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Rapid report

Phosphatidyl ethanolamine is essential for targeting the arginine transporter Can1p to the plasma membrane of yeast

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Abstract

In continuation of our previous study, we show that phosphatidyl ethanolamine (PE) depletion affects, in addition to amino acid transporters, activities of at least two other proton motive force (pmf)-driven transporters (Ura4p and Mal6p). For Can1p, we demonstrate that the lack of PE results in a failure of the permease targeting to plasma membrane. Despite the pleiotropic effect of PE depletion, a specific role of PE in secretion of a defined group of permeases can be distinguished. Pmf-driven transporters are more sensitive to the lack of PE than other plasma membrane proteins.

Keywords: Phosphatidyl ethanolamine (PE); Arginine permease; Secretory pathway retention

Phosphatidyl ethanolamine (PE) has been recognized as a non-bilayer lipid that evokes lateral pressure within membranes resulting in stabilization of membrane proteins in native conformations [1–3]. New insights into an additional role of this phospholipid in membrane transport processes were furnished by studies of the effect of PE on LacY permease in *E. coli* [4]. PE was shown to be necessary for the permease to couple transport with the electrochemical proton gradient across the membrane [5]. In addition, both in vivo and in vitro experimental evidence has been presented that PE acts as a molecular chaperone in the folding of this polytopic membrane protein [5–7].

Yeast triple mutant of *Saccharomyces cerevisiae*, RY200T, lacking all pathways for the biosynthesis of PE (*psd1*, *psd2*, *dpl1*), is able to grow only in the presence of ethanolamine. Its omission from the medium and replacing by choline led to PE depletion of the cells which, however, for a limited time period, retained the same viability, exogenous and endogenous respiration and plasma membrane ATPase activity as the control cells. On the other hand, the PE depletion had a dramatic effect on the accumulation of amino acids mediated by several distinct amino acid transport systems (Gap1p, Can1p, Put4p). Under the same conditions, the accumulation of hexoses mediated by facilitated diffusion was unaffected [8].

Two main questions were addressed in the present work:

(1) Is the observed effect of PE depletion restricted to amino acid transporters, or is this phospholipid required also for other proton motive force (pmf)-driven transport systems in the yeast plasma membrane?

(2) Is the observed decrease of amino acid accumulation caused by a selective decrease in the synthesis of the pertinent amino acid permeases or is the PE effect exerted at some different step?

(1) Activities of two other transport systems in yeast plasma membrane coupled to pmf were tested for their dependence on PE in the triple mutant of *S. cerevisiae* RY200T.

In yeast, the maltose transport system has been identified as being pmf dependent. The gene that encodes the maltose transporter is clustered with two other genes required for maltose utilization—maltase and a transcription activator (review Ref. [9]). Its expression is induced by maltose and repressed by glucose. However, many laboratory strains are unable to grow on maltose because they are deficient in the regulatory genes of MAL loci. Consequently, they do not express either the maltose transporter or maltase. Since this was the case of the triple mutant, we transformed this strain with a plasmid containing a complete *MAL1* locus [10]. The transformants were selected for their ability to grow on

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maltose and the initial rate of its transport was checked in PE-containing (ethanolamine-grown) and PE-depleted (choline-grown) cells. As shown in Fig. 1A, the accumulation of radioactive label from maltose was reduced by a factor of about 3 in PE-depleted cells as compared to PE-containing cells. An even more drastic effect (more than a 20-fold decrease) upon PE depletion was observed on the uptake of uracil (Fig. 1B) mediated by a specific proton symporter encoded by *FUR4* gene [11]. For comparison, the effect of PE depletion on arginine accumulation by Can1p, and a lack of the effect on glucose-derived radioactivity accumulation in the same batches of cells, are shown in Fig. 1C and D, respectively.

(2) For a detailed study of the PE-depletion effect on pmf-driven membrane processes, the specific arginine transporter Can1p [12] was used as a representative of yeast amino acid permeases.

For the sake of identification and quantification of Can1p, *CAN1* gene was cloned as a fusion product with the biotinylation peptide of *Klebsiella pneumoniae* and 6His tail. The fusion protein, Can1BioHisp, was proved to be equally active as the original one (data not shown). After expression in the triple mutant, the biotinylated permease was detected by a streptavidin–horseradish peroxidase conjugate. As shown in the Western blot analysis after SDS-PAGE resolution in Fig. 2, the protein is equally

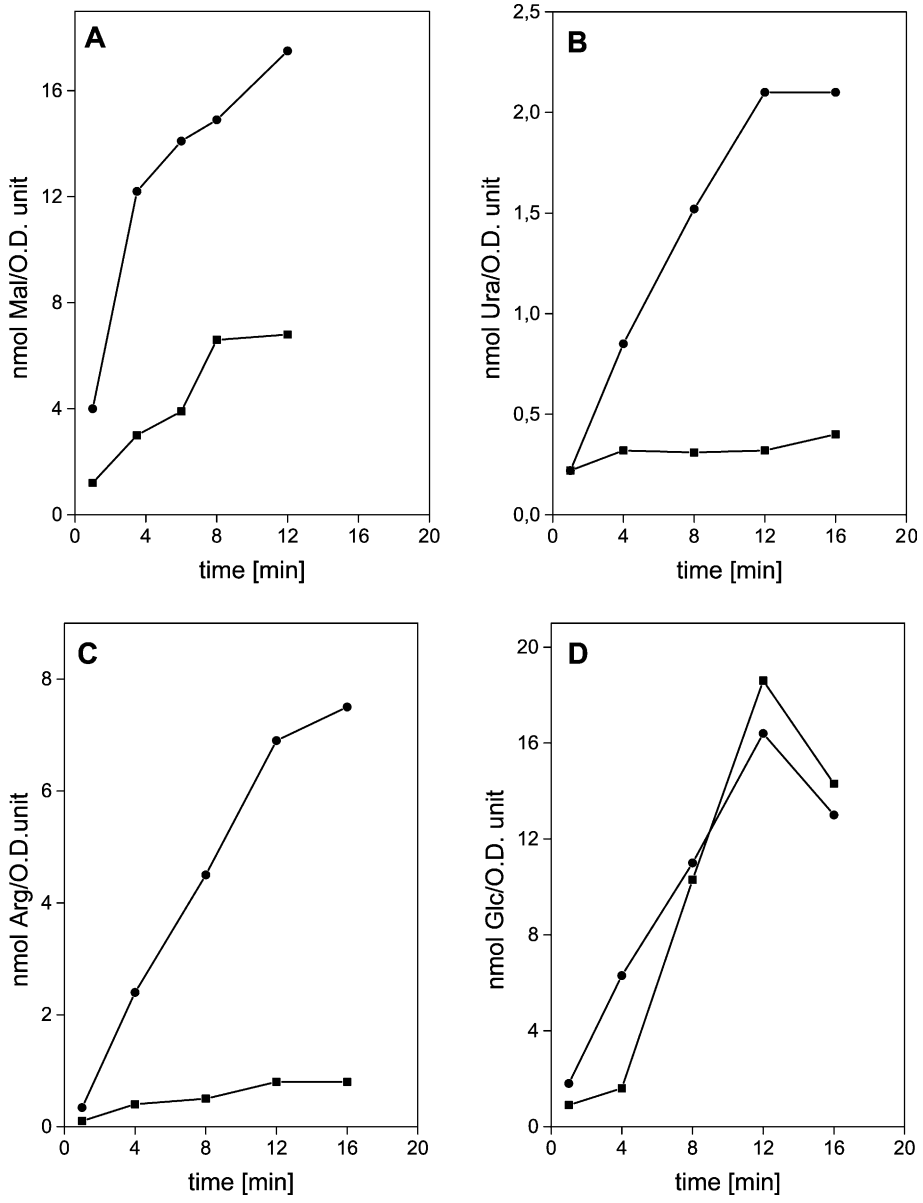

Fig. 1. Effect of PE depletion on activities of Mal6p, Ura4p, Can1p and Hxt1p measured as a substrate radioactivity accumulation in *S. cerevisiae* RY200T. Cells were grown on ethanolamine or choline medium as described previously [8]. To aliquots of yeast suspensions of ~ 2 OD/ml, radioactive substrates were added to final concentrations of 2 mM maltose (A), 50 μM uracil (B), 20 μM arginine (C) or 1 mM glucose (D). Samples were withdrawn at intervals, filtered on 0.8 μm pore-size cellulose nitrate filters and washed. The radioactivity was determined by scintillation counting.

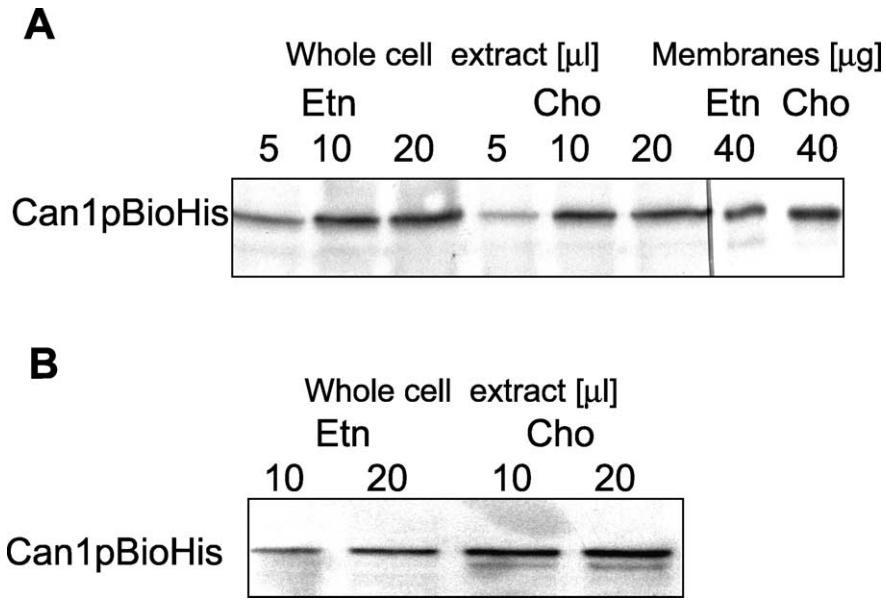

Fig. 2. Western blot analysis of Can1BioHisp content in PE-containing or PE-depleted cells. Whole cell extracts from 2×10^6 cells or extracts from total membrane fraction were prepared from PE-containing or PE-depleted cells by conventional methods. SDS-dissociated samples were subjected to SDS-PAGE and Western blot analysis. Can1pBioHisp was detected by streptavidin–peroxidase conjugate (Amersham) (A) or by anti-His antibody (Quiagen) as the primary, and by horseradish peroxidase-linked anti-mouse immunoglobulins (Sigma) as the secondary antibody (B). Enhanced chemiluminescence system (ECL, Amersham) was used for visualization.

expressed in PE-depleted and PE-containing cells; the amounts of the protein both in whole cell extracts and in total membrane fractions of choline- and ethanolamine-grown cells did not significantly differ. To avoid the possibility of misinterpretation due to possible different biotinylation efficiency in PE-containing and PE-depleted cells, the Can1BioHisp was also identified with the use of a specific antibody against the 6His tail engineered at the C-terminus of the Can1p construct. The same results as with streptavidin used for Can1BioHisp identification were obtained using anti-His antibody (Fig. 2B). The insignificant differences in the amounts of protein identified with the use of the two different antibodies do not correlate with the drastic decrease of Can1BioHisp activity observed in the corresponding cells (Fig. 1C).

If the total amount of permease in the cell membrane fraction is independent of the PE presence, it can be envisaged that the protein is either inactive in PE-depleted cells and/or it is not targeted correctly to the plasma membrane. The latter possibility was tested by fractionating total cell membranes on equilibrium density gradient (12–54% sucrose) according to the Ljungdahl Lab Protocol (kindly provided by F. Gilstring, Stockholm). After centrifugation, aliquots of each individual fraction were analysed for the presence of Can1pBioHisp and for the markers of plasma membrane and membrane organelles. As documented in Fig. 3, the pattern of the Can1BioHisp distribution in individual sucrose fractions is completely different in PE-containing and PE-depleted cells. The permease from PE-containing cells co-localized with the plasma membrane marker Pma1p, the plasma membrane ATPase, in the heavy membrane fractions. In contrast, the permease produced in PE-depleted cells was found in lighter fractions, where it co-localized with organelles of the secretory pathway—ER and Golgi apparatus—detected by specific membrane fraction markers Shr3p and Anp1p, respectively. This clearly proves that the dramatic decrease in arginine accumulation mediated by Can1BioHisp in PE-depleted cells is primarily caused by an inability of the permease to reach the plasma membrane and, most obviously, by its being retained in the organelles of the secretory pathway.

Can1p, like other amino acid permeases, is co-translationally inserted into the membrane of ER. To be subsequently delivered to the plasma membrane, it must be properly folded in its native conformation and, with the assistance of Shr3p, it has to be included into COPII coated transport vesicles [13–15]. As suggested earlier [16] and demonstrated recently for the general amino acid permease Gap1p, Shr3p does not participate in the co-translational insertion of amino acid permeases into ER [17].

PE in *E. coli* was shown to participate in a late step of LacY conformational maturation. The critical folding steps after membrane insertion are dependent on the interaction of LacY with PE [5–7]. It can be envisaged that events analogous to those occurring at the cell membrane of prokaryotes do take place at the level of subcellular membranes in eukaryotes. The PE is delivered to ER from mitochondria and from the Golgi/vacuole compartment (PE produced by phospatidyl serine decarboxylation mediated by Psd1p and Psd2p, respectively [18,19]) and/or from the Kennedy pathway [20] utilizing either external ethanolamine or ethanolamine–phosphate arriving from degrada-

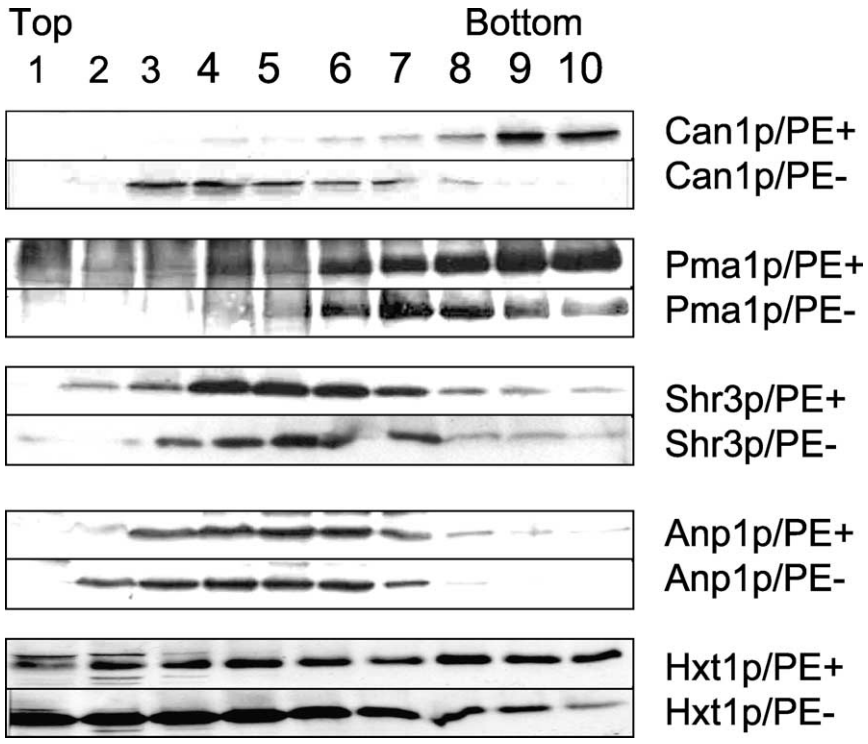

Fig. 3. Fractionation of cell membranes. Total cell membranes prepared from PE-containing and PE-depleted cells were fractionated on stepwise sucrose gradient (12–54%) by centrifugation for 3 h at 150,000 × *g* (SW-41) at 4 °C. An aliquot of 30 μl of each fraction was dissociated in SDS sample buffer at 50 °C for 5 min and resolved by SDS-PAGE and Western blot. The individual proteins were detected as above by the corresponding specific primary antibodies and by peroxidase–immunoglobulin conjugates (Sigma). Can1BioHisp was identified by streptavidin–peroxidase conjugate.

tion of sphingolipids (includes Dpl1p) [21]. Similarly as documented for LacY permease, proper Can1p folding might be crucially dependent on the presence of PE. The quality control machinery [22] would subsequently prevent the misfolded protein from being delivered to the correct place of its destination. Instead, it is retained in the compartments of the secretory pathway and/or eventually, rerouted for degradation into the vacuole [23,24].

Two other plasma membrane proteins were tested for their secretion to the plasma membrane under PE depletion. As shown previously and in this study, activities and total amounts of Pma1p (H^+–ATPase) and of a hexose transporter (Hxtp1p) are considerably less affected. Under the conditions used in this study (2% of glucose), the transport of glucose is mediated exclusively by the low-affinity/high-capacity transporter Hxt1p [25]. Though there was no difference in the accumulation of radioactive label originating from glucose (Fig. 1D), the pattern of Hxt1p distribution was slightly shifted toward lighter fractions in PE-depleted cells (Fig. 3), indicating thus that the trafficking of the protein toward the plasma membrane is somewhat impaired. The distribution of Hxt1p along all the membrane fractions (in contrast to distinct localization of other membrane markers) has been also observed by E. Boles, Düsseldorf (personal communication).

Plasma membrane ATPase is delivered to the cell surface by the secretory pathway [26]. Its distribution pattern in the membrane fractions from PE-depleted cells is, similarly as found for Hxt1p, partially shifted from the plasma membrane fractions toward the lighter ones (Fig. 3). All this indicates that the complete lack of PE in yeast interferes, to a certain degree, with the secretion of membrane proteins in general, and may reflect different demands of different nascent proteins for PE to undergo the complete secretion procedure. A family of permeases, sharing as common feature the coupling to pmf (amino acid permeases, Furp4p), is affected most seriously. For Can1p, the reason might be an incorrect folding of the permease in PE-less ER membrane and its consequent arresting in the secretory organelles. The Shr3p responsible for selective inclusion of amino acid permeases into transport vesicles [14] is an integral membrane component residing in ER membranes. It contains four membrane spanning segments and therefore it cannot be excluded that the PE depletion also affects Shr3p proper folding and/or function, which eventually leads to the same phenotype of Can1p retention in ER. This, however, would not explain the effect of PE depletion on Fur4p.

Requirement for PE is more stringent on nonfermentable than on fermentable carbon sources. Very recently, an essential role of PE was reported in the function of mitochondria-related processes in yeast [27] that is independent of its commonly accepted ability to form hexagonal phase structure [28]. The decreased amount of PE caused by deletion of pds1p located in the mitochondrial membrane

is not sufficient to support growth of *psd1Δ* cells on lactose and at elevated temperatures [27,29]. The specific requirement for PE in *psd1Δ*, *psd2Δ*, *dpl1Δ* strain could not be satisfied by phosphatidyl propanolamine, a structurally related lipid capable of forming the hexagonal phase [29]. This indicates that the essential function of PE is independent of its ability to form hexagonal phase structure. Disruption of two pathways leading to PE synthesis (psd1p, psd2p) results in an impairment of synthesis of glycosylphosphatidylinositol-anchored proteins (Gas1p), while the amount of carboxypeptidase and the activity of invertase are not affected [27]. This double mutant, however, still contains about 10–20% of PE (as compared to wild type) resulting from sphingolipid catabolism [18].

Using the triple mutant (*psd1Δ*, *psd2Δ*, *dpl1Δ*) devoid of all pathways leading to PE synthesis in endogenous metabolism, we show that the considerably decreased PE content in these cells grown on ethanolamine (about 5% as compared to the wild type) is sufficient to ensure the correct targeting of several (if not the majority) plasma membrane proteins. Further PE depletion caused by replacing the external ethanolamine by choline, however, results in severe defects in the traffic of a defined group of permeases to plasma membrane.

Secretion of membrane proteins, the activities of which are independent of the coupling to pmf, is considerably less affected.

Despite the pleiotropic consequences of PE depletion for an extended period of time, a primary specific effect on delivery of a pmf-driven plasma membrane permease Can1p can be distinguished. We demonstrated that the effect of PE depletion is exerted already at the level of protein secretion and/or targeting.

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