Chemical Modification Identifies Two Populations of Glycerophospholipid Flippase in Rat Liver ER†

Qing-long Chang, Sathyanarayana N. Gummadi,‡ and Anant K. Menon*

Department of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, Wisconsin 53706-1569

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ABSTRACT: Transbilayer flipping of glycerophospholipids in the endoplasmic reticulum (ER) is a key feature of membrane biogenesis. Flipping appears to be an ATP-independent, bidirectional process facilitated by specific proteins or flippases. Although a phospholipid flippase has yet to be identified, evidence supporting the existence of dedicated flippases was recently obtained through biochemical reconstitution studies showing that certain chromatographically resolved fractions of detergent-solubilized ER proteins were enriched in flippase activity, whereas others were inactive. We now extend these studies by describing two convenient assays of flippase activity utilizing fluorescent phospholipid analogues as transport reporters. We use these assays to show that (i) proteoliposomes generated from a flippase-enriched Triton X-100 extract of ER can flip analogues of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine; (ii) flipping of all three phospholipids is likely due to the same flippase(s) rather than distinct, phospholipid-specific transport proteins; (iii) functional flippases represent ∼1% (w/w) of ER membrane proteins in the Triton extract; and (iv) glycerophospholipid flippase activity in the ER can be attributed to two functionally distinct proteins (or classes of proteins) defined by their sensitivity to the cysteine and histidine modification reagents *N*-ethylmaleimide and diethylpyrocarbonate, respectively. Analyses of the *N*-ethylmaleimide-sensitive class of flippase activity revealed that the functionally critical sulfhydryl group in the flippase protein is buried in a hydrophobic environment in the membrane but becomes reactive on extraction of the protein into Triton X-100. This observation holds considerable promise for future attempts to isolate the flippase via an affinity approach.

Newly synthesized glycerophospholipids are located in the cytoplasmic leaflet of biogenic membranes such as the endoplasmic reticulum (ER)[1] and bacterial cytoplasmic membrane (bCM). These lipids must be transported across the bilayer to populate the exoplasmic leaflet for membrane growth. Transbilayer transport of glycerophospholipids and glycerophospholipid analogues has been assayed in the ER and bCM and is known to be a rapid, ATP-independent, bidirectional process facilitated by specific proteins (flippases) (*1*−*3*). Evidence supporting the requirement for specific proteins (i.e., dedicated flippases) in the flipping process was obtained through the recent development of a reconstitution system in which it was possible to measure flippase activity in chromatographically resolved fractions of detergent-solubilized ER or bCM proteins (*4*, *5*). Certain fractions replete with membrane proteins were found to be inactive, whereas flippase activity was enriched in other fractions (*4*−*6*). The reconstitution experiments also showed that phosphatidylcholine was rapidly exchanged (half-time for exchange ∼3 min) between the two leaflets of ∼250 nm diameter, unilamellar vesicles each equipped with a single functional flippase (*6*). This rate of exchange is much faster than that seen in vesicles containing transmembrane peptides or irrelevant membrane proteins, again arguing for the existence of dedicated flippases (*2*, *7*, *8*).

We were interested in characterizing the ER phospholipid flippase by testing the effect of protein modification reagents on flippase activity. Although chemical modification or protease treatment of the ER has been used historically in attempts to establish that proteins are required for phospholipid flipping, the results of these experiments have been generally inconsistent (*9*−*14*). Most reported experiments either show no effect or only partial reductions of flippase activity; typically, rate reductions have been noted with no evidence that the flippase could be destroyed by various treatments. The reconstitution system that we developed offers opportunities to reinvestigate this issue and characterize flippase in crude mixtures.

In this paper, we describe two assays in which acyl-NBD-labeled fluorescent phospholipid analogues are used as

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* To whom correspondence should be addressed. Tel: (608) 262-2913; fax: (608) 262-3453; e-mail: menon@biochem.wisc.edu.

‡ Present address: Department of Biotechnology, Indian Institute of Technology-Madras, Chennai 600 036, India.

[1] Abbreviations: bCM, bacterial cytoplasmic membrane; BSA, defatted bovine serum albumin; DEPC, diethylpyrocarbonate; ePC, egg PC; ER, endoplasmic reticulum; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-PL, phospholipid with an NBD-modified C_6-acyl chain; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TE, flippase-enriched Triton X-100 extract of salt-washed ER.

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reporters of flippase activity in reconstituted vesicles. We use the assays to show that glycerophospholipid flippase activity in the ER can be attributed to two functionally distinct proteins (or classes of proteins) defined by their sensitivity to the cysteine and histidine modification reagents *N*-ethylmaleimide (NEM) and diethylpyrocarbonate (DEPC), respectively. We go on to show that the functionally critical sulfhydryl goup in the NEM-sensitive flippase is buried in the membrane but becomes reactive when the activity is extracted into detergent. This latter observation holds considerable promise for future attempts to isolate the flippase by affinity methods.

EXPERIMENTAL PROCEDURES

Reagents. Egg phosphatidylcholine (ePC), diethylpyrocarbonate (DEPC), *N*-ethylmaleimide (NEM), and routine chemicals were obtained from Sigma Chemical Co. SM-2 Bio-beads were from Bio-Rad Laboratories. ULTROL grade Triton X-100 was from Roche Molecular Biochemicals. Fluorescent C_6-NBD-phospholipids were from Avanti Polar Lipids.

Preparation of a Triton X-100 Extract (TE) from Salt-Washed ER (SWER). SWER was prepared from a rat liver homogenate as previously described (*4*) and stored frozen (−80 °C) at a concentration of ∼20 mg/mL protein (corresponding to ∼5.4 μmol/mL phospholipid). A Triton extract was prepared by first diluting the SWER suspension with an equal volume of 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 0.1% (w/v) Triton X-100. The sample was mixed, left on ice for 30 min, and then ultracentrifuged (TLA 100.3 rotor, 70,000 rpm, 30 min, 4 °C). The supernatant was carefully removed, and the pellet was resuspended to the original volume of SWER in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100. The sample was thoroughly mixed by gentle vortexing and/or pipetting and left on ice for 30 min before being ultracentrifuged as stated previously. The resulting clear supernatant, or Triton extract (TE), was used directly for reconstitution or snap-frozen in aliquots that were stored at −80 °C for later use.

Reconstitution of Liposomes and Proteoliposomes. One milliliter mixtures of TE, ePC (4.5 μmol), and trace quantities (∼0.3 mol %) of fluorescent phospholipids solubilized in 10 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100 were treated with washed SM2 Bio-Beads to generate proteoliposomes as described previously (*4*, *6*, *15*). The amount of TE used was varied as required to prepare proteoliposomes with different protein/phospholipid ratios. Liposomes were prepared in parallel from identical ingredients except that TE was omitted. The protein/phospholipid ratio (PPR; mg of protein/mmol of phospholipid) of proteoliposome samples was determined as described (*16*−*18*). The average percent error was <3% for protein measurements and <2% for phospholipid measurements.

Assay for Flippase Activity using Dithionite Reduction. The assay was performed using a QuantaMaster Model C-60/2000 spectrofluorometer (Photon Technology International). A total of 50 μL of NBD-PL-containing liposomes or proteoliposomes were added to 1.95 mL of 10 mM HEPES pH 7.5, 100 mM NaCl in a cuvette. Sample fluorescence (excitation $\lambda = 470$ nm, emission $\lambda = 530$ nm) was monitored continuously at 22 °C with constant low-speed stirring. When the fluorescence intensity stabilized, 6 μL of 1 M sodium dithionite (freshly prepared in 1 M Tris Base) was added. When the fluorescence again reached a stable plateau, 50 μL of 10% (w/v) Triton X-100 was added to permeabilize the vesicles. The initial fluorescence (F_o) was taken as the average value of the fluorescence intensity of the first plateau; the fluorescence after dithionite reduction (F_{red}) was taken as the average value of the second plateau. The percent of NBD-PL that was reduced was calculated as

$$P_{red} = 100(1 - (F_{red}/F_0)). \quad (1)$$

The activity (A) of flippase in a proteoliposome preparation was taken as the difference between the percent reduced in the sample and the percent reduced in a liposome sample

$$A = (P_{red,proteoliposome} - P_{red,liposome}) \quad (2)$$

Specific activity was defined as A divided by the protein/phospholipid ratio (in mg/mmol). The proportion of functional flippases eliminated by treatment with NEM and/or DEPC (% inhibition) was calculated from the percent change in P_{red} after subtracting P_{red} for liposomes

$$\% \text{ inhibition} = (1 - (A_{(NEMand/orDEPC)}/A_{(mock)}))100\% \quad (3)$$

Assay of Flippase Activity using Albumin Extraction. The reduction in fluorescence that accompanies extraction of short-chain NBD-phospholipids by bovine serum albumin (BSA) was measured as described by Kubelt et al. (*19*). Fluorescently labeled liposomes and proteoliposomes were prepared as described previously except that 1-myristoyl-2-C_6-NBD-PLs were used instead of 1-acyl-2-C_6-NBD-PLs. The fluorescence of NBD-containing liposomes or proteoliposomes was measured on-line as for dithionite reduction, except that instead of dithionite, 150 μL of 27.6 mg/mL fatty acid free bovine serum albumin was added (final concentration of BSA ∼2 mg/mL). The accessible pool of fluorescent lipid, P_{ext}, was calculated as

$$F_{BSA} = (1 - P_{ext}) + 0.5P_{ext} \quad (4)$$

where F_{BSA} is the plateau value of fluorescence (normalized to initial fluorescence) of liposomes after extraction with BSA, P_{ext} is the proportion of labeled lipid that is extracted by BSA, and the relative quantum yield of BSA-bound fluorescent lipid is ∼0.5 as compared to a value of 1 for membrane-associated fluorescent lipid (*19*).

Treatment of TE and SWER with Protein Modification Reagents. Freshly prepared stock solutions of DEPC and NEM (typically 40 mM reagent in 10 mM HEPES pH 7.5, 100 mM NaCl) were combined with TE to yield the desired final concentration of protein modification reagent. The pH of the reaction was confirmed by paper to be ∼7.5. The number of free sulfhydryl groups in TE was measured using Ellman's reagent to ensure that all experiments utilizing NEM involved an excess of reagent over available −SH groups. For experiments such as those described in Figures 5 and 6 where NEM or DEPC are titrated, the concentration of protein (TE) was kept fixed while increasing the concentration of reagent.

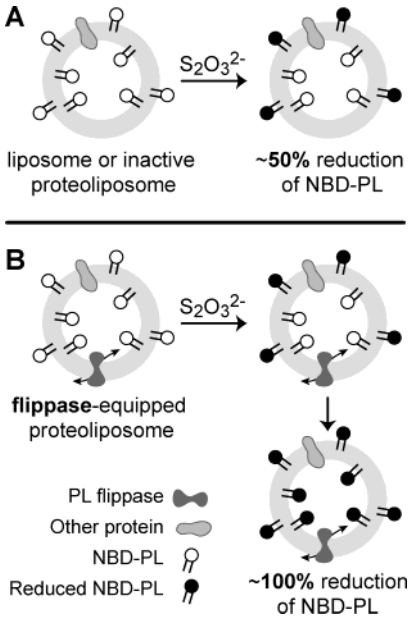

FIGURE 1: Flippase assay via dithionite reduction of NBD-phospholipids (NBD-PLs) in reconstituted vesicles. Assays are carried out with reconstituted vesicles consisting of ePC, 0.3 mol % 1-C_{16}-2-C_6-NBD-PL (symmetrically distributed in the two membrane leaflets) ± ER membrane proteins. When treated with dithionite ($S_2O_3^{2-}$), liposomes (or proteoliposomes without flippase) lose half their fluorescence due to reduction of NBD-PLs located in the outer leaflet (A). NBD-PL's in the inner leaflet are protected since dithionite does not permeate the membrane over the time frame of the experiment. Flippase-equipped proteoliposomes lose all their fluorescence upon dithionite treatment since NBD-PL's in the inner leaflet can be flipped out (panel B; the schematic shows flippase-facilitated mixing of NBD-PL's between the two membrane leaflets). For mixtures of flippase-equipped and inactive vesicles, the extent of fluorescence reduction is expected to be between 50 and 100%.

RESULTS AND DISCUSSION

Flippase Assay using Dithionite Reduction of NBD-Phospholipids. The flippase assay (Figure 1) makes use of the ability of the dianion dithionite to reduce the phospholipid-linked NBD fluorophore to nonfluorescent amine products (*20*, *21*). Briefly, 1-acyl-2-C_6−NBD-phospholipid (NBD-PL)-containing liposomes or proteoliposomes are treated with dithionite, and the fluorescence change accompanying dithionite addition is monitored. Since dithionite is not expected to permeate membranes significantly on the time-scale of our experiments (10−15 min), only the NBD-PLs located in the outer leaflet of the vesicles will be reduced, resulting in a predicted ~50% decrease in fluorescence (Figure 1A). If the vesicle contains a biogenic membrane flippase, then NBD-PLs will be able to exchange back and forth between the membrane leaflets. Thus, for flippase-equipped vesicles, the entire NBD-PL population will be susceptible to dithionite, causing the percent of NBD-PL reduced, P_{red}, to be ~100% (Figure 1B).

A continuously stirred suspension of NBD-PC-containing liposomes (~100 mM phospholipid containing NBD-PC at ~0.3 mol % of egg phosphatidylcholine (ePC)) was monitored on-line in a fluorescence spectrophotometer. Upon addition of 3 mM sodium dithionite, the fluorescence rapidly decreased ($t_{1/2}$ ~20s) to approximately half of the initial value (P_{red} ~50%) (Figure 2, top trace (labeled 0.0 mg/mmol)). Fluorescence remained at this level until 0.25% (w/v) Triton

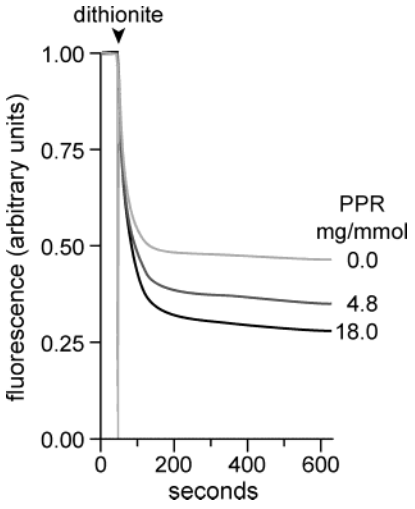

FIGURE 2: Dithionite reduction of NBD-PC in liposomes and proteoliposomes. Liposomes and proteoliposomes were reconstituted from TX-100-solubilized ePC, 1-C_{16}-2-C_6-NBD-PC (0.3 mol %) and different amounts of TE. Detergent was removed with SM2 Bio-Beads, and the resulting vesicles were diluted into buffer in a cuvette and placed in a fluorescence spectrometer set to measure NBD fluorescence (l_{ex} = 470 nm, l_{em} = 530 nm). When the fluorescence intensity stabilized, sodium dithionite was added, and loss in fluorescence was noted. The traces show that for liposomes, the extent of fluorescence reduction on adding dithionite (P_{red}) is ~50%, but for proteoliposomes P_{red} is higher (the exact value depending on the protein/phospholipid ratio (PPR) of the preparation; see also Figure 3A).

X-100 was added, at which time the signal immediately fell to zero (not shown). Thus, there is a pool of NBD-PC that is readily reduced by extravesicular dithionite, interpreted as the NBD-PC in the outer leaflet of the vesicle, and a pool that is reduced by dithionite only after the addition of membrane-permeabilizing concentrations of detergent, interpreted as the NBD-PC in the inner leaflet. The observations that the fluorescence signal stabilizes at ~50% of the starting value after adding dithionite and that it is eliminated after adding Triton X-100 confirm that dithionite does not leak into the vesicles and that the amount of dithionite used is sufficient to reduce all the NBD-PC present in the sample. Identical data were obtained for vesicles prepared with trace quantities of NBD-PE or NBD-PS instead of NBD-PC.

Rat Liver ER Membrane Proteins Facilitate Transbilayer Flipping of NBD-PC in Reconstituted Proteoliposomes. When proteoliposomes prepared from a mixture of ePC, NBD-PC, and a Triton extract (TE) of salt-washed rat liver ER (SWER) were treated with dithionite, P_{red} was greater than that seen for protein-free liposomes (Figure 2, lower traces). This suggests that NBD-PC in the inner leaflet of flippase-containing proteoliposomes can be flipped to the outer leaflet where it is reduced by dithionite (Figure 1B). Reconstitution of proteinase K-treated TE into vesicles yielded a liposome-like P_{red} of ~50% (data not shown). Also, proteinase K treatment of proteoliposomes caused a reduction in P_{red} relative to mock-treated vesicles (data not shown). Together with the absence of flipping noted for liposomes, these data reaffirm the involvement of proteins in flipping NBD-PC from the inner to the outer leaflet of proteoliposomes. Since the fluorescence traces obtained on dithionite treatment of NBD-PC-containing proteoliposomes display a similar time-course to the trace for a corresponding liposome sample (Figure 2), we suggest that the rate of flipping of

NBD-PC is similar to or faster than the rate of dithionite reduction of the NBD fluorophore. Indeed, preliminary stopped-flow kinetic experiments indicate that the half-time for NBD-PC and NBD-PE flipping in proteoliposomes reconstituted from TE is ~1 min (J. Kubelt and A.K.M., unpublished). We note that although it is possible to describe other scenarios (e.g., protein-mediated dithionite leakage into the vesicles or a protein-promoted, static asymmetric distribution of NBD-PC such that a greater fraction of NBD-PC is located in the dithionite accessible outer leaflet of proteoliposomes), to account for the TE-dependent increase in P_{red}, we view these explanations as unlikely based on data presented next.

Protein Dependence of the Extent of Reduction Indicates that Functional Flippases Capable of Flipping NBD-PC, -PE, and -PS Represent ~1 wt % of ER Proteins in the TE. Figure 2 shows that NBD-PC-containing proteoliposomes reconstituted at a protein/phospholipid ratio (PPR) of 18 mg of protein/mmol of phospholipid yield a greater P_{red} than proteoliposomes with less protein per vesicle. Indeed, as shown in Figure 3A, P_{red} increased linearly as the PPR increased from 0 to 12 mg/mmol, then plateaued at PPR >12 mg/mmol, irrespective of the NBD-PL used as transport reporter. We interpret these data as follows.

We suggest that for 0 < PPR < 12 mg/mmol, the vesicle ensemble contains vesicles with either 0 or 1 functional flippase (i.e., the ensemble is a mix of vesicles including those that lack protein, others that contain irrelevant proteins, and yet others that possess a mixture of proteins that includes a flippase). Of the vesicles in this ensemble, only those in the latter category (i.e., flippase-equipped vesicles) are expected to transport NBD-PLs from the inner to the outer leaflet (Figure 1B); the other vesicles are expected to behave essentially as liposomes with a pool of inner-leaflet NBD-PL protected from dithionite reduction (Figure 1A). As the protein concentration in the reconstitution mixture is increased to generate samples with a higher PPR (PPR is directly proportional to the amount of TE used in the reconstitution (Figure 3B)), the probability that a particular proteoliposome will contain a flippase increases. Thus, as the PPR tends toward 12 mg/mmol, more vesicles in the sample will be flippase-equipped, and the fraction of NBD-PL available for reduction will be greater. At a PPR of ~12 mg/mmol (Figure 3A), all vesicles are equipped with a flippase. Increases in PPR beyond this value result only in adding further functional flippases to vesicles already equipped with a flippase; thus, for PPR >12 mg/mmol, there is no increase in the fraction of accessible NBD-PL and P_{red} plateaus. In this regime, the rate but not the extent of flipping is expected to increase (*6*). We described a similar dependence of the amplitude of transport on PPR in previous work utilizing dibutyryl-PC and dipalmitoyl-PC as transport reporters (*4*−*6*).

The maximum P_{red} observed is ~80%, not 100% as suggested by the schematic in Figure 1B. Similar results have been reported previously in studies of outward translocation of NBD-PLs in membrane vesicles (*22*). A possible explanation is that a small fraction of NBD-PLs resides in a dithionite-resistant aggregate or domain leading to an assay efficiency of ~80%.

The dose−response plot shown in Figure 3A is comprised of data generated from a number of independent experiments using NBD-PC, NBD-PE, and NBD-PS as transport reporters. As is evident from the plot, transport of all three NBD-PLs displays the same protein dependence profile. This suggests that either all three lipids are transported by the same flippase or they are transported by different flippases that are similarly abundant in the TE (see next). An identical dose−response profile was obtained for NBD-PI (R. A. Vishwakarma and A.K.M., unpublished), indicating that our conclusion can be extended to all four major glycerophospholipids.

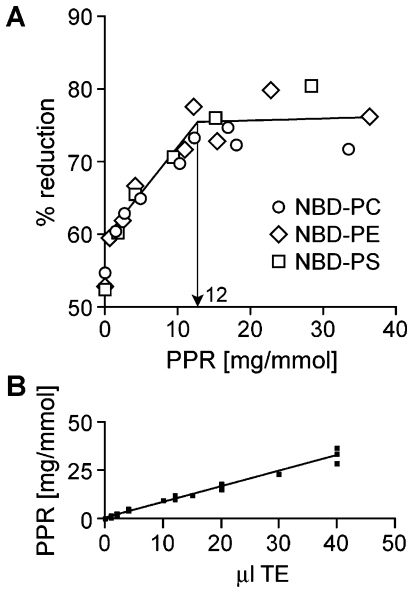

FIGURE 3: Extent of dithionite reduction of NBD-PLs in proteoliposomes depends on the protein/phospholipid ratio (PPR) and is similar for NBD-PC, NBD-PE, and NBD-PS. (A) Data such as those shown in Figure 2 were systematically acquired, in a series of experiments, for proteoliposomes prepared over a range of protein/phospholipid ratios (PPRs) using 1-acyl-2-C_6-NBD-PC, -PE, and -PS as transport reporters (the *y*-error (in the extent of reduction) associated with each data point is ~1%, and the *x*-error (in protein/phospholipid ratio determination) is ~3%). The percent reduction seen on dithionite addition is plotted as a function of the PPR. The inflection point is ~12 mg/mmol for all three phospholipids consistent with the proposal that a single flippase can transport different phospholipids. On the basis of assumptions described in the text, we conclude that the 12 mg/mmol inflection point corresponds to a population of 250 nm diameter vesicles, each with ~100 proteins (~50 kDa each) of which one is a flippase. (B) Plot demonstrating that the PPR of vesicles generated in our reconstitution protocol is directly proportional to the amount of TE used in the reconstitution mixture.

The plot shown in Figure 3A can be used to estimate the abundance of functional flippases in TE as described previously (*4*−*6*). If we assume that (i) each vesicle in a preparation with PPR ~12 mg/mmol is equipped with a single flippase (as discussed previously); (ii) the average external diameter of the vesicles is ~250 nm (as determined by dynamic light scattering and electron microscopy (*4*, *6*)), thickness of the membrane bilayer is ~4 nm, and the cross-sectional area of a phospholipid molecule is ~0.7 nm^2; and (iii) the average molecular weight of ER proteins is ~50 kDa, then at a PPR of ~12 mg/mmol, each vesicle has ~100 proteins of which one is a flippase. Thus, functional flippases represent ~1 wt % of ER proteins in TE. This number is higher than we reported previously because we used a flippase-enriched TE in these experiments, prepared from

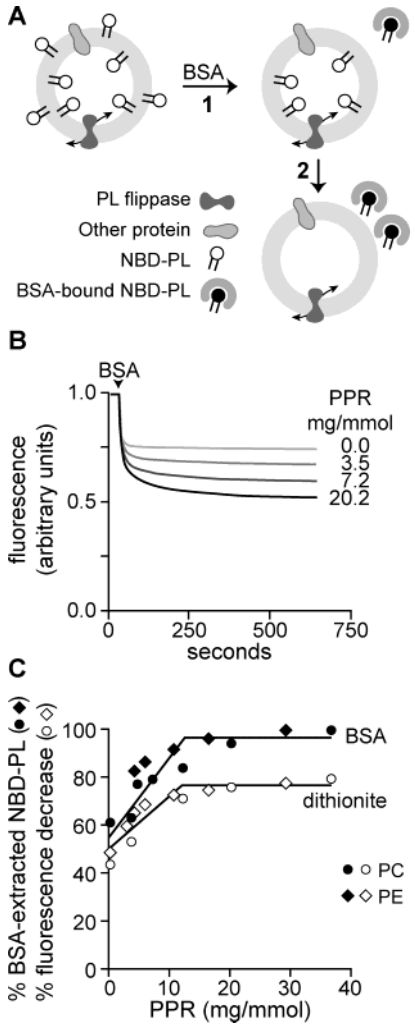

FIGURE 4: Flippase assay based on BSA back-extraction. (A) 1-C_{14}-2-C_6-NBD-PLs were reconstituted into liposomes or proteoliposomes, and flippase activity was assayed by using defatted BSA to extract the NBD-PLs from the outer leaflet of the vesicles. For liposomes or proteoliposomes lacking a flippase, only those NBD-PLs initially situated in the outer leaflet are extracted (step 1); for flippase-equipped proteoliposomes, NBD-PLs from the inner leaflet are flipped out and also extracted (step 2), leaving the proteoliposomes devoid of fluorescent lipids. (B) Fluorescence traces showing the reduction in signal obtained on adding BSA to 1-C_{14}-2-C_6-NBD-PC-containing liposomes or proteoliposomes. (C) Plot of the extent of BSA-extractable NBD-PC and NBD-PE as a function of the PPR (closed symbols). The data correspond to the extent of fluorescence reduction measured, scaled by a factor of 2 to compensate for the reduced quantum yield of NBD-PL bound to BSA (*19*). The same vesicle samples were also assayed by the dithionite method, and the corresponding data are shown by the open symbols.

SWER that had been preextracted with 0.05% (w/v) Triton X-100 before being processed to yield TE.

BSA Back-Extraction Assay Confirms Protein-Dependent Transbilayer Movement of NBD-PL in TE-Derived Proteoliposomes. As an additional test to establish that proteoliposomes derived from TE and ePC are capable of flipping NBD-PLs, we used a different assay procedure involving extraction of NBD-PLs by BSA (Figure 4A). Adding ~2 mg/mL defatted BSA to liposomes or proteoliposomes results in extraction of NBD-PLs from the outer leaflet of the vesicles and a decrease in the fluorescence of the sample (Figure 4B). Since BSA cannot cross the vesicle membrane, it extracts only NBD-PL molecules that are in the outer leaflet of the vesicle or those initially located in the inner leaflet that can gain access to the outer leaflet through flippase-mediated translocation (Figure 4A). The decrease in fluorescence on BSA extraction is because BSA-bound NBD-PL has a ~2-fold lower quantum yield than membrane-bound NBD-PL (*19*). Thus, extraction of all NBD-PLs from the outer leaflet of liposomes should yield a ~25% drop in fluorescence, whereas extraction of ~100% of NBD-PLs from flippase-equipped proteoliposomes should yield a ~50% reduction in fluorescence (Figure 4A).

Our experiments indicate that the amount of BSA-extractable NBD-PC and NBD-PE in proteoliposomes increases with the PPR, resulting in progressively larger decreases in fluorescence (traces for NBD-PC are shown in Figure 4B). When the data are corrected for quantum yield differences between BSA-bound and membrane-bound NBD-PL, the results from the BSA extraction assay show that ~50% of the NBD-PL is extracted from liposomes whereas ~100% of the NBD-PL is extracted from proteoliposome samples prepared at a PPR of ≥12 mg/mmol (Figure 4C, solid symbols). Dithionite reduction assays of the same vesicle preparations yielded comparable data (Figure 4C, open symbols). Both assays displayed the same dependence of transport amplitude on PPR, with an inflection point at PPR = 12 mg/mmol. We note that while the dithionite assay displays a built-in efficiency of 80% (see previously), the BSA assay achieves the theoretically predicted range of transport amplitudes (i.e., 50% for liposomes and 100% for flippase-equipped proteoliposomes), possibly because the back-extraction process drives the assay to completion. In any case, the coincidence of the results obtained with dithionite reduction and BSA back-extraction argues against the possibility that the results generated via the dithionite assay are due to protein-dependent permeation of dithionite into the interior of proteoliposomes. These data thus validate the use of the dithionite assay to measure flippase activity in reconstituted systems.

NEM Treatment Eliminates a Pool of Functional Flippases in TE. *N*-Ethylmaleimide has been used classically to probe structure−function relationships in a variety of proteins and membrane-localized transporters, in many instances yielding powerful new insights (e.g., refs *23*−*26*). We tested the functional consequences of chemically alkylating proteins in the TE prior to reconstituting the extract and assaying flippase activity by the dithionite method. When NBD-PC-containing proteoliposomes were reconstituted from TE that had been incubated with *N*-ethylmaleimide (NEM), P_{red} was lower than that seen for proteoliposomes reconstituted from mock-treated TE (Figure 5A, P-NEM vs P-mock). Since the proteoliposomes were prepared in a PPR regime (<12 mg/mmol) where each vesicle is expected to have 0 or 1 functional flippase, this result indicates that a pool of flippases is rendered inactive by NEM treatment while the remaining flippase molecules retain their ability to transport NBD-PC over the time frame of our experiment. The traces in Figure 5A indicate that ~40% of flippases are NEM-sensitive.

The effect of NEM was tested systematically to ensure that the knock-out of only ~40% of functional flippases was not due to insufficient reagent. Aliquots of TE were incubated with a range of concentrations of NEM (5−40 mM; protein concentration was kept fixed), then reconstituted and assayed using NBD-PC as transport reporter. The inhibitory effect

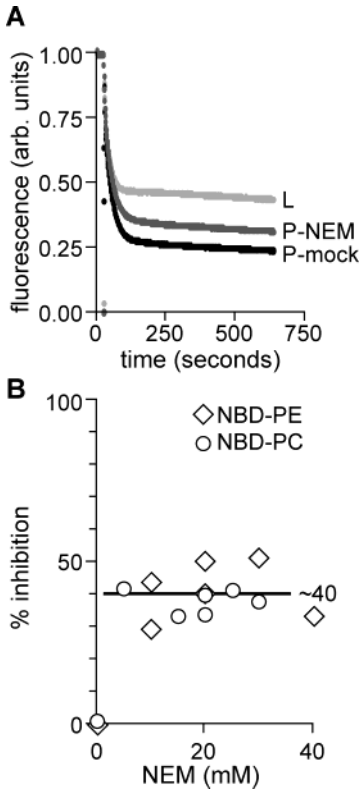

FIGURE 5: Inhibition of flippase activity by treating TE with NEM. (A) NBD-PC-containing proteoliposomes were generated from TE that had been mock-treated or incubated with NEM. The vesicles (labeled P-mock and P-NEM, respectively) were assayed for flippase activity using the dithionite method. Liposomes (labeled L) prepared in parallel were also assayed. PPR values for the P-mock and P-NEM samples shown were 6.1 and 6.6 mg/mmol, respectively. (B) Aliquots of TE were treated with a range of NEM concentrations, then used to generate proteoliposomes with PPR <12 mg/mmol. Mock-treated TE was used as a control. The extent to which NEM decreased P_{red} (% inhibition) was calculated from the percent change in P_{red} after subtracting P_{red} for liposomes (see Experimental Procedures for details) and adjusting for small variations in PPR. The average % inhibition, ~40%, is indicated by a line.

of NEM was evident at the lowest concentration tested and remained at ~30−50% inhibition over the entire concentration range (Figure 5B; the data are calculated from traces such as those shown in Figure 5A, corrected for small variations in PPR of the different proteoliposome preparations). Identical results were obtained when NBD-PE was used in the assay, instead of NBD-PC (Figure 5B). Thus, our results indicate that ~40% of the flippase-equipped vesicles in the sample have been rendered inactive and that the remainder of the flippase-active vesicles has been unaffected at this level of analysis (we would not be able to detect less than an order of magnitude change in translocation rate).

We conclude that the ER has two populations of glycerophospholipid flippase, operationally defined by sensitivity and resistance to NEM. This implies that either the glycerophospholipid flippase is a single protein that exists in NEM-sensitive and NEM-resistant states (the latter could result from complex formation or multimerization that protects the functionally critical cysteine) or that there are at least two different proteins in the TE that contribute to flippase activity.

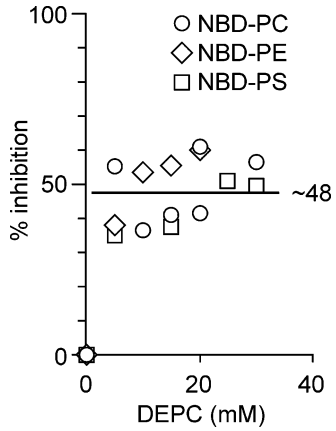

FIGURE 6: Elimination of a population of flippases by treating TE with DEPC. Aliquots of TE treated with a range of DEPC concentrations were used to generate proteoliposomes with PPR <12 mg/mmol. Mock-treated TE was used as a control. The extent to which DEPC decreased P_{red} (% inhibition) was calculated from the percent change in P_{red} after subtracting P_{red} for liposomes (see text for details) and adjusting for small variations in PPR. The average % inhibition, ~48%, is indicated by a line.

DEPC Treatment Eliminates ~50% of Functional Flippases in TE. We were interested in testing other protein modification reagents to see if they could be used to define further the two flippase populations deduced from the NEM experiments. Data on the effect of DEPC, a reagent that modifies histidine residues (*27*), are shown in Figure 6. Assay of DEPC-treated TE reconstituted into proteoliposomes with PPR <12 mg/mmol revealed ~50% reduction in the number of active flippases as compared to similarly reconstituted mock-treated TE. The data were similar regardless of whether NBD-PC, -PE, or -PS was used as a transport reporter. Also, the same level of inhibition was seen over the entire range of DEPC concentrations tested, suggesting that DEPC, like NEM, targets a particular population of flippases.

Combined NEM and DEPC Treatment Eliminates the Majority of Functional Flippases in TE. Since NEM and DEPC each target only a fraction of the functional flippases in TE, it was interesting to determine whether they targeted the same pool of flippases or whether their effects were complementary. Analyses of TE aliquots that were mock-treated or treated with NEM alone, DEPC alone, or NEM + DEPC (20 mM each) prior to reconstitution revealed that the effects of the two reagents on flippase activity were additive (Figure 7). Thus, a combination of NEM and DEPC is effective in eliminating essentially all functional flippases in the TE. We conclude that the flippase activity in the ER consists of two components, operationally defined by their sensitivity to NEM and DEPC.

NEM Treatment of Intact Vesicles Reveals that the Cysteine Residue Critical for Flippase Activity Is Located in an Apolar Environment. Preliminary studies indicated that proteoliposomes treated with NEM displayed essentially the same P_{red} as untreated or mock-treated samples (data not shown). These data contrast with those presented previously where we showed that it was possible to inactivate a population of flippases (~40% of the total pool) by chemical alkylation of TE prior to reconstitution of the extract into proteoliposomes. This suggests that the membrane environment protects the NEM-sensitive class of flippases from inactivation by NEM. We examined this issue in more detail

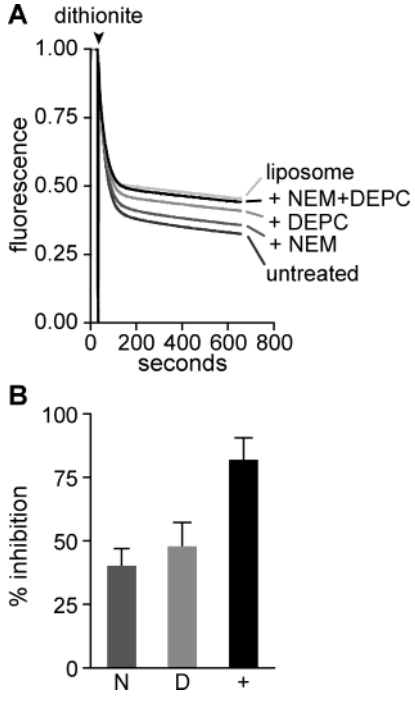

FIGURE 7: Effects of NEM and DEPC on TE are additive: two populations of glycerophospholipid flippase defined by sensitivity to NEM or DEPC. (A) Aliquots of TE were treated with NEM (20 mM), DEPC (20 mM), or a combination of the reagents (20 mM each), then reconstituted with NBD-PC and assayed for flippase activity using the dithionite assay. Liposomes and mock-treated TE were similarly reconstituted and assayed. The traces show that treatment of TE with a combination of NEM and DEPC reduces P_{red} to a liposome-like value. PPR values for untreated, +NEM, +DEPC, and +NEM+DEPC samples were 7.6, 8.3, 8.7, and 9.0 mg/mmol, respectively. (B) Summary of data culled from independent experiments with NBD-PC showing partial elimination of flippase pools by NEM and DEPC and close to complete elimination of flippase when the reagents are applied in concert. The data were similar regardless of whether NBD-PC or NBD-PE was used in the measurement.

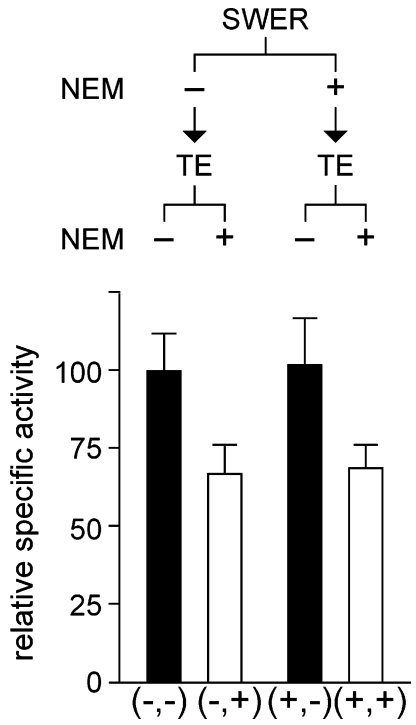

FIGURE 8: Cysteine critical for flippase activity is buried in the membrane. SWER was mock-treated (−) or incubated (+) with 20 mM NEM for 40 min at room temperature. The vesicles were washed by centrifugation, then extracted to generate TE. The TE from mock- and NEM-treated ER was divided into two aliquots, one of which was mock-treated (−) while the other (+) was incubated with 20 mM NEM. The four resulting TE preparations ((−,−), (−,+), (+,−), (+,+)) were reconstituted into proteoliposomes and assayed for flippase activity using the dithionite assay. Reconstitutions were done using at least two different PPRs per sample to calculate a specific activity measure (see Experimental Procedures). The average specific activity of the (−,−) sample was set at 100, and the values of the other samples were scaled accordingly. The data show that the (+,−) sample has the same specific activity as the (−,−) sample, indicating that NEM treatment of SWER has no effect on the number of functional flippases subsequently extracted into TE. However, NEM treatment of TE as seen in the (−,+) and (+,+) samples shows a loss of ∼35% of functional flippases, similar to data presented in Figure 5.

by treating SWER as well as TE with NEM as outlined in Figure 8.

SWER preparations were treated with 20 mM NEM (pH 7.5) for 40 min at room temperature; mock-treated samples were prepared alongside. The vesicles were washed by dilution and centrifugation and then extracted to generate TE. The TE samples from NEM-treated SWER and mock-treated SWER were each divided into two aliquots. One aliquot was treated with 20 mM NEM, while the other was mock-treated. The treated and mock-treated extracts were reconstituted (PPR <12 mg/mmol) and assayed for their ability to flip NBD-PC.

The data (Figure 8) clearly show that NEM treatment of SWER has no effect on the number of functional flippase molecules recovered in the TE (samples labeled (−,−) and (+,−)), whereas NEM treatment of TE prepared from either NEM-treated or mock-treated SWER (samples labeled (−,+) and (+,+)) eliminates ∼30−35% of functional flippases similar to the results shown in Figure 5B. We conclude that the functionally critical cysteine residue(s) is located in an apolar environment within the ER membrane bilayer where it exists predominantly in the nonreactive −SH form rather than in the reactive thiolate anion (S^-) form. In TE, this critical cysteine residue must become exposed to a polar environment and consequently reacts more rapidly with NEM.

CONCLUSIONS

We describe assays to measure phospholipid flip-flop in proteoliposomes reconstituted from a Triton X-100 extract of rat liver ER. The TE we use here is enriched in flippase activity as compared with TE that we described previously. The enrichment was achieved by rinsing salt-washed ER vesicles with 0.05% (w/v) Triton X-100, 100 mM NaCl, prior to extracting flippase with 1% (w/v) Triton X-100, 100 mM NaCl. Our results indicate that flippase represents ∼1 wt % of ER membrane proteins that are present in the enriched TE. The assays we describe provide further validation of our reconstitution studies of the ER glycerophospholipid flippase (*4*−*6*) while offering a simple means of measuring transport activity for the purpose of purifying the flippase(s).

We present three conclusions. First, the identical dose−response profiles for NBD-PC, -PE, and -PS suggest that all three phospholipids are flip-flopped by the same trans-

porter. It is, of course, conceivable that there exists a separate transporter for each of the lipids, but in this event, our data indicate that the different transporters would have to be similarly abundant in the TE. The only data in the literature that address this issue derive from a study of the flipping of spin-labeled PC and lyso PS in ER vesicles (*12*). In this earlier study, it was noted that the simultaneous presence of spin-labeled PC and lyso PS in the ER reduced the transbilayer diffusion rate of each analogue by the same factor, implying that the same protein transports PC and lyso PS. Second, we show that the glycerophospholipid flippase activity in the ER can be functionally divided into two independently active components defined by their sensitivity to NEM and DEPC.[2] The effect of the latter compound on flippase activity has not been reported previously, although it has been shown to slow the protein-mediated, ATP-independent transport of NBD-phospholipids across the plasma membrane of intestinal brush border cells (*28*). The data obtained with NEM are particularly interesting and offer hope for a direct approach to identifying flippase candidates. It is important to make the distinction between our observations on the effect of NEM on flippase activity and results reported in the literature—we report total inactivation of ~40% of the population of functional flippases on NEM treatment of TE, an observation made possible by reconstitution at a suitably low PPR. In contrast, published reports of the effect of NEM on ER glycerophospholipid flippase activity range widely in their conclusions. Backer and Dawidowicz (*10*) claimed that NEM had no effect on flippase activity in a reconstitution study, while Bishop and Bell (*9*), Kawashima and Bell (*11*), Herrmann et al. (*12*), and Buton et al. (*13*) claimed partial effects but no absolute inhibition. These mixed results can be explained by our data showing that the effect on transport amplitude is only detectable when reconstitutions are done at a suitably low PPR and that the effect of NEM on membrane vesicles is marginal. Third, we suggest that the noneffect of NEM on flippase activity in proteoliposomes or SWER vesicles is because the functionally critical cysteine is protected in an apolar environment in membrane vesicles, most likely within the hydrophobic interior of the bilayer. Exposure of this residue to a polar environment occurs once the flippase is Triton X-100-extracted. This observation affords the powerful opportunity to label specifically the NEM-sensitive class of flippase and to use the label to identify the relevant protein(s). Work along these lines is underway.

[2] Although our data are most easily interpreted as providing evidence for two separate flippase proteins in the ER, it could also be possible for NEM and DEPC to be modifying critical residues within the same protein to yield the partial and combined effects that we report in Figures 5−7 as follows. The NEM- and DEPC-inhibited components we identify would correspond to either of two possible topological orientations (↑ or ↓) of the flippase in the membrane. These orientations would come about in the process of reconstitution where the flippase protein could integrate into the forming bilayer in either of two, equally likely ways. If the flippase protein functions symmetrically, both the topological orientations would yield a flippase-active vesicle. Our assay, however, measures the transport—flop—of NBD-PLs from the inner leaflet to the outer leaflet. We suggest that NEM treatment blocks the ability of flippases integrated in one orientation (e.g., ↑) to flop NBD-PLs without affecting the ability of the oppositely oriented flippases (i.e., ↓) to function. We further suggest that DEPC affects the complementary orientations in the same way (i.e., DEPC affects the ability of ↓ but not ↑ oriented flippases to flop NBD-PLs). Since the experiments are performed in a PPR regime that we interpret to correspond to 0 or 1 flippase per vesicle, partial effects (~50% in each case) would be observed with either reagent with combined treatment eliminating all functional flippases.

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