Reconstitution of yeast microsomal lipid flip-flop using endogenous aminophospholipids

Teresa Nicolson[a], Peter Mayinger[b,*]

[a] *Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35, D-72076 Tübingen, Germany*
[b] *Zentrum für Molekulare Biologie, Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany*

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Abstract The molecular basis of transbilayer movement or flipping of phospholipids in the endoplasmic reticulum is largely unknown. To circumvent the problems inherent to studies with artificial phospholipid analogs, we studied microsomal flip-flop of endogenous phosphatidylethanolamine in yeast. The transbilayer transport of phosphatidylethanolamine was measured in reconstituted proteoliposomes derived from microsomal detergent extracts. Our results demonstrate that flipping is protease sensitive but does not require metabolic energy. Our assay is the first to use the endogenous substrate of the so-called 'flippase' to study phospholipid translocation in endomembranes and may therefore be crucial for the understanding of the catalytic properties of this elusive enzyme.

Key words: Membrane biogenesis; Phosphatidylethanolamine; Phospholipid flip-flop; *Saccharomyces cerevisiae*

1. Introduction

Assembly and maintenance of intracellular membranes is dependent on rapid bilayer translocation of newly synthesized phospholipids. Many enzymes involved in this process, like CDP-diacylglycerol synthase, phosphatidylserine (PtdSer) synthase, phosphatidylinositol (PtdIns) synthase and specific methyl transferases are associated with the cytoplasmic surface of the endoplasmic reticulum (ER) membrane [1,2]. In yeast, the majority of phosphatidylethanolamine (PtdEtn) is synthesized in mitochondria [3]. This reaction requires the transfer of PtdSer from the ER to the inner membrane of mitochondria where it is converted to PtdEtn by the mitochondrial PtdSer decarboxylase isoform [2–4]. Newly synthesized PtdEtn is rapidly translocated to the outer mitochondrial membrane in a not completely understood mechanism and then transported back to the ER. In the ER PtdEtn is first incorporated into the outer leaflet of the ER membrane where a fraction of it is used for phosphatidylcholine (PtdCho) synthesis. However, a substantial portion of PtdEtn has to be translocated across the ER lipid bilayer to enable proper assembly of these membrane structures. Similar transbilayer movement is also an essential part of the biogenesis of the other glycerophospholipids which are synthesized at the cytoplasmic side of the ER membrane.

A number of studies were performed to characterize phospholipid flip-flop activity in the ER. However, in most cases artificial lipid analogs with spin-labeled or fluorescent groups were used, which are structurally distinct from native phospholipid substrates [5–7]. In addition, short chain phospholipids were frequently employed for kinetic studies to avoid solubility problems inherent to natural lipids possessing long chain fatty acids [8–10]. As a consequence of these variations in experimental tools, a vast discrepancy exists in the literature with regard to the reported kinetic constants of phospholipid flip-flop [11,12]. Another controversial issue is the molecular mechanism of the microsomal phospholipid flip-flop reaction. The transbilayer movement of phospholipids across the ER membrane seems to be independent of an energy source [13,14], yet there is substantial evidence that this process is protein-mediated [10,14].

In this study we used a novel strategy to measure transbilayer movement of endogenous PtdEtn in reconstituted proteoliposomes from yeast microsomal extracts. We show that transbilayer translocation of PtdEtn in these proteoliposomes is protein-mediated and we determined the half time for this reaction. Our results define yeast as a promising model system for identifying components involved in this transport process.

2. Materials and methods

2.1. Materials and reagents

2,4,6-Trinitrobenzenesulfonic acid (TNBS), 1,5-difluoro-2,4-dinitrobenzene (DFDNP), phospholipase D (type I from cabbage) and phospholipids were purchased from Sigma. Zymolyase was obtained from ICN. Radiochemicals were purchased from Amersham. Silica gel 60 plates for thin layer chromatography were obtained from Merck.

2.2. Preparation of subcellular fractions

For preparing crude membrane fractions, wild-type yeast cells (SEY6210 [15]) were grown to early log phase. The cells were digested with zymolyase and lysed as described in [16]. The cell lysate was centrifuged at $3000\times g$ for 15 min to remove non-lysed cells and nuclei. The supernatant was pelleted by a 30 min spin at $27\,000\times g$. The pellet was resuspended in a buffer containing 250 mM sucrose, 50 mM KOAc, 20 mM HEPES, pH 7.4. Isolation of ER-derived microsomal membranes was performed as published in [16].

2.3. Preparation of reconstituted proteoliposomes

As starting material for proteoliposomes an enriched microsomal fraction from yeast cells [16] was used. The membranes were solubi-

*Corresponding author. Fax: (49)-6221-545892.
E-mail: mayingerp@sun0.urz.uni-heidelberg.de

Abbreviations: ER, endoplasmic reticulum; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdOH, phosphatidic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP-PtdEtn, trinitrophenyl-derivative of PtdEtn; FDNP-PtdEtn, fluorodinitrophenyl-derivative of PtdEtn

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lized by incubation of 2.5 mg of membranes for 15 min on ice in 250 μl of solubilization buffer containing 3% Triton X-100, 150 mM NaCl, 20 mM HEPES, pH 7.4 and 1 mM phenylmethylsulfonyl fluoride. After clearing the detergent extract by centrifugation at 20 000×*g* for 10 min the supernatant was used in the reconstitution protocol. The following lipid mixtures were used for reconstitutions: PtdCho; PtdCho/PtdEtn (7/1); PtdCho/PtdIns/PtdEtn/PtdSer (40/28/12/7). The lipid mixtures were dried in a speedvac and resuspended in buffer (100 mg/ml total lipid, in 100 mM MOPS, pH 7.4) by sonication in an ice bath. For reconstitution, the microsomal extract containing 10 mg/ml of protein was supplemented with a total amount of 48 mg/ml Triton X-100. To this solution 24 mg/ml of the lipid mixture was added in small portions and the mixture was adjusted to 250 mM sucrose. After the solution became translucent, the mix was incubated for 30 min on ice. Unilamellar proteoliposomes were generated by removal of detergent accomplished by repeated passage over Bio Beads SM2 columns (1 g SM2 beads/24 mg Triton X-100) [17]. The proteoliposomes were separated from non-reconstituted material by gel filtration on a Sephadex G-150 column. Protein free liposomes were generated using the same protocol by substituting buffer for microsomal extracts. For assessing protease sensitivity of lipid translocation, proteoliposomes were treated with 0.5 mg/ml of proteinase K for 30 min on ice. Digestion was stopped by addition of 1 mg/ml of PMSF.

2.4. Labeling of membranes with [^{14}C]serine

Membranes (final concentration 10 mg/ml) were incubated with 0.05 mM of [^{14}C]serine (1 μCi) in 20 mM borate buffer pH 8.0, 250 mM sucrose, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 mM CTP for 15 min at 25°C. Subsequently 1 mM non-labeled serine was added and aliquots were removed at different time points for TNBS modification.

2.5. Labeling of proteoliposomes with [^{14}C]ethanolamine

The labeling of proteoliposomes was based on the transphosphatidylation reaction catalyzed by phospholipase D [18]. 2 mg/ml protein were incubated in 100 mM borate buffer pH 5.7, 250 mM sucrose 0.5 mM $CaCl_2$, 0.35 mM [^{14}C]ethanolamine (2 μCi) and 1 U/ml of cabbage phospholipase D at 25°C. After 30 min labeling, the mixture was adjusted to pH 7.4 with borate buffer. Aliquots were removed from the reaction at different time points for TNBS modification.

2.6. Modification of PtdEtn by TNBS and lipid analysis

Fifty μl aliquots of labeled membranes or proteoliposomes were supplemented with 50 μl of 1% TNBS, 250 mM sucrose, and 100 mM borate pH 9.0. The samples were incubated at 20°C for 10 min and then supplemented with 5 μl of 5 mg/ml bovine serum albumin and 20 μl of ice cold 30% trichloroacetic acid which immediately quenched the TNBS reaction. The mixtures were incubated for 5 min on ice and centrifuged for 10 min at 20 000×*g*. The precipitate was resuspended in 80 μl of 0.1 M KCl, 0.01 N HCl. To this suspension 300 μl of $CHCl_3$/methanol/1 N HCl (495:990:15) and 100 μl of $CHCl_3$ was added. Then 100 μl of 2 M KCl, 0.01 N HCl was added, the suspension was vigorously mixed for 1 min and centrifuged for 2 min. The lower organic phase was washed once with 300 μl of 2 M KCl, 0.01 N HCl by 1 min vortexing and centrifugation. The resulting lower organic phase was dried under a nitrogen stream. The dried extracted lipids were resolubilized in 20 μl $CHCl_3$/methanol (2:1) and spotted on silica 60 gel plates. The plates were developed in $CHCl_3$/methanol/acetic acid (65:25:10). [^{14}C]Ethanolamine-containing lipids were visualized by fluorography using a phosphoimager equipped with MacBas software from Fuji. The different lipid species were identified by comparison with the following lipid standards: PtdEtn, trinitrophenyl-derivative of PtdEtn (TNP-PtdEtn), PtdSer. To determine the amount of TNBS-protected PtdEtn, the non-derivatized level of PtdEtn in Triton X-100-treated samples was subtracted as background.

3. Results and discussion

To avoid the problems inherent to experimental approaches using lipid analogs, it was important to establish an accurate method for following the bilayer localization of an endogenous phospholipid species. This approach required a precise

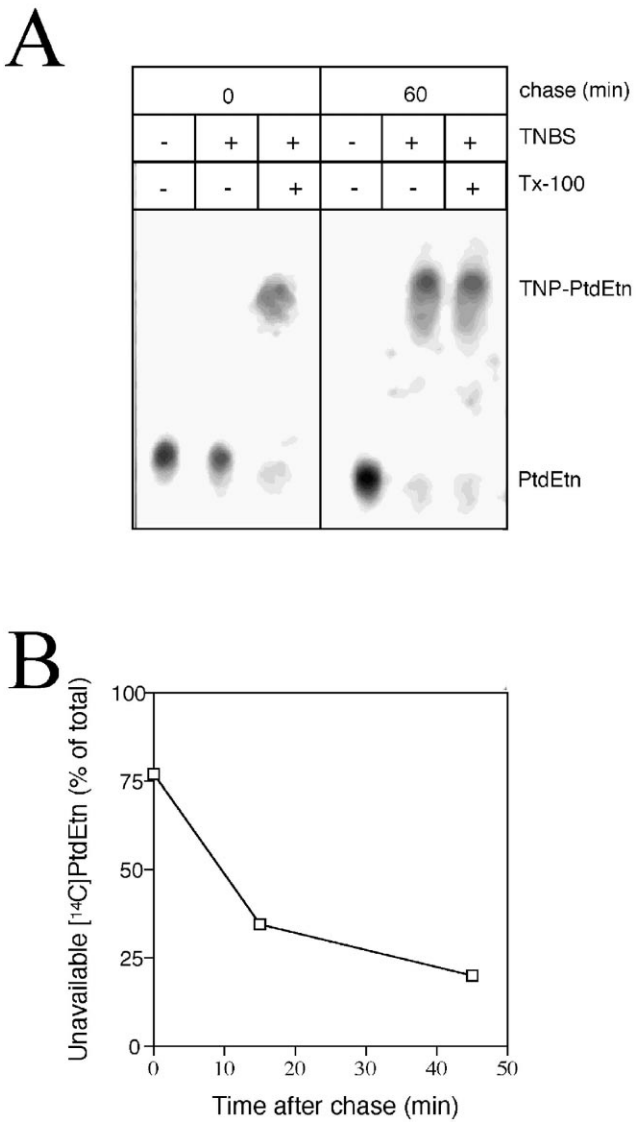

Fig. 1. Relocation of newly synthesized PtdEtn to the outer mitochondrial membrane. A: A crude yeast membrane fraction was labeled with 1 μCi [^{14}C]serine for 15 min at 25°C. Subsequently 1 mM unlabeled serine was added. At the indicated times after addition of the chase solution, aliquots were removed and analyzed. B: Kinetics of PtdEtn relocation. Labeling and analyses of lipids was conducted as described for A.

determination of leaflet location of a specific phospholipid at a given time point. In previous studies, the bilayer distribution of aminophospholipids was determined with sufficient accuracy by modification of the outer surface leaflet with the membrane impermeable reagent, TNBS [19–21]. To test whether such a protocol was applicable for the resolution of transport kinetics, we first used a TNBS modification protocol to study an already well-established biosynthetic process: the conversion of PtdSer to PtdEtn at the inner mitochondrial membrane and the subsequent rapid relocation of this phospholipid to the outer mitochondrial membrane. In studies using permeabilized cells and in cell homogenates, PtdSer was shown to be transferred to a mitochondrial fraction where it is converted to PtdEtn at the inner mitochondrial membrane in an ATP- and cytosol-independent manner [3,22]. Newly synthesized PtdEtn is rapidly transported to the outer mito-

chondrial bilayer and becomes accessible to the cytosol [23]. Though it is not clear whether flippases are involved in this process, this reaction provided the necessary control experiment for testing our TNBS modification protocol. We used a crude yeast membrane fraction containing microsomes as well as mitochondria for analyzing the conversion of PtdSer to PtdEtn in vitro. Since PtdEtn is synthesized at the inner mitochondrial membrane, the newly synthesized PtdEtn pool should be protected from modification with TNBS and only become available to this membrane impermeable reagent after its translocation to the outer mitochondrial membrane.

We used [^{14}C]serine to specifically label the outer leaflet of the isolated membrane fraction. To determine the conversion of ^{14}C-labeled outer leaflet PtdSer to PtdEtn, we conducted a pulse/chase experiment. To test the orientation of newly synthesized PtdEtn, aliquots were taken at different time points and treated with TNBS at 20°C for 10 min. TNBS penetrates the phospholipid bilayers very slowly and specifically modifies outer leaflet aminophospholipids in short incubations at 20°C [21]. As a control, identical aliquots were incubated with TNBS in the presence of the detergent Triton X-100, rendering all lipids accessible to TNBS. Newly synthesized PtdEtn was nearly completely protected from TNBS modification after 15 min of [^{14}C]serine labeling (Fig. 1A), which is consistent with previous reports that showed that PtdSer decarboxylase activity is located at the inner mitochondrial membrane [24]. Subsequently, when the reaction was chased with unlabeled serine for 60 min, virtually all labeled PtdEtn became available to modification with TNBS (Fig. 1A). This result indicated that PtdEtn was translocated to the outer leaflet of the outer mitochondrial membrane. In detergent control experiments, we observed that PtdEtn was converted almost quantitatively to TNP-PtdEtn (Fig. 1A). In further experiments we used this experimental set-up to monitor the kinetics of PtdEtn biosynthesis and translocation to the outer mitochondrial membrane in vitro (Fig. 1B). A half-time for this reaction of 5 min was extrapolated from these data which is similar to data obtained previously using a different assay [23].

Since TNBS modification could be used successfully to study the time course of PtdEtn biosynthesis in vitro, we employed the same technique to examine microsomal phospholipid flip-flop in reconstituted proteoliposomes and in protein-free liposomes. For labeling of the outer surface of the liposomes with [^{14}C]ethanolamine we used the transphosphatidylase activity of phospholipase D, which is known to recognize ethanolamine as an acceptor substrate in a phospholipid head group exchange reaction [18]. Phospholipase D requires Ca^{2+} for enzymatic activity, but since Ca^{2+} ions also trigger lipid phase separation and fusion in lipid vesicles containing anionic phospholipids such as PtdSer and phosphatidic acid (PtdOH) [25], the Ca^{2+} concentration in our assay had to be carefully controlled. Therefore, we used 0.5 mM Ca^{2+} in our experiments which is suboptimal but sufficient for phospholipase D-mediated labeling of the liposomes and precludes Ca^{2+} dependent vesicle fusion reactions. We found that [^{14}C]ethanolamine was efficiently incorporated into liposomes in a 30 min labeling reaction at the pH optimum of cabbage phospholipase D, which is pH 5.7. Under these conditions no transbilayer translocation of [^{14}C]PtdEtn was observed, as indicated by the almost complete conversion of [^{14}C]PtdEtn to [^{14}C]TNP-PtdEtn (Fig. 2A). However, when

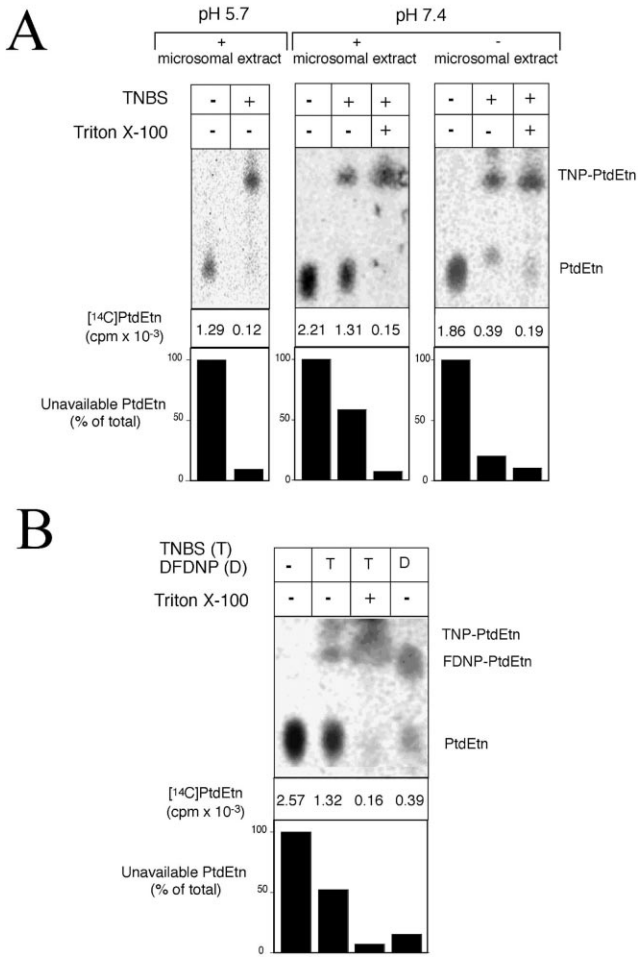

Fig. 2. Transbilayer translocation of [^{14}C]PtdEtn in reconstituted proteoliposomes. A: The outer membrane leaflets of unilamellar liposomes (PtdCho/PtdIns/PtdSer/PtdEtn) reconstituted either with or without a detergent extract from purified yeast microsomes were labeled with 2 μCi [^{14}C]ethanolamine and phospholipase D for 30 min at pH 5.7. Where indicated the reactions were adjusted to pH 7.4, further incubated for 60 min and then analyzed. B: Proteoliposomes prepared from microsomal extracts were labeled for 30 min as described for A and then adjusted to pH 7.4. After 60 min of further incubation the sample was analyzed as indicated.

the liposomes were adjusted to pH 7.4 to stop the labeling reaction and further incubated for an additional 60 min, a TNBS resistant pool of [^{14}C]PtdEtn appeared (Fig. 2A). This result was critical for kinetic experiments, since it showed that the labeling reaction could be separated from the transbilayer translocation of [^{14}C]PtdEtn. Significantly, accumulation of [^{14}C]PtdEtn unavailable to TNBS modification was strictly dependent on the presence of microsomal proteins in the liposomes (Fig. 2A). Furthermore, addition of detergent to the liposomes before adding TNBS resulted in an almost complete derivatization of [^{14}C]PtdEtn to [^{14}C]TNP-PtdEtn (Fig. 2B). Alternatively, the membrane permeable reagent DFDNB was used instead of TNBS, which resulted in conversion of the majority of labeled [^{14}C]PtdEtn to [^{14}C]FDNP-PtdEtn (fluorodinitrophenyl-derivative of PtdEtn) even in the absence of detergent (Fig. 2B). These controls demonstrated that TNBS only reacted with outer leaflet PtdEtn and that the TNBS resistant pool of PtdEtn was sequestered to the inner

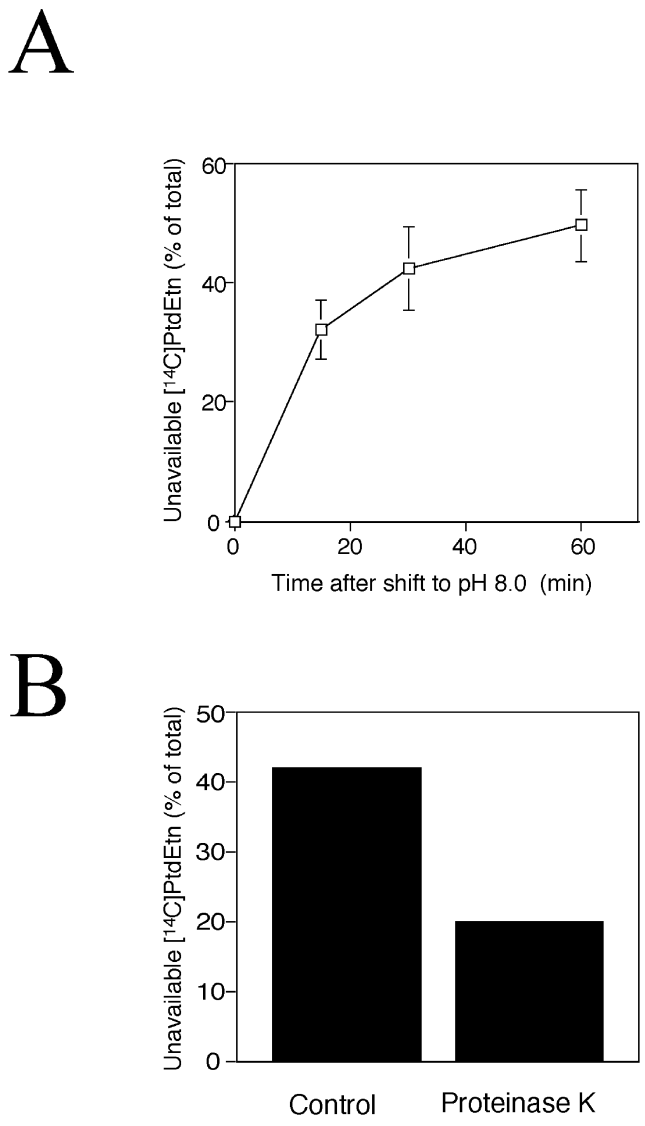

Fig. 3. Kinetics and protease sensitivity of PtdEtn flip-flop in reconstituted proteoliposomes. A: Proteoliposomes prepared from microsomal extracts were labeled for 30 min, adjusted to pH 7.4 and further incubated. At indicated time points aliquots were removed and analyzed. The amount of [^{14}C]PtdEtn unavailable to TNBS derivatization was determined and plotted. The fraction of non-derivatized [^{14}C]PtdEtn in TNBS/Triton X-100 treated samples was subtracted as background. B: Proteoliposomes prepared from microsomal extracts were either incubated with control buffer or with 0.5 mg/ml proteinase K for 30 min on ice followed by inactivation of the protease by PMSF. Afterwards the proteoliposomes were labeled for 30 min, adjusted to pH 7.4 and further incubated for 30 min. The amount of protected [^{14}C]PtdEtn was calculated and plotted. The fraction of non-derivatized [^{14}C]PtdEtn in TNBS/Triton X-100-treated samples was subtracted as background.

leaflet of the liposome lipid bilayer. Taken together these results indicated that active transbilayer transport of endogenous PtdEtn was measured in our assay.

In further experiments we determined the time course of PtdEtn translocation in the reconstituted system. The proteoliposomes were labeled for 30 min with [^{14}C]ethanolamine at pH 5.7 as above and then shifted to pH 7.4. From this reaction aliquots were taken at different time points, subjected to TNBS modification and analyzed by TLC. The respective spots representing [^{14}C]PtdEtn and TNP-[^{14}C]PtdEtn were quantified and the results were plotted as shown in Fig. 3A. The transbilayer reaction of PtdEtn occurred with a $t_{1/2}$ of 10 min. From these data a PtdEtn translocation rate of 8 μmol/min × g protein was calculated. In addition, no bilayer asymmetry of PtdEtn between the outer and inner membrane leaflet was generated in the reconstituted systems since about 50% of the labeled PtdEtn was protected from TNBS derivatization at equilibrium. Finally, we examined whether the transbilayer translocation of PtdEtn in the reconstituted system was protein-mediated. Proteoliposomes were incubated in the absence or presence of 0.5 mg/ml proteinase K for 30 min on ice. Subsequently, the protease was inactivated by addition of PMSF. These pretreated proteoliposomes were then used in the lipid translocation assay. Treatment with proteinase K had a pronounced effect on flippase activity. However, a high residual activity of about 50% of PtdEtn transbilayer translocation was observed in protease-treated samples (Fig. 3B). Similar protease-protected flippase activities were also observed in a number of studies that employed lipid analogs in intact microsomes [8,10]. This protease-protected fraction of flippase activity may be explained by the possibility that the responsible lipid transporter is deeply embedded in the phospholipid bilayer and not readily accessible to proteolytic attack.

In summary, the work presented here describes the first successful reconstitution of microsomal transbilayer translocation of endogenous aminophospholipids. We have described a simple assay for measuring the kinetics of this phospholipid flip-flop in reconstituted proteoliposomes from yeast. Our data provide strong evidence that the majority of the transbilayer translocation of PtdEtn in yeast microsomes depends on specific protein factors located in the microsomal membrane. Finally, the use of yeast as a model system for reconstituting microsomal flippase activity allows the combination of genetic analysis with a powerful biochemical assay which should foster the identification of long sought after components involved in endomembrane phospholipid translocation.

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