Flip-Flop of Fluorescently Labeled Phospholipids in Proteoliposomes Reconstituted with Saccharomyces cerevisiae Microsomal Proteins

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A phospholipid flippase activity from the endoplasmic reticulum (ER) of the model organism Saccharomyces cerevisiae has been characterized and functionally reconstituted into proteoliposomes. Analysis of the transbilayer movement of acyl-7-nitrobenz-2-oxa-1,3-diazol-4-yl (acyl-NBD)-labeled phosphatidylcholine in yeast microsomes using a fluorescence stopped-flow back exchange assay revealed a rapid, ATP-independent flip-flop (half-time, <2 min). Proteoliposomes prepared from a Triton X-100 extract of yeast microsomal membranes were also capable of flipping NBD-labeled phospholipid analogues rapidly in an ATP-independent fashion. Flippase activity was sensitive to the protein modification reagents N-ethylmaleimide and diethylpyrocarbonate. Resolution of the Triton X-100 extract by velocity gradient centrifugation resulted in the identification of a ~4S protein fraction enriched in flippase activity as well as of other fractions where flippase activity was depleted or undetectable. We estimate that flippase activity is due to a protein(s) representing ~2% (w/w) of proteins in the Triton X-100 extract. These results indicate that specific proteins are required to facilitate ATP-independent phospholipid flip-flop in the ER and that their identification is feasible. The architecture of the ER protein translocon suggests that it could account for the flippase activity in the ER. We tested this hypothesis using microsomes prepared from a temperature-sensitive yeast mutant in which the major translocon component, Sec61p, was quantitatively depleted. We found that the protein translocon is not required for transbilayer movement of phospholipids across the ER. Our work defines yeast as a promising model system for future attempts to identify the ER phospholipid flippase and to test and purify candidate flippases.
siue and mammalian cells, the tools of yeast genetics and molecular biology offer promising means to identify and dissect molecular components involved in the flipping process. A single report documents the ability to reconstitute flipping of in situ-labeled phosphatidylethanolamine (PE) in proteoliposomes prepared from a detergent extract of yeast microsomes (28). Extending this approach to investigate membrane proteins that might play a role in the transblayer movement of phospholipids in the ER, we now describe the results of experiments in which we assayed flip-flop activity in yeast microsomal membranes as well as in proteoliposomes reconstituted from a detergent extract of yeast microsomes. We used 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled short-chain phospholipid analogues as probes to monitor flip-flop, making use of their ability to be extracted from the membrane by bovine serum albumin (BSA) as well as to be chemically reduced by dithionite, a membrane-impermeant dianion. Our experiments reveal fast flip-flop of NBD-phospholipids (NBD-PLs) in yeast microsomes and demonstrate that flipping is facilitated by a protein(s) that sediments operationally at ~4S and whose activity is compromised by treatment with the protein modification reagents N-ethylmaleimide (NEM) and diethylpyrocarbonate (DEPC). Fractionation of the detergent extract prior to reconstitution yielded protein mixtures enriched in flip-flop activity and other fractions devoid of activity, indicating unambiguously that a specific protein (or a specific subset of ER proteins) is required to facilitate flip-flop. We also show clearly that the protein translocon, a promising flip-flop candidate, is not required for the efficient transblayer movement of phospholipids across the ER. Our results define yeast as a promising model system for future attempts to identify flip-flop proteins by isolating and probing candidate proteins.

MATERIALS AND METHODS

Lipids. Egg phosphatidylcholine (egg PC) and all NBD-PLs were purchased from Avanti Polar Lipids (Alabaster, AL).

Yeast strains and culture conditions. S. cerevisiae strain sce61 (MATa ura3-52 leu2-3,112 ade2-10 sec61ts), obtained from J. Holteus (Utrecht University, Utrecht, The Netherlands), was used. Cells were grown at 27°C in liquid YPD buffer T supplemented with 100 mM NaCl. Immunoblot analysis was performed by immunoblotting. Pellets from the 100,000 g av supernatant (S100) in homogenizing buffer) and membrane pellets were resuspended in buffer T supplemented with 100 mM NaCl. Immunoblot analysis was performed using antibodies against Dpm1p (Molecular Probes), Aac2p (H. Rottensteiner, Bochum University, Germany), Yae4p (M. Veit, Free University Berlin, Germany), Tgl2p (J. Holtius, Utrecht University, Utrecht, The Netherlands), Sec61p and Sec62p (T. Sommer, Max-Delbrück-Center Berlin, Berlin, Germany), and Wbp1p (35). Protein blots were probed with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad), which were detected using ECL (Amersham). Chemiluminescent bands were quantified using a GS-710 calibrat- ing imaging densitometer (Bio-Rad) with Quantity One software. The intactness of the microsomes (6 mg of protein/ml) was tested by a protease protection assay using trypsin (0.25 mg/ml). After termination of protease digestion by addition of a trypsin inhibitor (1 mg/ml), samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Preparation and fractionation of the Triton X-100 extract. Fp100,000 microsomal membranes (20 mg/ml protein) were solubilized by diluting with an equal volume of 6% (wt/vol) Triton X-100 (Roche Diagnostics) in buffer T. After centrifugation for 45 min on ice, insoluble material was removed by centrifugation (177,000 × g av, 30 min, 4°C), resulting in a supernatant fraction designated the Triton extract (TE). By this procedure, 10 to 20% of the protein and 90% of the phospholipid were solubilized from the microsomes. For TE fractionation, a linear gradient of 10 to 35% (wt/vol) glycerol in buffer TX (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.8% [wt/vol] Triton X-100) was prepared, loaded with 250 µl TE, and centrifuged to equilibrium in a swing-out rotor (8 km). Fractions (300 µl) were collected, pooled pairwise, discarding the first one and the very bottom part. The obtained samples I to VI (60 µl each) were desalted using BioGel-P6 spin columns (200 µl sample per 1 ml [bed volume] of resin [Bio-Rad] to remove glycerol; aliquots of each sample were taken for protein determination and SDS-PAGE analysis before the majority of the material was used for reconstitution and flip-flop activity measurement. Sedimentation standards (150 µg each of ovalbumin [36], BSA [4.2S], β-amylase [8S], and catalase [11S]) were loaded onto a parallel gradient; fractions (300 µl) were harvested from the gradient and ana- lyzed by SDS-PAGE and Coomassie staining to locate the migration positions of the sedimentation standards.

Reconstitution of liposomes and proteoliposomes. Proteoliposomes were pre- pared from a mixture of crude or fractionated TE and Triton X-100-solubilized egg PC and NBD-PL as previously described (26). Briefly, a mixture of egg PC (4.5 µmol) and NBD-PL (22.5 nmol or 0.5 mol% of total phospholipid) in chloroform was dried under nitrogen in a glass screw-cap tube and then dissolved in buffer TX. Various amounts of TE or TE-derived fractions were added to generate proteoliposomes with different protein/phospholipid ratios (PPR). The proportion of reconstituted protein correlated linearly with the amount of TE used for the reconstitution (data not shown, but see reference 10). The final phospholipid concentration was 4.5 µmol. Protein-free liposomes were prepared similarly by replacing TE with buffer TX. To remove detergent and generate vesicles, 40 mg of SM (no TE) or 50 mg of TE (Bio-Rad) was added. After 3 to 4 h of rotation at room temperature, the samples were supplemented with an additional 200 mg/ml of Bio-Beads and then transferred to 4°C for a further 14 to 16 h of mixing. The resulting turbid suspensions were withdrawn carefully to avoid collecting any beads and were adjusted to an 8 to 10-fold excess of buffer T. The vesicles were collected by centrifugation (200,000 × g av, 50 min, 4°C) and reus- pended at 9 mM phospholipid using a Dounce homogenizer. For some experi- ments, the vesicle suspension was used directly after removal of the Bio-Beads, without centrifugation and resuspension. Protein recovery in the reconstituted vesicles was ~40%; phospholipid recovery was 75 to 90%. More than 99.98% of the initial Triton X-100 amount was removed, as determined by extraction with 4 volumes of chloroform-methanol (1/2, vol/vol) and measurement of the absorbance of the supernatant at 275 nm (15).

Protein modification. The sensitivity of flip-flop activity to protein modification was assessed by treating proteoliposomes with proteasome K as described previously (38). NBD-PL and NBD-PL-supplemented natural samples were treated as described previously (10) except that 200 mM stock solutions or reagent was used (freshly prepared in 10 mM HEPES-NaOH [pH 7.4]–100 mM NaCl) and incubations were carried out for 45 min at room temperature.

Preparation and labeling of liposomes. Egg PC dissolved in chloroform-meth- anol (1:1, vol/vol) was mixed with NBD-PLs (1 mol%) and dried under a stream of nitrogen. The dried lipid film containing NBD-PLs was hydrated by vortexing in buffer T supplemented with 0.5 µg of protein/ml NaCl to give a final lipid concentration of 1 mM. The resulting aqueous phospholipid dispersion was subjected to five freeze-thaw cycles and was then extruded 10 times through 200-nm-pore-size polycarbonate filters (excluder from Lipex Biomembranes; filters from Costar Nuclepore). This procedure gives liposomes with a symmetrical transblayer distribution of NBD-PLs. To prepare vesicles with NBD-PLs located exclusively...
on the outer leaflet (asymmetrically labeled vesicles), liposomes were labeled after preparation.

**Stopped-flow analysis of C₆-NBD-PC flip-flop.** Microsomes were adjusted to a phospholipid concentration of 1 mM in buffer T supplemented with 100 mM NaCl. Aliquots were incubated at room temperature with 1-palmitoyl-2-C₆-NBD-PC (added from stock solutions of 160 μM in buffer T supplemented with 100 mM NaCl) for 30 or 90 min such that the final amount of the NBD-PL was ~1 mol% of total phospholipid. Fluorescence measurements were performed in quartz cuvettes at 20°C using an Aminco Bowman series 2 spectrofluorometer (SLM Instruments, Rochester, NY) equipped with a stopped-flow accessory (RX 1000; Applied Photophysics, Leatherhead, United Kingdom).

The transfer of C₆-NBD-PC from the aqueous phase into the membranes of microsomes was determined by monitoring the increase in fluorescence emission at 540 nm (excitation, 470 nm; slit widths, 4 nm; resolution, 1 s); insertion was essentially complete within 2 min. In order to determine background scattering and efficiency of label extraction from membranes by BSA, emission scans were recorded (wavelength, 490 to 600 nm; excitation, 470 nm; slit widths, 4 nm) and values at 540 nm for two independent experiments were taken for quantification. For stopped-flow measurements, a 100-μl aliquot of labeled vesicles was mixed with an equal volume of fatty acid-free BSA dissolved in the same buffer by simultaneous injection into the chamber of a stopped-flow accessory to give final concentrations of 1% (wt/vol) BSA and 75 μM membrane phospholipid containing 1 mol% of NBD-PL. Fluorescence decay traces were recorded at 540 nm for 500 s (excitation, 470 nm; slit widths, 4 nm; resolution, 1 s).

**Kinetic analysis.** The stopped-flow data were evaluated according to a three-compartment model as described previously (20, 23). This kinetic model describes transbilayer movement as well as the transfer of phospholipid analogues between the outer leaflet of the membrane vesicle and BSA. The outward and inward movements of phospholipid analogues are described by the rate constants $k_{1}$ and $k_{-1}$, respectively. The movement of the analogues from microsomal membranes to BSA is characterized by the rate constant $k_{e}$ (extraction of the analogues by BSA), and $k_{-e}$ describes the movement of analogues back from BSA to the vesicle membrane. As previously shown (20, 23), due to the excess of BSA used, the exchange process described by $k_{-e}$ did not contribute to the kinetics, and the values for this time constant were very small (typically 10⁻¹² s⁻¹), as expected. Furthermore, being aware that the phospholipid analogues used are partially water soluble, one has to consider that analogues partition between membranes and the aqueous compartments, including the vesicular lumen. However, since the analogue concentration is very low with respect to that of micromolar lipids or nonlabeled lipids (reconstitution experiments), the fraction of analogues in the aqueous compartments is negligible. This was verified by measuring the NBD fluorescence associated with the microsomes and supernatant after centrifugation and addition of Triton X-100, showing that essentially all analogues were incorporated into membranes. Therefore, the following model can be applied.

$$[\text{PL}_{\text{ext}}] = [\text{PL}_{\text{int}}] + [\text{PL}_{\text{in}}] + [\text{PL}_{\text{out}}]$$

The concentration of analogue transferred to BSA ([PL]ₑ) is taken to be zero at the time of addition. The model is represented by the following system of differential equations:

$$d[\text{PL}]_t = -k_{1}[\text{PL}]_t + k_{-1}[\text{PL}]_e$$

$$d[\text{PL}]_t = k_{-1}[\text{PL}]_e + (k_{1} + k_{2})[\text{PL}]_t + k_{-2}[\text{PL}]_o$$

$$d[\text{PL}]_o = -k_{1}[\text{PL}]_e - k_{-2}[\text{PL}]_o$$

$$[\text{PL}]_e = C - [\text{PL}]_t - [\text{PL}]_o$$

Experimental data were fitted to the model by the Marquardt-Levenberg algorithm using SigmaPlot for Windows (Systat Software, Inc.). This algorithm seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable (fluorescence intensity). Three time traces of two independent experiments were fitted in order to derive rate constants.

**Analysis of NBD-PL flip-flop by albumin extraction.** To assay flip-flop of 1-myristoyl-2-C₆-NBD-PL in reconstituted vesicle preparations by albumin extraction, the fluorescence of NBD-containing liposomes or proteoliposomes was measured at 540 nm (excitation, 470 nm; slit width, 4 nm) before and after BSA treatment for 5 min (final concentration, 2 mg/ml). Fluorescence was measured at 25°C using quartz microcuvettes and an Aminco Bowman series 2 spectrofluorometer (SLM Instruments, Rochester, NY). The accessible pool of fluorescent lipid ([PL]ₑ) was calculated as

$$\frac{[\text{F}]_{\text{BSA}}}{{[\text{F}]_{\text{BSA}}} + 1/[1 - 0.55]} \times 100$$

where [F]_{BSA} is the plateau value of fluorescence of vesicles after extraction with BSA, $F_{\text{BSA}}$ is the initial fluorescence of vesicles in buffer without BSA, and $F_{\text{BSA}}$ is the proportion of labeled lipid that is extracted by BSA, and the relative quantum yield of BSA-bound fluorescent lipid is 0.55 compared with a value of 1 for membrane-associated fluorescent lipid (20).

**Analysis of NBD-lipid flip-flop by dithionite reduction.** To assay the flip-flop of NBD-PLs (1-myristoyl-2-C₆-NBD-PC, 1-acetyl-2-C₆-NBD-PC, 1-acetyl-2-C₁₂-NBD-PC, and PE) in reconstituted vesicle preparations by dithionite reduction (10, 38), the fluorescence of NBD-containing liposomes or proteoliposomes was measured as for BSA extraction, except that instead of BSA, 1 M sodium dithionite (freshly dissolved in 1 M Tris base) was added to a final concentration of 5 mM. For complete reduction of all analogues by dithionite, Triton X-100 was then added to a final concentration of 0.5% (wt/vol). The percentage of NBD-PL that was reduced ($P_{\text{red}}$) was calculated as

$$\frac{[\text{F}]_{\text{BSA}}}{[\text{F}]_{\text{BSA}} + [\text{F}]_{\text{dithionite}}} \times 100$$

where $F_{\text{dithionite}}$ is the initial fluorescence of vesicles in buffer without dithionite, and $F_{\text{BSA}}$ is the plateau value of fluorescence of vesicles after incubation with dithionite for 5 min, and $F_{\text{BSA}}$ is the final fluorescence after addition of Triton X-100.

**Collisional quenching experiments.** To assay the steady-state distribution of the NBD-PLs across the membranes of liposomes and proteoliposomes, collisional quenching of NBD probes with potassium iodide (KI) was performed according to the method of Lakowicz (21) as follows. NBD-PL-containing liposomes or proteoliposomes were reconstituted using buffer TX supplemented with 0.25 M KCl. Excitation and emission wavelengths were set at 470 and 530 nm, respectively. Fluorescence intensity was measured for samples consisting of 50 μl of vesicles diluted into 1.95 ml of buffer containing 10 mM HEPES-NaOH (pH 7.4), 40 mM Na₂S₂O₃, and the quencher KI (0 to 0.25 M, as indicated); KCl was used to adjust the ionic strength to 0.25 M so that the osmolarity of the solution inside and outside the vesicles was the same. Na₂S₂O₃ was included to prevent production of I₂ by keeping the iodide ion reduced; it had no detectable effect on NBD fluorescence. Parallel samples were measured in which KCl was used instead of KI. Data were analyzed according to the modified Stern-Volmer equation (21): $F_o/F = (1 + K_o [Q]) + (1 + K_o [Q])$, where $F_o$ is the fluorescence intensity in the absence of the quencher, $F$ is the fluorescence intensity in the presence of the quencher at concentration [Q], $K_o$ is the fraction of fluorescence which is accessible to the quencher, and $K$ is the Stern-Volmer quenching constant.

**Other methods.** Protein content was quantified following trichloroacetic acid precipitation or delipidation according to the procedure of Wessel and Flugge (40) using either modified Lowry reagent (Sigma-Aldrich) or Micro BCA protein assay reagent (Pierce, Rockford, IL) with BSA as a standard. In some experiments, the Kaplan-Pedersen method for protein determination was used (16). The phospholipid content was determined by extracting lipids according to the method of Bligh and Dyer (7) and measuring the amount of phosphorus (53). NBD-PLs were checked for purity and stability during the course of reconstitution experiments by thin-layer chromatography on Silica Gel 60 plates (Merck, Darmstadt, Germany) using chloroform-methanol-water (65/25/4, vol/vol) as the solvent system.

**RESULTS AND DISCUSSION**

**Flip-flop of a fluorescent PC analogue in yeast microsomes.** Yeast microsomes were isolated by differential centrifugation and characterized by immunoblotting using antibodies directed against organelle-specific proteins. Figure 1A shows that the P₁₀₀ microsome fraction is enriched in ER membranes; it contains the ER marker Dpm1p but is almost entirely depleted of mitochondrial (Aac2p), vacular (Vac8p), and Golgi (Tlg2p) markers. The integrity of the ER vesicles in the P₁₀₀ fraction was determined by testing the susceptibilities of ER membrane protein markers (Wbp1p and Dpm1p) to exogenously added trypsin in the presence or absence of detergent. Wbp1p is a type I ER-resident membrane protein with a large luminal domain and a small cytoplasmic tail (17); in contrast, the bulk of the Dpm1p protein is oriented toward the cytoplasm (29).
liposomes, the fluorescence rapidly decreased to 75% of the initial value (Fig. 2B, trace a), indicating complete extraction of C6-NBD-PC by BSA. For symmetrically labeled liposomes, fluorescence rapidly decreased to 50% of the initial value (Fig. 2B, trace b), consistent with the extraction of ~50% of the analogues by BSA (C6-NBD-PC molecules residing in the inner leaflet of the liposomes cannot be extracted, since phospholipid flip-flop does not occur in liposomes during the time scale of the experiment [6]). The fluorescence decrease in both cases could be described well by a monoeXponential function, consistent with a single rate process, i.e., the essentially irreversible extraction of C6-NBD-PC from the outer leaflet of the vesicles by BSA.

The luminal domain of Wbp1p remained unaffected when trypsin was added to P100 vesicles, while Dpm1p was rapidly degraded under the same conditions (Fig. 1B). Both proteins were cleaved when trypsin was added to vesicles that had been permeabilized with Triton X-100. These results indicate that ER vesicles in the P100 fraction are sealed and right side out.

To measure phospholipid flip-flop in the P100 vesicles, we used NBD-PLs with a short C6 acyl chain in the sn-2 position. C6-NBD-PLs are readily extracted from membranes by defatted BSA, and since the quantum yield of BSA-bound NBD-PLs (20), extraction is readily monitored by following the decrease in the fluorescence intensity of the sample. In preliminary experiments we prepared asymmetrically labeled liposomes in which C6-NBD-PC was located exclusively in the outer leaflet, as well as symmetrically labeled liposomes that contained C6-NBD-PC in both membrane leaflets. We used a stopped-flow accessory to rapidly mix defatted BSA with the liposome preparations and measured the kinetics of fluorescence decay. When BSA was added in excess to asymmetrically labeled liposomes, the fluorescence rapidly decreased to ~50% of the initial value (Fig. 2B, trace a), indicating complete extraction of C6-NBD-PC by BSA. For symmetrically labeled liposomes (C6-NBD-PC molecules residing in the inner leaflet of the liposomes cannot be extracted, since phospholipid flip-flop does not occur in liposomes during the time
We next labeled P100 vesicles with C6-NBD-PC for 30 min and used stopped-flow kinetic analysis to determine the accessibility of the NBD-PL to BSA extraction. Upon mixing of the labeled P100 vesicles with BSA, the fluorescence intensity dropped to ~50% of its initial value, indicating complete extraction of C6-NBD-PC by BSA (Fig. 2B, trace c). However, in contrast to that for asymmetrically labeled liposomes, the decay of fluorescence intensity occurred more slowly and displayed biphasic kinetics. We interpret these data as follows.

During the 30-min labeling period, C6-NBD-PC equilibrates across the two leaflets of the P100 membrane vesicles as a result of the action of the ER phospholipid flippase. This results in symmetrically labeled vesicles. Upon incubation of the labeled vesicles with BSA, the pool of C6-NBD-PC molecules in the outer leaflet is rapidly extracted; however, C6-NBD-PC molecules initially located in the inner leaflet must first be translocated across the membrane before gaining access to BSA. The rate of transbilayer translocation is lower than the rate of NBD-PL extraction by BSA, as also found for other membranes (20, 23, 38), accounting for the second (slower) phase of fluorescence decay. Although the data in Fig. 2B (trace c) refer to measurements on P100 vesicles labeled for 30 min with C6-NBD-PC, essentially the same results were obtained with vesicles labeled for 90 min. This indicates that the NBD-PLs are already equilibrated between the two leaflets of the microsomal membrane after 30 min of labeling. To determine the half-time of NBD-PL flip-flop in microsomes, the data were fitted to a three-compartment model as diagramed in Fig. 2A (residuals for the fit to the model versus a monoeponential fit are shown in Fig. 2C). The results (Table 1) indicate that C6-NBD-PC is rapidly translocated across yeast microsomal membranes (half-time, <2 min) and that the rate of flipping resembles that seen for C6-NBD-PC and -PE in rat liver microsomes (23, 38). The movement of C6-NBD-PC to the luminal leaflet of the microsomes was slightly slower than that to the cytoplasmic layer. This difference might be related to the insensitivity of the stopped-flow assay or to a slightly nonsymmetric transbilayer distribution of the C6-NBD-PC.

Reconstitution of phospholipid flippase activity in proteoliposomes generated from Triton X-100-solubilized P100 microsomal membranes. To further characterize the yeast ER phospholipid flippase activity, we reconstituted flippase activity according to previously described procedures (10, 11, 15, 20, 22, 26, 38, 39). P100 microsomal membranes were solubilized in Triton X-100 to generate TE. Different amounts of TE were combined with Triton X-100-solubilized egg PC and trace quantities of NBD-PL (0.5 mol%). The solution was then treated with SM2 Bio-Beads to remove detergent and form proteoliposomes. Liposomes lacking protein were prepared in parallel by the same method except that TE was omitted. We used NBD-PLs with either a C6* or a C12-NBD-labeled fatty acyl chain at the sn-2 position. As reported previously, we found that the majority of vesicles formed by this procedure are unilamellar (data not shown) (15, 22, 26). Liposomes had an average diameter of ~150 nm, while proteoliposomes displayed a larger average diameter of ~175 nm. A comparison of the protein profiles of TE versus proteoliposomes by SDS-PAGE and silver staining revealed that microsomal membrane proteins in the TE were represented in the reconstituted vesicles without apparent bias (Fig. 3A).

Before proceeding with the flippase assay, we tested whether the NBD-PLs were incorporated symmetrically into the liposomal and proteoliposomal membranes. To do this, we used a collisional quenching approach in which membrane-impermeant iodide ions were used to eliminate the fluorescence contribution of NBD-PLs located in the outer (accessible) leaflets of the vesicles. We note that the collisional quenching approach reports on the pool size of NBD-PLs in the outer leaflets of the vesicles and does not provide information on whether or not NBD-PL flipping occurs in the vesicles. We first

| Organism and NBD-PL | Outward movement (s) | Inward movement (s) |
|---------------------|----------------------|---------------------|
| Yeast C6-NBD-PC     | 103 ± 20             | 176 ± 39            |
| Rat liver           |                      |                     |
| C6-NBD-PC           | 98 ± 9               | 148 ± 12            |
| C6-NBD-PE           | 81 ± 8               | 62 ± 4              |

* Experimental traces (see Fig. 2) were fitted according to the model to obtain the rate constants of the outward and inward movements of phospholipid analogues, k1 and k−1, respectively (see Materials and Methods). Half-times of the outward and inward movements across the ER membrane were derived from k1 and k−1, respectively, according to the formula t1/2 = (ln2)/k, where t1/2 is the half-time. Data shown are means ± standard deviations for two independent experiments. For each experiment, three single kinetics were averaged and subsequently analyzed.

* Data are from Marx et al. (23).

FIG. 3. Characterization of reconstituted proteoliposomes. Proteoliposomes were reconstituted from mixtures of Triton X-100-solubilized egg PC and TE; liposomes were prepared similarly except that TE was omitted. (A) Silver-stained SDS-PAGE (12% acrylamide) gel of TE and reconstituted proteoliposomes (P). Molecular mass markers are indicated on the left. Samples of TE (30 μl) and proteoliposomes (prepared from 60 μl TE) were precipitated with trichloroacetic acid and washed with acetone before being dissolved in an SDS-containing sample buffer for PAGE analysis. (B) Collisional quenching of NBD fluorescence with iodide ions to determine the fraction of NBD-PL accessible on the outer leaflets of vesicles. Proteoliposomes (open squares; protein/phospholipid ratio, 4.5 mg/mmol) and liposomes (open circles) were reconstituted from Triton X-100-solubilized mixtures containing C12-NBD-PC. Asymmetrically labeled liposomes (filled circles) were prepared by adding C12-NBD-PC to preformed vesicles. The data are presented as modified Stern-Volmer plots (see Materials and Methods). F0, is the fluorescence intensity of the sample in the absence of quencher, whereas ΔF is the fluorescence intensity at a given iodide ion concentration. The connecting lines were obtained by linear regression. The inverse of the y-intercept represents the fraction of C12-NBD-PC that is accessible to the quencher.
tested asymmetrically labeled liposomes generated by addition of NBD-PC after vesicle preparation. Analysis of the quenching data via a modified Stern-Volmer plot (21) (the fraction of NBD-PL accessible to iodide quenching is calculated as the inverse of the y-intercept) indicated that the majority (>80%) of the NBD-PC in these vesicles was accessible to iodide ions, as expected (Fig. 3B). For “symmetrically labeled” liposomes and proteoliposomes generated by our reconstitution procedure, ~50% and ~56% of NBD-PC, respectively, was accessible to iodide ions. We conclude that the NBD-PLs are symmetrically distributed across the membranes of the reconstituted vesicles and that this distribution is largely unaffected by the presence of membrane proteins.

Next, C6-NBD-PC-containing proteoliposomes were assayed for flippase activity by using defatted BSA to extract the NBD-PLs from the outer leaflets of the vesicles. On liposomes, BSA depleted ~50% of the C6-NBD-PC, consistent with a symmetric distribution of the NBD-PL across the liposomal membrane and the absence of lipid flip-flop in protein-free vesicles (Fig. 4A). In similar experiments carried out with proteoliposomes, a larger fraction of C6-NBD-PC was accessible to BSA, suggesting that C6-NBD-PC molecules located in the inner leaflets of proteoliposomes can be translocated to the outer leaflets, where they are extracted by BSA. The accessible proportion of C6-NBD-PC increased as the PPR of the vesicle preparation increased until a plateau maximum was reached at a PPR of ~10 mg/mmol (determined by fitting the dose-response data with two linear segments as described in reference 11). This can be explained as follows. As the PPR increases, the number of flippase-competent vesicles in the sample increases to a stage where each vesicle in the ensemble is equipped at least with a single flippase. At this point (corresponding to a PPR of ~10 mg/mmol), all NBD-PLs in the preparation should be accessible to BSA. In practice, more than 90% of the NBD-PL is extracted from proteoliposome samples prepared at a PPR of >10 mg/mmol.

As an additional test to establish that proteoliposomes derived from TE and egg PC are capable of flipping NBD-PLs, we used a different assay procedure involving dithionite, a membrane-impermeant dianion that reduces the NBD moiety and destroys its fluorescence. Upon addition of dithionite to NBD-PL-containing liposomes, fluorescence was reduced by ~50%, whereas for proteoliposomes the accessible proportion of NBD-PLs increased as the PPR of the vesicle preparation increased, irrespective of the PPR used as a transport reporter (Fig. 4B). These data reinforce our conclusion that ER-derived proteoliposomes are competent to flip NBD-PLs.

Both the BSA and dithionite assays displayed the same dependence of transport amplitude on PPR, with an inflection point at a PPR of ~10 mg/mmol. We note that while the dithionite assay displays a built-in efficiency of ~70 to 75%, the BSA assay approaches the theoretically predicted range of transport amplitudes (i.e., 50% for liposomes and ~90% for flippase-equipped proteoliposomes). The reason for this is unclear, but similar results have been reported previously in studies of outward translocation of NBD lipids in membrane vesicles (10, 38) and in assays of outward translocation of natural phospholipids (11). We established that dithionite itself had no unduly minimizing effect on flippase activity, since treatment of microsomes with dithionite prior to solubilization did not alter flippase activity in reconstituted vesicles (data not shown). The PPR at which the reduction amplitude reaches its maximum can be used to infer that flippase constitutes ~2% by weight of proteins in the TE (see reference 26 for details of the calculation). This is higher than the estimate of flippase abundance in TE from rat liver ER (20), possibly reflecting selective solubilization of the flippase in cold Triton X-100 (2).

Effects of protein modification reagents on flippase activity. To confirm that a membrane protein(s) is required for NBD-PL flip-flop in reconstituted vesicles, the effects of the protein modification reagents NEM (directed at cysteine residues [3, 24]) and DEPC (directed at histidine residues [27]) were tested. Treatment of TE with these reagents prior to reconstitution of proteoliposomes (prepared with PPR in the range of 2.9 to 4.1 g/mol, providing for vesicles with a maximum of 1 flippase) resulted in ~60% and ~40% losses of flippase-active vesicles, respectively (the PPR of inhibitor-

![FIG. 4. Reconstitution of phospholipid flippase activity in proteoliposomes. Vesicles were prepared from Triton X-100-solubilized egg PC, TE, and either 1-C14-2-C6-NBD-PC, 1-acyl-2-C6-NBD-PC, 1-acyl-2-C12-NBD-PC, or PE. Different amounts of TE were used to generate vesicle populations with different PPR (expressed in milligrams per millimole). (A) Plot of the extent of BSA-extractable 1-C14-2-C6-NBD-PC as a function of the PPR. (B) Plot showing the percentage of reduction in fluorescence obtained upon addition of dithionite to the reconstituted vesicle preparations. The data are fitted by a monoequilibrium function that is simplified by deconvolution into two linear segments: a line of positive slope generated by linear regression of data points from the rising section of the graph and a plateau determined by the monoequilibrium curve fit. The point where the rising linear segment intersects the plateau (PPR, ~10 mg/mmol) is interpreted as the point where each vesicle contains a single functional flippase. The fatty acid composition at the 1 position of the acyl-NBD-PLs used in panel B is as follows: palmitic acid, 62%; stearic acid, 29%; oleic acid, 5.5%.

- **A** BSA EXTRACTION
  - 1-C14-2-C6-NBD-PC
  - 1-acyl-2-C6-NBD-PC
  - 1-acyl-2-C12-NBD-PC
  - PE

- **B** DITHIONITE REDUCTION
  - 1-C14-2-C6-NBD-PC
  - 1-acyl-2-C6-NBD-PC
  - 1-acyl-2-C12-NBD-PC
  - PE

The PPR at which the reduction amplitude reaches its maximum can be used to infer that flippase constitutes ~2% by weight of proteins in the TE (see reference 26 for details of the calculation).
treated and mock-treated vesicles were similar). However, when TE was treated with NEM and DEPC simultaneously, the effect was roughly additive: >80% of flippase-active vesicles were inactivated (Fig. 5). These results can be explained by postulating two classes of flippase protein, each defined by its sensitivity to one of the two inhibitors. Alternatively, NEM and DEPC could modify critical residues within the same protein to yield the partial and combined effects that we observe (see reference 10 for further discussion of this issue).

NEM did not affect flippase activity when applied to intact membranes of yeast microsomes or proteoliposomes prepared from yeast microsomes. Although NEM is membrane permeant and should be able to access cysteine residues within and on either side of intact membrane vesicles, it reacts more rapidly (by orders of magnitude) with the thiolate (S⁻) ion than it does with protonated thiols (SH) (3). Our data therefore suggest that a functionally critical cysteine residue in the flippase is located in a hydrophobic environment, where it is less reactive with NEM (data not shown). We did not test the effect of DEPC on intact vesicles.

We were interested to know whether flippase activity could be reduced by protease-treatment of proteoliposomes. Proteoliposomes containing different amounts of protein (PPR ranging from 2.9 to 7.3 g/mol) were treated with proteinase K and subsequently assayed for their ability to mediate flip-flop of C12-NBD-PC. This treatment resulted in a 42.5% ± 8.5% decrease in the amount of reduced analogues relative to that for mock-treated proteoliposomes. Since the vesicles were prepared with a PPR range where each vesicle has no flippases or at least 1 flippase, this result implies that proteinase K treatment destroys flippase activity in ~40% of the vesicles while having no effect on the remaining ~60% of the flippase-containing vesicle population. The ability to eliminate some but not all flippase-containing vesicles by protease treatment requires explanation. One possibility is that at least two distinct flippases with different protease sensitivities might be present in the TE, giving rise to at least two classes of flippase-containing vesicles, as discussed above. An alternative possibility is that the flippase protein is reconstituted symmetrically in the vesicle population, i.e., some vesicles contain the flippase oriented such that its “proteinase K-sensitive segment” is on the outside and accessible to the protease, whereas in other vesicles, the cleavage site is in the vesicle interior. If the flippase functions symmetrically, both topological orientations would yield a flippase-active vesicle, but only the activities of those proteoliposomes containing the flippase oriented with its proteinase K-sensitive segment on the outside would be destroyed by proteinase K treatment (10).

Taken together, these data confirm that transbilayer movement of NBD-PLs requires the participation of membrane proteins and that the flippase protein(s) contains functionally critical cysteine and histidine residues. Similar results have been reported and discussed previously for flippase activity in a TE prepared from rat liver ER vesicles (10).

Role of the ER protein translocon in transbilayer phospholipid movement. It has been speculated that the protein translocon could act as a phospholipid flippase (26, 39). The translocon is an oligomeric structure with a transverse aqueous channel that opens laterally toward the lipid bilayer (8, 36). Such a molecular architecture could provide a transverse diffusion conduit for phospholipids (39). We used two different approaches to test whether the presence of Sec61p, an essential component of the protein translocon in yeast (41), would contribute significantly to phospholipid flippase activity in the yeast ER. Sec61p was depleted in vivo by using a temperature-sensitive sec61Δ mutant. In contrast to wild-type Sec61p, mutant versions of this protein are selectively degraded at 38°C, resulting in reduced cellular Sec61p levels (4). Microsomal membranes were prepared by differential centrifugation of total-cell lysates derived from 38°C-shifted wild-type and sec61Δ cells as described in Material and Methods. Depletion of Sec61p in preparations from 38°C-shifted sec61Δ cells was verified by SDS-PAGE and immunoblotting (Fig. 6A), showing that membranes from the sec61Δ cells contained less than 2% of the Sec61p in wild-type cells, whereas levels of Dpm1p, an unrelated ER protein, were unchanged.

We assayed NBD-PL flip-flop in wild-type and sec61Δ-depleted membranes by labeling the membrane vesicles with C6-NBD-PC and using stopped-flow kinetic analysis to determine the accessibility of the fluorescent phospholipid to BSA extraction. As shown in Fig. 6B, a rapid, two-component decay of fluorescence was observed with both wild-type and Sec61p-depleted membranes. The fluorescence decay curve in both cases could not be described as a monoeponential process (see the plots of residuals in the Fig. 6B inset) but fitted well with the kinetic model outlined in Fig. 2A. Although the rate constants \(k_{+2}, k_{-1}, k_{-2}\) obtained from data fitting were of the same order for both wild-type and Sec61p-depleted membranes, they were slightly higher for Sec61p-depleted membranes than for wild-type membranes. This could be a result of changes in the lipid and protein compositions of the ER membrane during Sec61p depletion.

We next assayed flippase activity in proteoliposomes reconstituted from Triton X-100 extracts of wild-type versus Sec61p-depleted membranes. The protein dependence of NBD-PL flipping was identical in Sec61p-depleted and Sec61p-equipped proteoliposomes (data not shown). We conclude that flippase activity in Sec61p-depleted membranes is comparable to that
seen in wild-type membranes. Thus, the protein translocation apparatus is not required for the efficient transbilayer movement of phospholipids across the ER membrane of yeast.

**Flippase activity sediments slowly in velocity gradients.** TE prepared from wild-type P100 membranes was fractionated on a linear glycerol velocity gradient, yielding six pools of separated ER membrane proteins (Fig. 7B). SDS-PAGE resolution of the different fractions revealed successful separation of solubilized microsomal membrane proteins, with clear differences in protein profiles between pools I to VI. Reconstitution of these pools into proteoliposomes resulted in vesicles with different flippase activities, as assayed using C12-NBD-PC and the dithionite method. The majority of flippase activity was recovered in pool II, corresponding to proteins that sediment at 4S (Fig. 7A). Little or no activity was detected in pools IV, V, and VI, which are enriched in Sec61p (Fig. 7A and D). The latter result reinforces the conclusion from the preceding section that the protein translocon plays no role in phospholipid flip-flop in the ER. Importantly, it also indicates that not all membrane proteins promote rapid phospholipid flip-flop and that distinct proteins are required.

**Conclusions.** We used a combination of fluorescence-based methods to characterize phospholipid flippase activity in microsomes of the model organism *Saccharomyces cerevisiae*. Using BSA back-extraction in conjunction with stopped-flow kinetic analyses, we demonstrated rapid, ATP-independent flip-flop (half-time, \(100 \text{s}\)) of NBD-PL analogues in intact P100 microsomal vesicles. We also biochemically reconstituted flippase activity in proteoliposomes generated from a Triton X-100 extract of P100 membranes. Using this approach, we demonstrated that flippase activity requires a membrane protein(s) that is sensitive to proteinase K, NEM, and DEPC and that sediments slowly, as assessed by velocity gradient centrifugation analyses (operational sedimentation coefficient, \(4S\), consistent with data reported for the flippase from rat liver ER [26] and *Bacillus subtilis* [15]). Our experiments also allowed us to estimate that the flippase protein represents 2% (wt/wt) of membrane proteins in the TE. Using ER membrane protein-containing proteoliposomes such as those described here, we recently succeeded in reconstituting flippase activity in giant unilamellar vesicles (31), allowing us to study the time scale of flippase-mediated transbilayer movement of unlabeled phospholipids.
phospholipids. These studies revealed half-times of phospholipid flip-flop on the order of few minutes, similar to those that have been determined here for flip-flop of fluorescently labeled phospholipid analogues.

The reconstitution approach described here and elsewhere (10, 11, 15, 20, 26, 28, 38, 39) provides a basis with good prospects for the identification of the flipflopase through conventional protein purification. Attempts to do this are under way. Our experiments also present a general method for identifying the flipflopase by testing promising candidates, in particular, to check whether a protein contributes at all to the number of flip-flop activities (11, 26). Likewise, protein pools with higher specific flipflopase activities were obtained in this study by fractionation; conversely, other fractions replete with membrane proteins were inactive. In addition, a recent study clearly demonstrated that the presence of membrane proteins in giant unilamellar vesicles per se is not sufficient to facilitate the flip-flop of phospholipids (31). These results reinforce the conclusion that specific proteins are required to facilitate phospholipid flip-flop; the data presented in this paper indicate that the identification of these proteins is feasible.

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