models of NBD-$C2_{Lact}$ and NBD-PH_{FAPP} based on the crystal structure of the C2 domain of bovine lactadherin (PDB ID: 3BN6[48]) and the NMR structure of the PH domain of the human FAPP1 protein (PDB ID: 2KCJ[46]). An *N,N′*-dimethyl-*N*-(thioacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine moiety, built manually and energetically minimized, was grafted onto the thiol function of C352 (NBD-$C2_{Lact}$) and C13 (NBD-PH_{FAPP}) residues (represented as spheres, with carbon in green, nitrogen in blue, oxygen in red, sulfur in yellow, and hydrogen in white). The surface of the lipid-binding site of each probe was colored in blue. (**B**) Extraction assays. In the PS extraction assay, PC/PS liposomes (98/2 mol/mol) were incubated with 250 nM NBD-$C2_{Lact}$. In the absence of extraction, the probe strongly bound to the liposomes, resulting in a blue shift of NBD fluorescence and an increase of its emission intensity. If PS extraction occurred in the presence of an LTP (*e.g.,* Osh6p), the probe dissociated from the liposomes, and its fluorescence was lower. In the PI(4)P extraction assay, liposomes were doped with 2 mol% PI(4)P, and 250 nM NBD-PH_{FAPP} was used. (**C**) FRET-based lipid transport assays. In the PS transport assay, PC/PS/Rhod-PE liposomes (93/5/2 mol/mol, L_A) were incubated with 250 nM NBD-$C2_{Lact}$. PC liposomes (L_B), doped or not with 5 mol% PI(4)P, and Osh6p were sequentially added at t = 1 min and t = 4 min, respectively. If PS transport occurs, this elicits a dequenching of the NBD signal corresponding to the translocation of NBD-$C2_{Lact}$ from L_A to L_B liposomes. In the PI(4)P transport assay, L_B liposomes doped with 5 mol% PI(4)P were incubated with 250 nM NBD-PH_{FAPP}. PC liposomes (L_A) doped or not with 5 mol% PS were added. If PI(4)P transport occurs, this causes a quenching of the NBD signal due to the translocation of NBD-PH_{FAPP} from L_B to L_A liposomes. Abbreviations: NBD = 7-nitrobenz-2-oxa-1,3-diazol fluorophore; NMR = nuclear magnetic resonance; FAPP1 = four-phosphate-adaptor protein 1; PDB = Protein Data Bank; PS= phosphatidylserine; PC = phosphatidylcholine; LTP = lipid transfer protein; Osh6p = oxysterol-binding protein (OSBP) homolog 6 protein; PI(4)P = phosphatidylinositol 4-phosphate; FRET = fluorescence resonance energy transfer; Rhod-PE = rhodamine-labelled phosphatidylethanolamine; L_A liposomes = liposomes composed of PC, doped with 2 mol % Rhod-PE, and containing or not 5 mol% PS; L_B liposomes = liposomes incorporating 5 mol% PI(4)P. Please click here to view a larger version of this figure.

Figure 2A shows an SDS-PAGE analysis of the products of different steps leading to the purification of $C2_{Lact}$. Lane 1 shows the protein profile of the lysed bacteria expressing GST-$C2_{Lact}$ (~44.8 kDa), whereas lanes 2 and 3 respectively show the protein profiles of the supernatant and bacterial debris after ultracentrifugation. The comparison of these lanes shows that GST-$C2_{Lact}$ has been recovered in the supernatant and thus can be isolated using glutathione-linked agarose beads. Lanes 4 and 5 show the protein profiles of the supernatant after incubation with beads and washes recovered by gravity flow, whereas lane 6 shows the profile of proteins that have been retained on the beads. An analysis of these lanes indicates that almost all GST-$C2_{Lact}$ has been recovered from the beads.

Lanes 8-12 show the presence of a major band corresponding to $C2_{Lact}$ (~17.9 kDa) in the supernatants recovered through successive washes of the beads after thrombin treatment. Lane 13 indicates that non-cleaved GST-$C2_{Lact}$, along with GST (~26.9 kDa), remained bound to beads after this treatment. The comparison of these lanes indicates that the cleavage procedure, albeit not 100% efficient, did yield $C2_{Lact}$ that was then fluorescently labelled. **Figure 2B** shows an ultraviolet (UV)-visible absorbance spectrum of $C2_{Lact}$ labelled with NBD. As the construct is 100% pure, these results confirm that all $C2_{Lact}$ molecules were labelled with an NBD group based on the optical density measured at 280 nm (Trp) and 495 nm (NBD). The purity of NBD-$C2_{Lact}$ and its fluorescence were determined by SDS-PAGE analysis (**Figure 2C**).

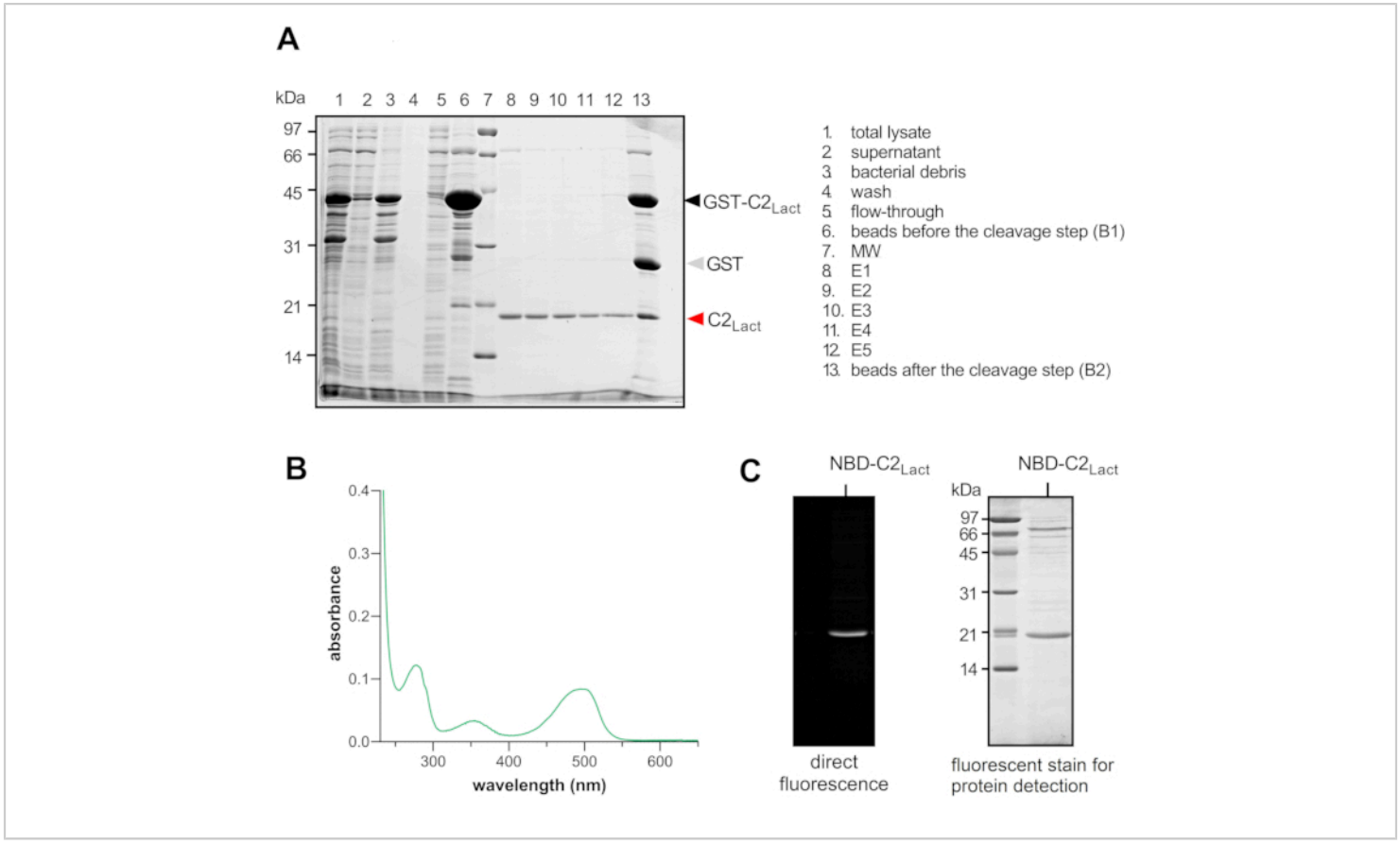

Figure 2: NBD-C2$_{Lact}$ purification. (**A**) SDS-PAGE analysis was used to check the presence of the protein at different steps of the purification procedure before labeling. The arrowheads indicate the position of the C2$_{Lact}$ domain (red arrowhead), of the GST alone (grey arrowhead), and of the GST-C2$_{Lact}$ construct (black arrowhead). **(B)** UV-visible absorbance spectrum of NBD-C2$_{Lact}$. **(C)** SDS-PAGE analysis of the purified NBD-C2$_{Lact}$. The first image was acquired under UV illumination without staining and reveals the presence of the NBD-C2$_{Lact}$ construct as it emits fluorescence. The second image shows the same gel after a protein staining procedure (see the **Table of Materials**). Abbreviations: NBD = 7-nitrobenz-2-oxa-1,3-diazol fluorophore; NBD-C2$_{Lact}$ = *N,N′*-dimethyl-*N*-(thioacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine moiety linked to the thiol function of C352 residue of a reengineered version of the C2 domain of bovine lactadherin (PDB ID: 3BN6[48]); PDB = Protein Data Bank; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; GST = glutathione S-transferase; MW = molecular-weight size marker; UV = ultraviolet. Please click here to view a larger version of this figure.

Figure 3 shows the results from PS and PI(4)P extraction assays using Osh6p. When only incubated with liposomes containing 2 mol% PS, the fluorescence of NBD-C2$_{Lact}$ was maximal as the NBD fluorophore was inserted in the membrane (*i.e.,* a hydrophobic context), indicating that the sensor was membrane-bound. In the presence of Osh6p, the fluorescence was lower and comparable to that measured with pure PC liposomes **(Figure 3A)**. The normalization of

intensity values at 536 nm indicated that ~75% of accessible PS was extracted. In the second assay, NBD-PH_{FAPP} was mixed with liposomes containing 2 mol% PI(4)P. The NBD signal was high without Osh6p, but low when Osh6p was present and was similar to that measured with PI(4)P-free liposomes (**Figure 3B**). An analysis of the intensity revealed that ~100 % of accessible PI(4)P was extracted by the LTP.

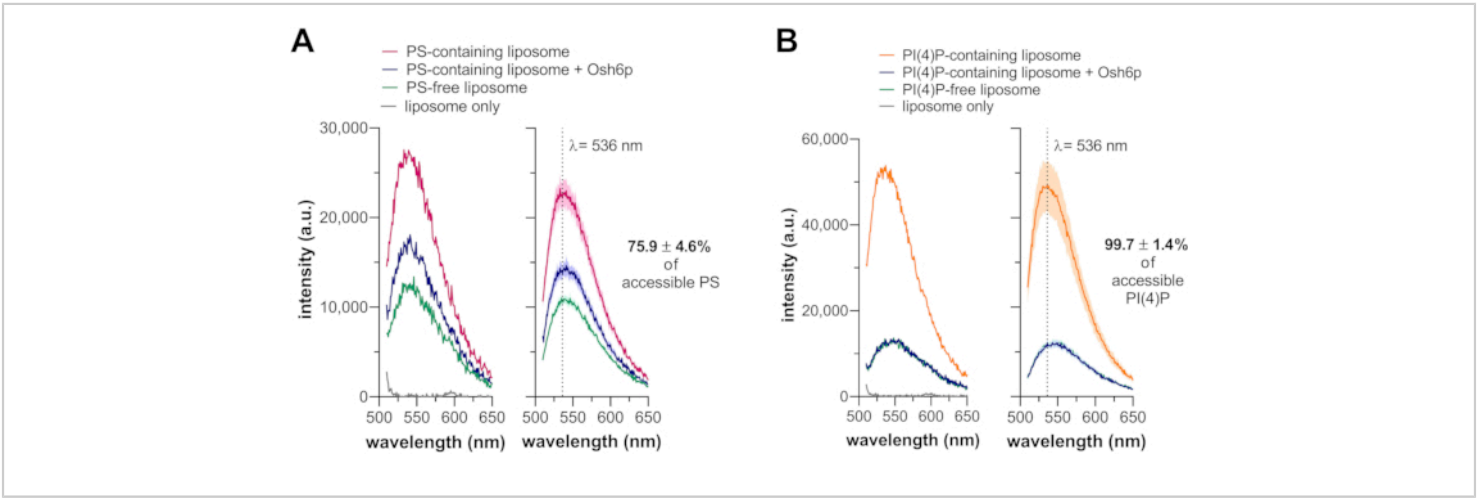

Figure 3: Extraction assays. (**A**) Fluorescence spectra of NBD-$C2_{Lact}$ (250 nM) measured upon excitation at 460 nm in the presence of liposomes (80 µM, 2 mol% PS) in the absence or presence of 3 µM Osh6p. Reference spectra were recorded with pure PC liposomes incubated or not with NBD-$C2_{Lact}$ (left panel). Several spectra were recorded from different series of wells, corrected by subtracting the background scattering signal from DOPC liposomes alone and averaged (n=4, ± SEM). The percentage of accessible PS that was extracted is indicated (right panel). (**B**) Fluorescence spectra of NBD-PH_{FAPP} (250 nM) mixed with liposomes (80 µM, 2 mol% PI(4)P) in the absence or presence of 3 µM Osh6p. Reference spectra recorded with PC liposomes in the presence and absence of the sensor are shown (left panel). Several spectra were recorded from different series of wells, corrected by subtracting the background scattering signal from DOPC liposomes alone and averaged (n=4, ± SEM). The percentage of accessible PI(4)P that was extracted is indicated (right panel). Abbreviations: NBD = 7-nitrobenz-2-oxa-1,3-diazol fluorophore; NBD-$C2_{Lact}$ = *N,N′*-dimethyl-*N*-(thioacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine moiety linked to the thiol function of C352 residue of a reengineered version of the C2 domain of bovine lactadherin (PDB ID: 3BN6[48]); PDB = Protein Data Bank; PS= phosphatidylserine; PC = phosphatidylcholine; DOPC = dioleoylphosphatidylcholine; Osh6p = oxysterol-binding protein (OSBP) homolog 6 protein; PI(4)P = phosphatidylinositol 4-phosphate; SEM = standard error of the mean; a.u. = arbitrary units. Please click here to view a larger version of this figure.

Figure 4A shows typical results from a PS transfer assay using Osh6p as an LTP. At time zero, NBD-$C2_{Lact}$ was mixed with L_A liposomes containing 5 mol% 16:0/18:1-PS (POPS) and 2 mol% Rhod-PE in a volume of 570 µL of HKM buffer at 30 °C. As the probe bound to L_A liposomes, its signal was quenched due to FRET with Rhod-PE present

in these liposomes. After one minute, Rhod-PE-free L_B liposomes (30 µL) were added; this was expected to only elicit a slight change in the signal due to light diffusion by this second liposome population and/or a dilution effect. The signal intensity after the addition of the L_B liposomes corresponds to F_0. The injection of a few µL of a stock solution of Osh6p (typically 40 µM) to dilute 200 nM of the protein in the reaction mix elicited a slow increase in the NBD signal due to the dequenching of the fluorophore as PS was transported from L_A to L_B liposomes, thereby promoting the translocation of NBD-$C2_{Lact}$.

When L_B liposomes contained 5 mol% PI(4)P, the dequenching was much faster, as PS was transferred more rapidly by Osh6p to L_B liposomes due to the counterexchange of PS with PI(4)P by the LTP (second curve). The third curve corresponds to an experiment in which NBD-$C2_{Lact}$ was mixed with equal amounts of $L_{A\text{-}Eq}$ and $L_{B\text{-}Eq}$ liposomes. The signal was higher than F_0 and corresponded to a situation where the probe was evenly bound to L_A and L_B liposomes, thus reflecting a situation where PS was fully equilibrated between the two populations of liposomes. F_{Eq} was calculated by averaging the value of the signal measured in the last 5 min of the experiment.

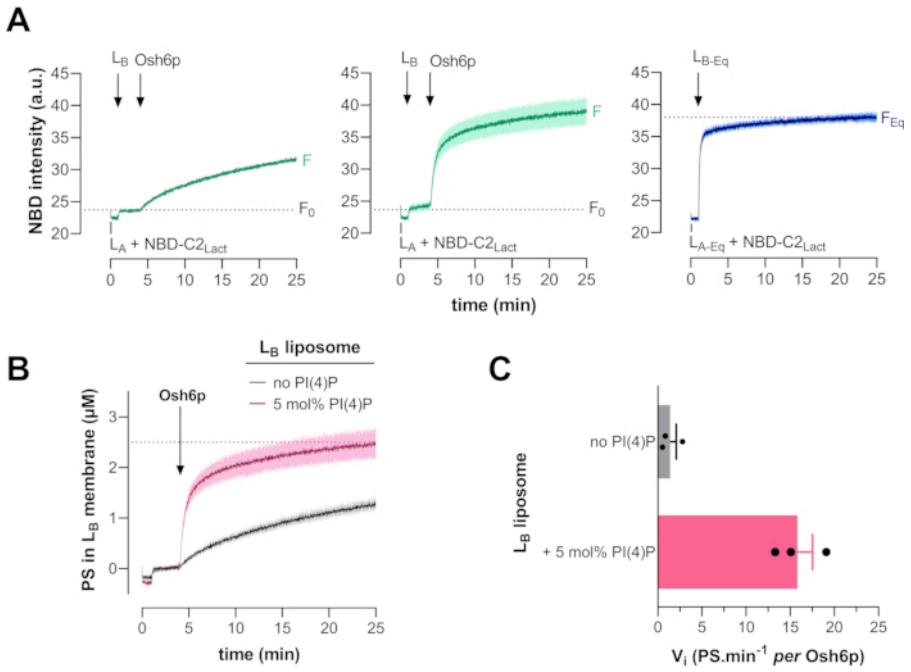

Figure 4: Typical PS transport kinetics measured with Osh6p and reference curve. (**A**) L_B liposomes devoid of PI(4)P (200 µM total lipids) and Osh6p (200 nM) were sequentially added to a cuvette containing NBD-C2$_{Lact}$ (250 nM) and L_A liposomes (200 µM) doped with 5 mol% PS and 2 mol% Rhod-PE (left curve). The same experiment was performed with L_B liposomes doped with 5 mol% PI(4)P (middle curve). To determine F_{Eq}, NBD-C2$_{Lact}$ (250 nM) was premixed with L_{A-Eq} liposomes; then, L_{B-Eq} liposomes were added (right curve). (**B**) Averaged PS transport curves determined after the normalization of several measurements done using L_B liposomes with or without PI(4)P (mean ± SEM, n=3). **(C)** Initial PS transfer rates (mean ± SEM, n=3). Abbreviations: NBD = 7-nitrobenz-2-oxa-1,3-diazol fluorophore; NBD-C2$_{Lact}$ = *N,N′*-dimethyl-*N*-(thioacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine moiety linked to the thiol function of C352 residue of a reengineered version of the C2 domain of bovine lactadherin (PDB ID: 3BN6[48]); PDB = Protein Data Bank; PS= phosphatidylserine; Osh6p = oxysterol-binding protein (OSBP) homolog 6 protein; PI(4)P = phosphatidylinositol 4-phosphate; Rhod-PE = rhodamine-labelled phosphatidylethanolamine; L_A liposomes = liposomes composed of phosphatidylcholine, doped with 2 mol% Rhod-PE, and containing or not 5 mol% PS; L_B liposomes = liposomes incorporating 5 mol% PI(4)P; F = fluorescence; F_0 = fluorescence corresponding to NBD before addition of Osh6p; F_{Eq} = fluorescence signal if PS is fully equilibrated between L_A and L_B liposomes by a transfer process; SEM = standard error of the mean. Please click here to view a larger version of this figure.

Figure 4B shows average kinetic curves of PS transfer from L_A liposomes to L_B liposomes, doped or not doped with PI(4)P after the normalization of F data using F_0 and F_{Eq} as reference values. The initial transport rate for each experiment was calculated by fitting the initial data points measured after the injection of the protein with a linear

function. **Figure 4C** shows the mean initial PS transport rate determined from three distinct experiments using L_B liposomes with or without PI(4)P. When L_B liposomes contained 0 and 5 mol% PI(4)P, the rates were respectively equal to 1.4 and 15.8 $PS.min^{-1}$ per Osh6p molecule. **Figure 5A** shows typical results from a PI(4)P transfer assay using Osh6p as an LTP, which was carried out with the same materials and conditions as those used for the PS transport assay.

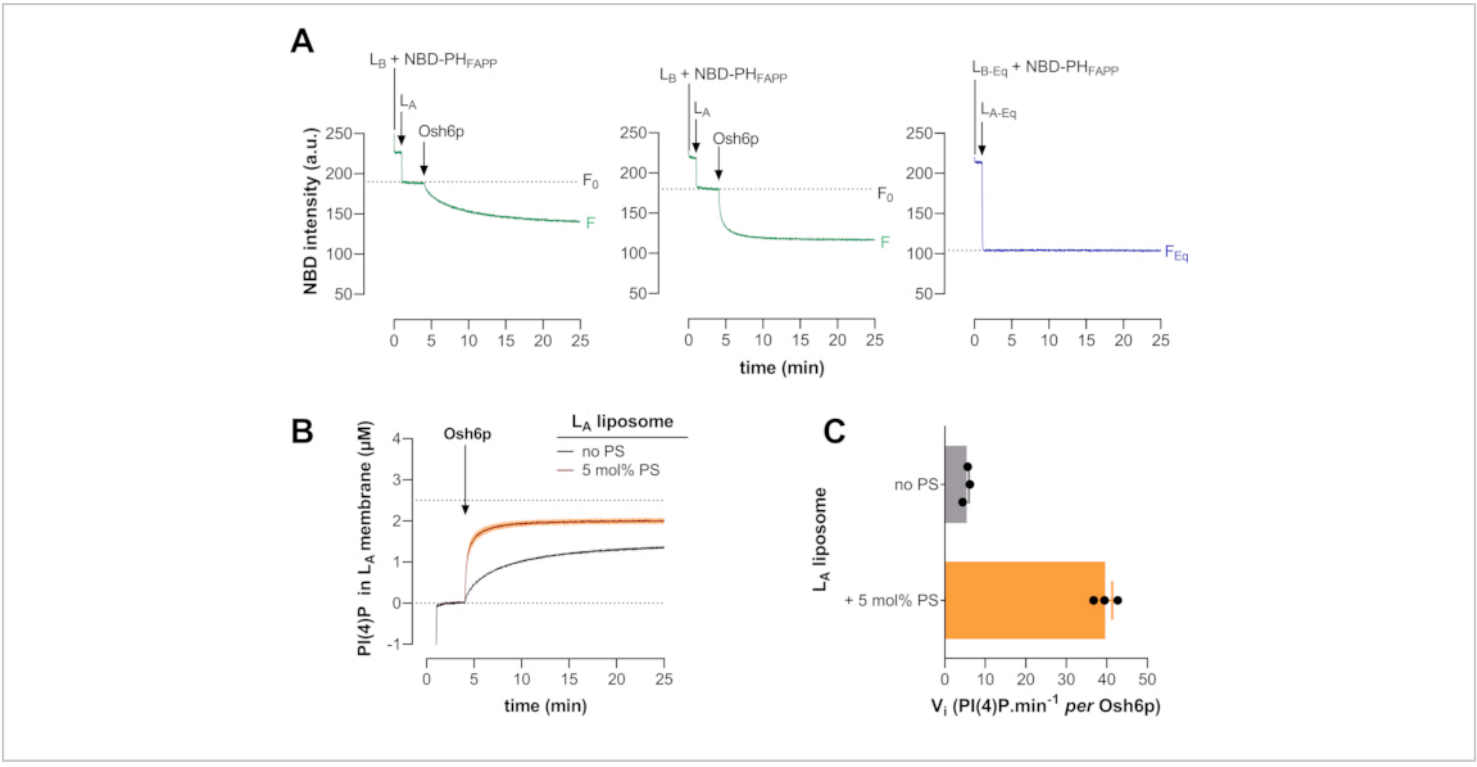

Figure 5: Typical PI(4)P transport kinetics measured with Osh6p and reference curve. (**A**) PS-free liposomes containing 2 mol% Rhod-PE (L_A, 200 µM total lipids) and Osh6p (200 nM) were sequentially added to a cuvette containing NBD-PH_{FAPP} (250 nM) and L_B liposomes (200 µM) doped with 5 mol% PI(4)P (left curve). The same experiment was performed with L_A liposomes doped with 5 mol% PS (middle curve). To determine F_{Eq}, NBD-PH_{FAPP} (250 nM) was premixed with L_{B-Eq} liposomes; then, L_{A-Eq} liposomes were added (right curve). (**B**) PI(4)P transfer kinetics determined after normalization of several measurements of L_A liposomes with or without PS (mean ± SEM, n=3). (**C**) Initial PI(4)P transfer rates (mean ± SEM, n=3). Abbreviations: NBD = 7-nitrobenz-2-oxa-1,3-diazol fluorophore; NBD-PH_{FAPP} = NBD-labeled Pleckstrin homology domain of the human four-phosphate-adaptor protein 1 (FAPP1, UniProt: Q9HB20, segment [1-100]); PS= phosphatidylserine; Osh6p = oxysterol-binding protein (OSBP) homolog 6 protein; PI(4)P = phosphatidylinositol 4-phosphate; Rhod-PE = rhodamine-labelled phosphatidylethanolamine; L_A liposomes = liposomes composed of phosphatidylcholine, doped with 2 mol% Rhod-PE, and containing or not 5 mol% PS; L_B liposomes = liposomes incorporating 5 mol% PI(4)P; F = fluorescence; F_0 = fluorescence corresponding to NBD before addition of Osh6p; F_{Eq} = fluorescence signal if PI(4)P is fully equilibrated between L_A and L_B liposomes by a transfer process; SEM = standard error of the mean. Please click here to view a larger version of this figure.

At time zero, NBD-PH_{FAPP} was mixed with L_B liposomes containing 5 mol% PI(4)P in a volume of 570 µL of HKM buffer. Because NBD-PH_{FAPP} was bound to L_B liposomes, its signal was high. After one minute, L_A liposomes (30 µL) were added, which was expected to only elicit a slight change in the signal. The intensity then corresponded to F_0. Injecting Osh6p (200 nM final concentration) into the reaction mix triggered a quenching of the NBD signal

due to the translocation of NBD-PH_{FAPP} molecules to L_A liposomes as PI(4)P was transferred from L_B liposomes to L_A liposomes. When L_A liposomes contained 5 mol% POPS, the dequenching was much faster owing to a faster PI(4)P transfer resulting from PS/PI(4)P exchange. The third curve corresponds to an experiment in which NBD-PH_{FAPP} was mixed with equal amounts of $L_{A\text{-}Eq}$ and $L_{B\text{-}Eq}$ liposomes.

The signal was lower than F_0 as it corresponded to a situation where the probe was uniformly bound to L_A and L_B liposomes, thus indicative of a full equilibration of PI(4)P between the liposomes. F_{Eq} was calculated by averaging the value of the signal measured in the last 5 min of the experiment. **Figure 5B,C** show averaged kinetics curves obtained after signal normalization and mean PI(4)P initial transfer rates measured with L_A liposomes that were doped or not doped with 5 mol% PS. Here, it is worth noting that both lipid ligands were transported faster and with similar velocities when each lipid was initially present in L_A and L_B membranes, respectively, which indicates that Osh6p is a PS/PI(4)P exchanger.

Discussion

The outcomes of these assays directly rely on the signals of the fluorescent lipid sensors. Thus, the purification of these probes labelled at a 1:1 ratio with NBD and without free NBD fluorophore contamination is a critical step in this protocol. It is also mandatory to check whether the LTP under examination is properly folded and not aggregated. The amount of LTP tested in the extraction assays must be equal to or higher than that of accessible PS or PI(4)P molecules to properly measure whether this LTP efficiently extracts these lipids. Indeed, NBD-$C2_{Lact}$ and NBD-PH_{FAPP} bind to PS and PI(4)P, respectively, following a classical saturation binding curve. Given their respective affinities for PS and PI(4)P, these probes remain largely bound to the liposomes even if the liposomes contain residual traces of these ligands. This can lead to the erroneous conclusion that an LTP does not efficiently extract these lipids. The protocol relies on standard and commercially available PC, PS, and PI(4)P subspecies that are 18:1/18:1-PC, 16:0/18:1-PS, and 16:0/16:0-PI(4)P, respectively. Performing experiments using lipid species with other acyl chains can give different results, as previously reported[12,35].

In the transfer assays, if the bulk lipid composition (not considering PS or PI(4)P) of L_A and L_B liposomes has to be modified, it is key to also perform the control experiments using $L_{A\text{-}Eq}$ and $L_{B\text{-}Eq}$ liposomes with a bulk composition similar to that of L_A and L_B liposomes, respectively. The same principle applies for the extraction assays. Genetically encoded fluorescent lipid-binding domains are broadly used to analyze lipid distribution inside cells[44]. Caution is recommended when analyzing such experiments as the association of these lipid probes with the membrane, although primarily driven by the presence of the targeted lipid, can be influenced by other parameters (density of anionic lipids, lipid-packing) that differ between cell compartments. In these *in vitro* assays, the composition of liposomes is much simpler than that of cellular membranes: they are mostly made of PC, a zwitterionic lipid, and thereby expose a rather inert surface that does not impact how PS and PI(4)P are recognized by their corresponding sensor. PH_{FAPP} can be used in the presence of anionic lipids, such as PS, phosphatidic acid (PA), and PI[32], and $C2_{Lact}$ does not recognize PI(4)P[12]; both these observations allow unbiased measurements of PS/PI(4)P exchange. NBD-PH_{FAPP} is not influenced by sterol[43].

However, it is not known whether these probes, in particular, NBD-$C2_{Lact}$, can be used with liposomes with

extreme features (*e.g.,* very low or high lipid-packing, highly negatively charged surface, presence of lipid domains). Despite this potential limitation with respect to liposome composition, these transfer assays based on fluorescent sensors have tremendous advantages compared to other methods. First, they do not rely on fluorescently labeled PS and PI(4)P that bear an extra bulky group and are likely not properly accommodated by the binding pocket of LTPs. Second, these assays offer far better time resolution than methods based on liposome separation (radioactivity-based assays[45] or mass spectrometry[33]), and it should be noted that radiolabeled PI(4)P and PS are not commercially available. The capacity of a protein to transfer PS or PI(4)P is not impacted by the association of the fluorescent sensors with liposomes. Indeed, if the amount of NBD-$C2_{Lact}$ or NBD-PH_{FAPP} and that of PS and PI(4)P in the outer leaflet of liposomes that is accessible to the LTP is considered, only 5% of PS or PI(4)P are associated with probe during a kinetics measurement.

Moreover, only 0.55-0.86% of the membrane surface is covered by the probe, considering the total surface of liposomes (L_A+L_B liposomes; 5.1 × 10^{16} nm^2 with an area of 0.7 nm^2 per lipid), the membrane surface that one individual C2 or PH molecule can occupy (≈3.1 and 4.8 nm^2, respectively, estimated from references[46,47,48]) and their concentration (250 nM). Thus, the principles underlying these assays are adapted to properly analyze the kinetic and mechanistic aspects of LTPs. As mentioned in the introduction, alterations in the activity of ORP5/8 can lead to cellular dysfunctions[49]. For example, the invasiveness of pancreatic cancer cells seems to rely on the level of ORP5 expression. Moreover, a causality exists between high levels of ORP5 expression and the poor prognosis of human pancreatic cancer. A high level of ORP5 expression is also detected in lung tumor tissues, and more particularly, in metastatic cases. Interestingly, the ability of ORP5 to transfer lipids might explain why it promotes cell proliferation and migration.

ORP5 upregulates the mammalian target of rapamycin complex 1 (mTORC1) complex, which plays a central role in cell survival and proliferation, probably because it enhances the activity of Akt, an upstream activator of mTORC1, by supplying the PM with PS. Overall, these observations suggest that ORP5 might be an interesting pharmacological target, and that these *in vitro* assays might serve to screen molecules that are able to inhibit its activity. This assay can also help to better define whether other members of the ORP/Osh family are PS/PI(4)P exchangers. Notably, ORP10 was found to encapsulate PS *in vitro* and transfer PS in ER-Golgi contact sites[27,50], but it is still unknown whether it acts as a PS/PI(4)P exchanger. Furthermore, these protocols can serve to explore the ability of LTPs belonging to other families to transport PI(4)P, as was recently shown with steroidogenic acute regulatory transfer-like proteins[10], or PS. Additionally, NBD-PH_{FAPP} has been used to follow the ability of LTP to transport PI(4,5)P_2 between liposomes, as it recognizes this PIP[10,35]. Finally, this strategy could be adapted to measure other extraction or transport processes *in vitro* by using other lipid-binding domains (*e.g.,* a non-catalytic PI-PLC to detect PI[51], sporulation-specific protein 20 fragment to detect PA[52], domain 4 (D4) of perfringolysin O to detect sterol[53]).

Disclosures

The authors declare that there are no conflicts of interest.

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