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Differential effect of phosphatidylethanolamine depletion on raft proteins Further evidence for diversity of rafts in *Saccharomyces cerevisiae*

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Abstract

A considerable amount of evidence supports the idea that lipid rafts are involved in many cellular processes, including protein sorting and trafficking. We show that, in this process, also a non-raft lipid, phosphatidylethanolamine (PE), has an indispensable function. The depletion of this phospholipid results in an accumulation of a typical raft-resident, the arginine transporter Can1p, in the membranes of Golgi, while the trafficking of another plasma membrane transporter, Pma1p, is interrupted at the level of the ER. Both these transporters associate with a Triton (TX-100) resistant membrane fraction before their intracellular transport is arrested in the respective organelles. The Can1p undelivered to the plasma membrane is fully active when reconstituted to a PE-containing vesicle system in vitro. We further demonstrate that, in addition to the TX-100 resistance at 4 °C, Can1p and Pma1pa exhibit different accessibility to nonyl glucoside (NG), which points to distinct intimate lipid surroundings of these two proteins. Also, at 20 °C, these two proteins are extracted by TX-100 differentially. The features above suggest that Pma1p and Can1p are associated with different compartments. This is independently supported by the observations made by confocal microscopy. In addition we show that PE is involved in the stability of Can1p–raft association.

Keywords: Lipid raft; Trafficking; *Saccharomyces cerevisiae*; Can1p; Pma1p

1. Introduction

The lipid bilayer constituting biological membranes is no longer viewed as a homogeneous fluid. Instead, biological membranes are currently viewed as a mosaic of different compartments formed by domains of distinct lipid and protein composition. Domains enriched in sphingolipids and sterols are called lipid rafts. Originally, rafts have been proposed to function as a sorting platform for the apical delivery of plasma membrane proteins in epithelial cells [1,2]. Recent increasing evidence documents that lipid rafts reflect the planar organization common to plasma membrane of most if not all eukaryotic cells.

So far, four plasma membrane transporters of yeast plasma membrane were shown to be associated with lipid rafts. These include plasma membrane ATPase, Pma1p [3–6] uracil permease, Fur4p [7,8] and two amino acid permeases, Can1p [9] and Tat2p [10].

The trafficking of Pma1p was shown to be conditioned by the formation of oligomers before exiting the ER [6]. The oligomerization is linked to ceramide synthesis. Pma1p does not form oligomers in mutant cells containing lower level of sphingolipids (*lcb1-100*), and after entering the COPII vesicles it is rerouted to the vacuole for degradation [6]. A dramatic reduction of Pma1p–raft association was observed in mutants with altered plasma membrane lipid composition (sterol/sphingolipid ratio) due to combined defects in acyl-chain elongation (*elo3*) and ergosterol synthesis (*erg6*) [11]. On the other hand, raft-integration of another raft-associated protein Gas1p was not affected under the same conditions. Judged from its localization to the floating fraction of a density gradient performed after TX-100 treatment, arginine permease Can1p also behaves like a typical raft-associated protein.

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As shown by its decreased transport activity and by the visualization of Can1pGFP in living cells, defects in ergosterol or sphingolipid syntheses result in the cessation of Can1p delivery to the plasma membrane [9].

In general, the depletion of the typical raft lipids, sterols and/or sphingolipids results in the impairment or a complete cessation of the trafficking of raft-residing proteins. In the present study, we document that also a non-raft phospholipid, phosphatidylethanolamine (PE), is directly involved in the sorting and trafficking of at least two raft-inhabiting integral plasma membrane proteins Can1p and Pma1p.

The physiological function of PE in yeast has been studied in mutants deleted in one, two or all three key enzymes (*PSD1*, *PSD2*, and *DPL1*) leading to PE biosynthesis. The analysis of different mutants or their combinations revealed that a minimum level of PE is essential for growth [12,13]. Strains with decreased content of PE could not grow on non-fermentable carbon sources and were sensitive to elevated temperature. A specific function of PE was demonstrated in prokaryotic cells of *E. coli* where it acts as a specific chaperon assisting the LacY permease folding [14]. In yeast, PE exhaustion was shown to cause retention of several plasma membrane transporters in internal membranes while the trafficking of some others was unaffected [15,16]. In this study we document that the trafficking of two raft-residing proteins, Can1p and Pma1p, is controlled not only by their raft association but, independently, also by the availability of PE. The lack of PE causes the transporter's retention in the membranes of the secretory pathway; interestingly, each transporter is arrested in a distinct compartment. PE depletion does not prevent the protein–raft association in cells as evaluated by the standard criterion of resistance to solubilization by TX-100, neither does it seem to affect the protein folding.

2. Materials and methods

2.1. Materials

2.1.1. Antibodies

Can1BioHis p was detected with the use of streptavidin–peroxidase conjugate (Amersham Biosciences). Specific primary antibodies against the Pma1p, Anp1, a component of the M-Pol II complex embedded in the Golgi membranes, and Wbp1p-dolichyl-diphosphooligosaccharide-protein glycosyltransferase beta subunit precursor embedded in the ER membranes were generous gifts of R. Serrano (Valencia), S. Munro (Cambridge) and L. Lehle (Regensburg) respectively. Horseradish peroxidase-linked anti-rabbit or anti-mouse immunoglobulins were from Amersham Biosciences. Phospholipids L-α-phosphatidyl ethanolamine (type IX from *E. coli*, approximately 50%), cardiolipin, phosphatidyl serine and phosphatidyl choline were obtained from Sigma. Detergents Triton X-100, *n*-Nonyl-β-D-glucoside (NG) and Fos-choline-16 were from Anatrace. Arginine (11.26 GBq/mmol) was from Amersham Biosciences.

2.2. Yeast strains, media and growth conditions

The strain of *Saccharomyces cerevisiae* RY200T disrupted in all biosynthetic pathways leading to phosphatidyl ethanolamine (PE) synthesis [16] was used in this study. Its final genotype is (Mat α, *ura3-52, leu2-3, 112, his3-* Δ*200, trp1-* Δ*901, lys2-801, suc2-* Δ*9, GAL, psd1* Δ::*TRP1, psd2* Δ::*HIS3, bst1=dpl1* Δ:: *KANR*) and it is referred to as a "triple mutant" in further text. For confocal microscopy studies or for detection by specific antibody, the strain was transformed by plasmid pVTU100 bearing a *CAN1* gene tagged with a sequence coding for GFP [9] or RFP [8]. The ER membranes were visualized with the use of an expression of centromeric plasmid pJK53 bearing N-terminally tagged GFP Shr3p fusion protein that is expressed under the control of the endogenous SHR3 promoter (generally provided by P. Ljungdahl, Sweden; not published). For Western blot detection of Can1p, the strain was expressing *CAN1BioHis* [15]. The cells were grown at 30 °C on rich (YPD) or yeast minimal (YNB — yeast nitrogen base w/o amino acids) media containing 2% glucose and either 2 mM ethanolamine (Etn) or 4 mM choline (Cho). YNB media were enriched with a "drop-out" mixture containing all amino acids and bases with the exception of arginine and marker amino acids that were added when required.

2.3. PE starvation scheme

Cells grown on yeast minimal medium with ethanolamine were collected, washed and inoculated to either the same fresh medium or to the medium where ethanolamine was replaced by choline. Overnight cultures were collected in the logarithmic/stationary transition phase, washed, transferred to fresh corresponding media and used for the transport assays and immunological estimation of membrane proteins. As shown previously, this procedure led to a substantial decrease of phosphatidyl ethanolamine in the crude membrane fraction of choline-grown cells (PE-depleted) as compared with ethanolamine-grown cells (PE-containing), while the viability of the cells remained largely unaffected [15,16].

2.4. Preparation of crude membrane fractions

Yeast were grown to OD_{578} not exceeding 1.2 and harvested. The pellets (50 OD_{578}) were chilled on ice and all the other steps were carried out at 4 °C. Pellets were resuspended in 1 ml of lysis buffer (0.8 M sorbitol, 10 mM TNE (Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM Na_4 EDTA)

containing 1 mM PMSF, leupeptin, pepstatin and aprotinin, 1 μg/ml each). Cells were broken with glass beads in FastPrep FP120 Bio101, Thermo Savant) twice for 30 s. The unbroken cells and heavy cell debris were removed by two successive 5-min low-speed centrifugation at 3400 × *g* and 4800 × *g*. Crude membranes from the supernatant were collected by centrifugation at 40,000 × *g* for 20 min.

2.5. Detergent-resistant membrane isolation

2.5.1. Detergent resistant membranes

Lipid rafts were isolated according to [4] with modifications described in [9]. In short, crude membrane preparation (200–300 μg of protein) in TNE buffer with protease inhibitors were adjusted to a volume of 300 μl, TX-100 was added to a final concentration of 1% and the mixture was incubated for 30 min on ice. The samples were mixed with Optiprep (Nycomed, Vienna, Austria) to final a concentration of 40% (wt/vol.), transferred to centrifuge tubes and overlaid with 1.32 ml of 30% Optiprep in TNEX (TNE plus 0.1% TX-100) followed by 220 μl of TNEX. After centrifugation for 2 h at 50,000 rpm in Beckman SW60 rotor at 4 °C, six equal fractions were collected from the top of each gradient and the protein content in each fraction was analyzed by SDS-PAGE and Western blotting either directly or after TCA precipitation.

2.6. Extraction of membranes with TX-100

Aliquots of membranes (200 μg protein) were incubated at 20 °C for 30 min in 100 μl 10 mM TNE with protease inhibitors containing the varying indicated concentrations of TX-100. The suspensions were subjected to low-speed centrifugation (10,000 × *g*, 30 min) at 4 °C. Each of the pellets was resuspended in 100 μl of the buffer containing corresponding TX-100 concentration and the same centrifugation was performed. The resulting pellets were analyzed by Western blotting.

2.7. Sucrose gradient fractionation

Cell membranes were fractionated on equilibrium density gradients according to the Ljungdahl Lab Protocol (kindly provided by F. Gilstring) as adapted from [17–19]. Briefly, amounts of cells corresponding to OD_{578}=50 were collected, washed and resuspended in 1 ml of a breaking buffer (0.8 M sorbitol, 10 mM MOPS, pH 7.2) supplemented with either 4 mM Na_4EDTA or 4 mM $MgCl_2$. The breaking buffer was complemented by a protein inhibitor mixture as above. The crude membranes were layered onto a stepwise sucrose gradient (12%–54% sucrose) containing either 4 mM Na_4EDTA or 4 mM $MgCl_2$. After centrifugation for 3 h at 150,000 × *g* (Beckman rotor SW-41) at 4 °C, 1-ml fractions were collected from the top and 30-μl aliquots were analyzed for the presence of individual proteins: Can1p, Pma1p, Anp1 and Wbp1p.

2.8. Western immunoblotting and protein detection

The resolved proteins were blotted from gels onto a PVDF membrane (Amersham Biosciences) for 25 min at 360 mA. The membranes were blocked in TBS-T buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing either 2% BSA or 2% milk, and probed with various antibodies. For the detection of Pma1p, Wbp1p and Anp1p, the membranes were incubated at 4 °C with the corresponding antibodies overnight. Goat anti-rabbit peroxidase conjugate, diluted 1/10,000, was used as a secondary antibody. Can1p Bio His was detected by streptavidin horseradish–peroxidase conjugate. For repeated detection with a different antibody, the membranes were incubated in 50 ml of stripping buffer (62.5 mM Tris–HCl, pH 6.7; 2% SDS) supplemented with 350 μl β-mercaptoethanol for 30 min at 50 °C. After repeated washing in TBS, the membranes were blocked and re-probed with another antibody as required. The proteins were visualized by ECL kit (Amersham Biosciences) and their quantity evaluated by the AIDA software, 3.28 version.

2.9. Reconstitution of Can1p

Can1p was reconstituted into vesicles as described in [20]. Briefly, *E. coli* phospholipid or a mixture of cardiolipin, phosphatidyl serine and phosphatidyl choline (1:1:1) purified according to [21] was resuspended by probe sonication in 0.3% NG in 50 mM potassium phosphate, pH 6.3, and supplemented with cytochrome *c* oxidase (0.2 mg protein). The mixed suspensions were incubated on ice for 10 min and then dialyzed overnight against 5 l of 50 mM potassium phosphate and 2 mM $MgSO_4$, pH 6.3. Resulting proteoliposomes (6 mg of phospholipid) were mixed with crude membranes (300 μg protein), frozen in liquid nitrogen, slowly thawed at room temperature and briefly sonicated with a probe-type ultrasonic homogenizer.

2.10. Transport assays

For testing the arginine uptake into whole cells, a mixture of radioactive and non-radioactive arginine was added to aliquots of 0.6 ml of PE-containing or PE-depleted cells. Samples of 100 μl were withdrawn at intervals, diluted in 2 ml of water, filtered on 0.8 μm pore-size cellulose acetate filters (Schleicher & Schuell) and washed with another 2 ml of water. The radioactivity was determined by scintillation counting.

The accumulation of arginine into reconstituted vesicles was measured after energizing the system with 22 mM ascorbate, 260 μM *N*,*N*,*N*′-tetramethyl-*p*-phenylenediamine and 26 μM cytochrome *c*. Aliquots of 50 μl were withdrawn at intervals, diluted with 2 ml of 100 mM LiCl, filtered through 0.22-μm-pore-size cellulose acetate filters (Schleicher & Schuell) and washed with another 2 ml of 100 mM LiCl.

2.11. Determination of transmembrane electrical potential $\Delta\Psi$

The membrane potential (inside negative) was determined from the distribution of tetra-[^{3}H]phenylphosphonium cation as described in Opekarová and Tanner [22].

3. Results and discussion

3.1. Visualization of Can1p in PE-containing and PE-depleted cells

Our previous studies with confocal microscopy of GFP-labeled proteins revealed that Can1p and Pma1p are not distributed homogeneously in the plasma membrane but occupy distinct membrane domains characteristic for each protein. The depletion of the raft lipids resulted in the alternation of the typical distributions of the proteins mainly due to the failure of their delivery to the plasma membrane [9]. Recently we showed that also the depletion of a non-raft lipid, PE, resulted in a defect of Can1p delivery to the plasma membrane [15].

To visualize the changes of Can1p distribution in living cells due to the PE depletion, the GFP fusion protein [9] was expressed in *S. cerevisiae* RY200T bearing deletions in all pathways leading to PE biosynthesis [16]. The deletant cells can grow only in the presence of ethanolamine that is converted to PE by the Kennedy pathway [23]. In ethanolamine-grown cells (PE-containing cells), Can1p was distributed in the plasma membrane in the characteristic patchy pattern (Fig. 1), which was observed in wild type cells [9]. When the ethanolamine in the growth medium was replaced by choline, the cell viability was preserved for at least 14 h while their internal PE was largely depleted [16]. In these PE-depleted cells, Can1p was observed to accumulate in dot-like formations inside the cells (Fig. 1B). This distribution resembles the localization of the Golgi markers (see localization database, [24]). A further indication that PE-depletion results in the retention of Can1p in the Golgi membranes was obtained by the fractionation of the total membranes isolated either from the PE-depleted or PE-containing cells (see below).

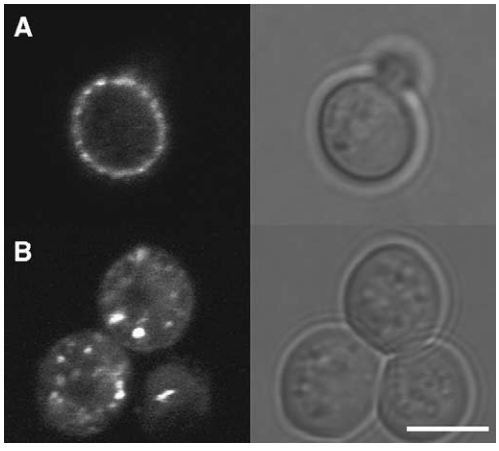

Fig. 1. Localization of Can1GFP depends on PE. Can1GFP was expressed in strain RY200T, and the cells were grown in the presence 2 mM ethanolamine (A) (PE-containing cells) or 4 mM choline (B) (PE-depleted cells). Transversal optical sections (left) and differential interference contrast images (right) are also shown. Bar: 5 μm.

3.2. Raft association is not affected by PE-depletion

We checked whether Can1p and Pma1p associate with rafts already in the membranes of the secretory pathway. For this purpose, the crude membranes were isolated from both PE-containing and PE-depleted cells. After treatment with TX-100 at 4 °C and Optiprep density gradient centrifugation, the majority of Can1p and Pma1p from both types of membranes was detected in the upper raft—containing floating fractions 1 and 2 (Fig. 2A, B). The TX-100 treatment and subsequent density gradient centrifugation is a method generally used for testing the protein–raft association. As evident from Fig. 1B, in the PE-depleted cells, Can1p is exclusively located in internal membranes. Thus, according to the criterion above, it has to be concluded that Can1p is incorporated into rafts already in the internal membranes en route to the plasma membrane. Hence, most obviously, the PE depletion does not affect the Can1p–raft association and thus, in this case, the raft association is not the bottleneck for Can1p trafficking to the plasma membrane.

3.3. PE depletion interrupts the trafficking of Can1p and Pma1p at different points of the secretory pathway

As shown above, Can1p associates with rafts already in the internal membranes of PE-depleted cells. Therefore, PE seems to be required for the protein trafficking in the steps subsequent to its raft association. To characterize the trafficking defect of Can1p in more detail, we attempted to determine the cell compartments in which Can1p is accumulated due to PE depletion. For this purpose, resolution of membranes from PE-containing and PE-depleted cells was performed on sucrose density gradients in the absence or presence of Mg^{2+} ions, i.e., under conditions which have been shown to influence the separation of different types of membranes. In the presence of EDTA (Mg^{2+} ions were removed from the lysate by chelation), the ER and Golgi membranes have a low buoyant density and are well resolved from the denser plasma membrane [25]. The presence of magnesium ions in the lysate produces ER membranes with a much higher buoyant density than the Golgi membranes, due to their association with ribosomes remaining in the preparation [26]. Membranes prepared from PE-containing or PE-depleted cells were resolved on sucrose gradients containing either EDTA or Mg^{2+} (see Materials and methods). As shown in Fig. 3A and B, independently of the presence of Mg^{2+}, internal

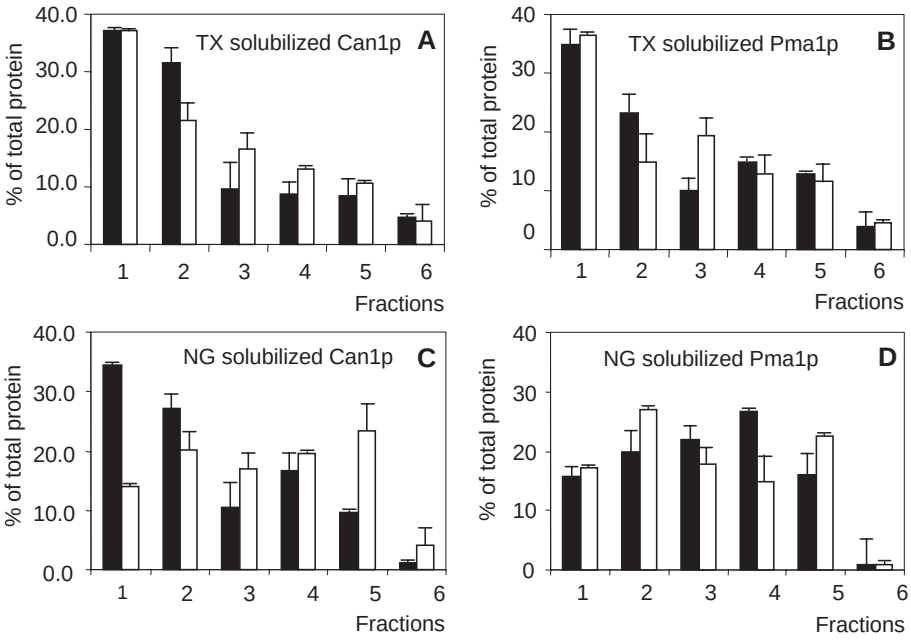

Fig. 2. PE effect on Can1p and Pma1p distributions in fractions of floating density gradient. Crude membranes from PE-containing (full columns) or PE-depleted (empty columns) cells (250 μg of protein of each) were solubilized by 1% TX-100 (A, B) or 0.3% NG (C, D), and the solubilizates were centrifuged on Optiprep gradients. Resulting fractions of 0.4 ml were collected from the top, TCA precipitated, resolved by SDS PAGE and, after Western blotting, immunoanalyzed for individual proteins. The spots were evaluated by AIDA software. The mean values of three experiments are presented.

membranes of the Golgi (marker Anp1p) and the ER (marker Wbp1p) separated well from the denser plasma membrane (markers Can1p and Pma1p) of the control PE-containing membranes. Within the PE-depleted membranes, however, the protein distributions differed. In the absence of Mg^{2+}, virtually all Can1p co-localized with internal membranes and only around 20% of Pma1p reached the heavy fractions of the plasma membrane. The peaks of

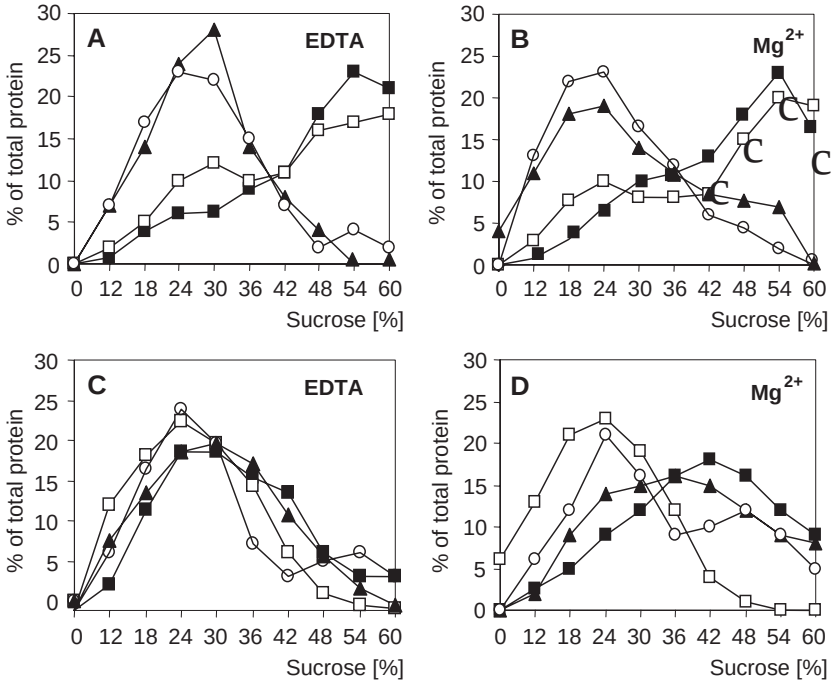

Fig. 3. Distribution of Can1p (□), Pma1p (■), Wbp1p-ER marker (▲), and Anp1-Golgi marker (○) in membranes resolved on sucrose density gradient. Proteins in individual fractions of sucrose gradient (12–54%) were resolved on SDS-PAGE, immunodetected by specific antibodies and their amounts evaluated by AIDA software. The distribution of the proteins from PE-containing membranes in the presence of EDTA (A) or Mg^{2+} (B) is shown. The distribution of the proteins from PE-depleted membranes in the presence of EDTA (C) or Mg^{2+} (D) is also shown. Similar results were observed in three independent experiments.

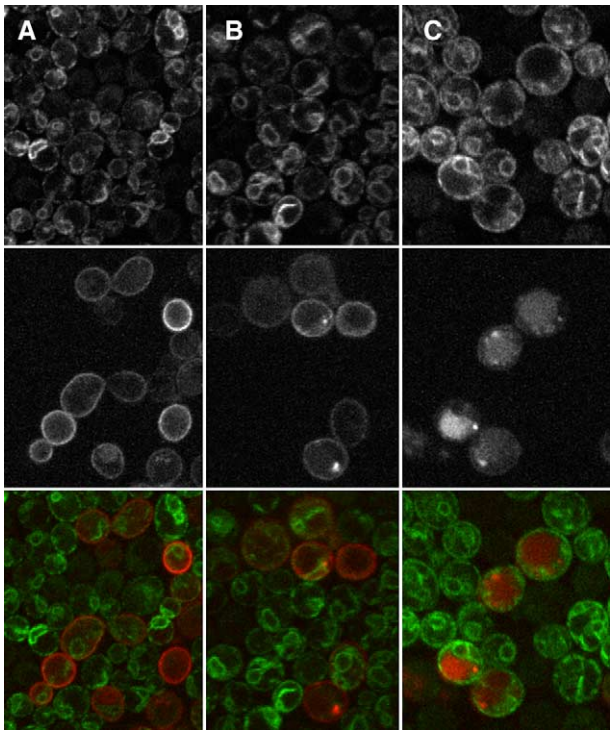

Fig. 4. Can1 RFP in PE-depleted cells localizes in compartments different from the ER. Can1pRFP and GFPShr3p were expressed in strain RY200T, and the cells were grown in the presence 2 mM ethanolamine (A) (PE-containing cells) or 4 mM choline (PE-depleted cells) for 5 (B) or 15 (C) h. Transversal optical sections of green fluorescence signal (up), red fluorescence signal (middle) and merge image (down) are shown. Bar: 5 μm.

Can1p and Pma1p overlapped with those of the internal membrane markers (Fig. 3C). In contrast, the presence of Mg^{2+} resulted in a clear shift of the ER membranes (Wbp1p) towards heavier fractions, while the distribution of the Golgi membranes (Anp1p) was clearly less affected. Interestingly, the distribution of Pma1p followed the shift of the ER marker, while the distribution of Can1p remained unaffected similarly as the Golgi marker (Fig. 3D). Thus, we conclude that PE depletion interrupts the trafficking of Can1p in the Golgi membranes, while the Pma1p trafficking stops in the membranes of the ER.

The validity of the approach above for the resolution of the internal membranes was sustained by confocal microscopy studies in living cells. Cells expressing simultaneously Can1p-RFP and -GFP-Shr3 (resident protein of the ER membranes) were grown either on ethanolamine or choline and examined for the changes in the distribution of the fluorescence (Fig. 4). After 6 h starvation for ethanolamine, the red fluorescence of Can1p started appearing in the cell interior where it accumulated in dot-like structures distinct from the green fluorescing membranes of the ER (Fig. 4B). With prolonged PE depletion, red fluorescence could be detected also in the vacuolar compartment (Fig. 4C).

Bagnat et al. [3] proposed that Pma1p associated with lipid rafts in the Golgi. However, the observations of Lee et al. [6] suggest that the Pma1p entry into rafts and its oligomerization begin before arrival to the Golgi. Our finding that the Pma1p retained in the ER due to the PE depletion is already raft associated is consistent with the latter conclusion. The question why Can1p, under the same conditions, is able to reach the Golgi compartment, neither the molecular basis of PE depletion effect, can be satisfactorily answered at the moment. However, the very fact that the trafficking of these two transporters is affected at different points of the secretory pathway argues for an explicit function of PE in the process and rules out an interpretation of an unspecific growth cessation due to an essential constituent depletion.

3.4. Is phosphatidyl ethanolamine required for the oligomerisation of Can1p?

Lee et al. [6] showed that Pma1p forms a large oligomeric complex of >1 MDa in the ER, which is packed into COPII vesicles. The oligomerization is linked to membrane lipid composition; Pma1p is rendered monomeric in cells depleted of ceramide. Monomeric Pma1p can be exported from the ER in COPII vesicles but is subsequently rerouted to the vacuole instead of being delivered to the plasma membrane. To learn whether PE depletion has an analogous effect on arginine permease oligomerization and its subsequent trafficking, we used BN-PAGE to examine the oligomeric state of Can1p in PE-containing and PE-depleted cells. Membrane fractions prepared from both types of cells were solubilized with 1% TX-100 or 0.1% Fos-choline-16 (a detergent efficient in solubilization of Can1p without affecting its activity after in vitro reconstitution — our unpublished results), and the proteins were separated under non-denaturating conditions. Irrespective of PE depletion and the applied detergent, Can1p migrated as a distinct band of a mass ~60 kDa, similarly as the permease dissociated by SDS (Fig. 5). Since that no evidence for

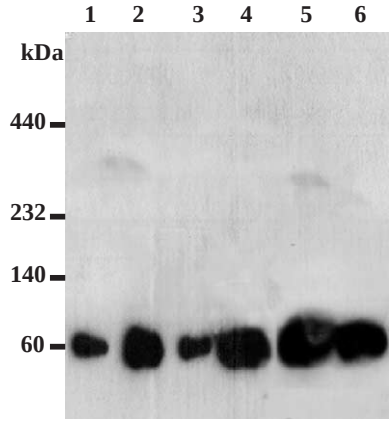

Fig. 5. Can1p occurs in cells in a monomeric form. Membranes prepared from PE-containing and PE-depleted cells were solubilized by TX-100 (lanes 1 and 3) or Foscholine16 (lanes 2 and 4) and analyzed by BN-PAGE and immunoblotting. As a control, the migration of SDS-dissociated samples of PE-containing (lane 5) and PE-depleted (lane 6) membranes are shown.

multimer was found by this method, we reason that, unlike Pma1p, Can1p occurs as a monomer in the cells and need not to be oligomerized prior to its delivery to the plasma membrane.

3.5. Is phosphatidyl ethanolamine required for proper folding of Can1p?

An incorrect folding of Can1p in the absence of PE may offer one explanation why this protein is not able to reach the plasma membrane. In fact, a role of PE as molecular chaperon assisting in the folding of a membrane protein was described for LacY permease of *E. coli* [27]. If PE played an analogous role in yeast, the misfolded protein could be recognized by a mechanism of quality control and re-routed for degradation [28].

As a rule, the misfolding of a protein results in its partial or total inactivation. To check whether Can1p arrested in the internal membranes is folded correctly and is active, we attempted to reconstitute this protein into an in vitro system. We prepared crude membranes from PE-depleted cells where Can1p is located in the Golgi membranes (see above). Membranes prepared from PE-containing cells (Can1p is active and located in the plasma membrane) served as a control. Fig. 6A shows the accumulation of arginine mediated by Can1p in whole cells which served as a starting material for the membrane fractions preparation: No accumulation of arginine is observed in PE-depleted cells. The reconstitution of Can1p activity was performed by the fusion of the membranes prepared from the respective cells with the proteoliposomes energized by cytochrome *c* oxidase as described in [20]. When the membranes isolated from PE-containing or PE-depleted cells were reconstituted into proteoliposomes prepared from the phosphatidylethanolamine of *E. coli* (approximately 50%; pure PE does no form liposomes), the activity of Can1p was fully recovered in both cases (Fig. 6B, full symbols). The Can1p that originated from the internal membranes of PE-depleted cells proved to be even more active than the permease reconstituted from PE-containing cells (Can1p in the plasma membrane). The same results were obtained when the liposomes were prepared from pure PE in mixture with PS and PG (1:1:1). This means that either the permease is correctly folded already in the internal membranes, even before reaching the plasma membrane, or, if not, the misfolded state is reversible and the addition of PE to the in vitro system restores fully its active conformation. To distinguish between these two possibilities, we attempted to reconstitute the protein into a system lacking PE. PE-containing and PE-depleted membranes were fused with proteoliposomes prepared from mixtures of PS/PG/CL (1:1:1) or PS/PG/PC (1:1:1). Regardless of the Can1p origin, no accumulation of arginine could be detected in this PE-free system (Fig. 6B, empty symbols). The failure of Can1p to accumulate arginine can be attributed to an insufficient energization of the system under these conditions. The membrane potential (the component of proton motive force driving predominantly the arginine uptake in the system [20]) formed in these proteoliposomes never exceeded −50 mV which is considerably lower than the value of −190 mV detected in vesicles prepared from *E. coli* phospholipid. Thus, it was not possible to decide experimentally between the two possibilities indicated above. It seems more likely, however, that Can1p is already active in the internal membranes, since a reactivation in vitro in PE-containing liposomes would not be expected to proceed with such a high efficiency.

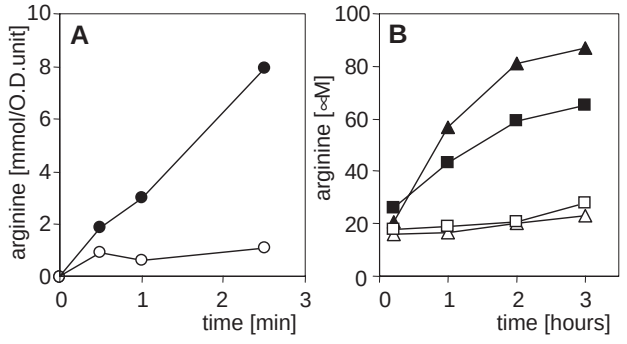

Fig. 6. (A) Arginine uptake into triple mutant cells mediated by Can1p. The cells were grown in yeast minimal medium with 2% glucose supplemented with either 2 mM ethanolamine (● — PE-containing cells) or 4 mM choline (○ — PE-depleted cells). A mixture of non-radioactive and ^{14}C(U)-arginine was added to a final concentration of 30 μM at time 0 and samples were withdrawn at intervals as indicated. (B) Reconstitution of Can1p from PE-containing (■, □) or PE-depleted (▲, △) membranes. The membranes were fused with cytochrome *c* oxidase-containing proteoliposomes prepared either from *E. coli* phospholipid (■, ▲) or a mixture of PC/PS/CL, 1:1:1 (□, △). The energization was started as described in Materials and methods 5 min before the addition of a mixture of non-radioactive and ^{14}C(U)-arginine to a final concentration of 20 μM. Accumulated arginine was measured in samples withdrawn at intervals as indicated. All results are representative of at least three separate experiments.

3.6. PE affects the raft association of proteins as determined by different detergent solubility

Ever since the definition of rafts as detergent resistant membranes (DRMs) enriched in sterols and sphingolipids, and since the suggestion that rafts play a role in many cellular processes, a considerable activity has aimed at identifying different membrane proteins associated with rafts. The most widely used method for these studies is TX-100 treatment, though recently a wider range of non-ionic detergents has been employed. Different patterns of raft-associated proteins detected in floating fractions of density gradients after treatment with different detergents have led to the suggestion that the different protein solubility reflects different types of rafts. Most recent studies, however, document that, on its own, the differential association of proteins or lipids with different DRMs is insufficient to define distinct membrane domains [29]. In detergents, smaller rafts (possibly bearing different proteins) coalesce and form large sheets [30], which eventually are found in

the floating fraction misindicating thus the occurrence of different proteins in the same type of rafts.

To contribute to the dispute on the adequacy of the methods currently used for the identification of raft associated proteins and their assignment to different types of rafts, we performed a simple experiment with Pma1p and Can1p, which were previously shown to be associated with rafts [3,4,9]. As shown in Fig. 2A and B, PE depletion did not affect Can1p and Pma1p localization to the floating raft-containing fractions of density gradient. However, when the membranes were treated by 0.3% NG, the Can1p distribution in fractions did not change substantially in PE-containing membranes, while in PE-depleted membranes, Can1p shifted to more soluble fractions (Fig. 2C). The same treatment affected the distribution of Pma1p differently: The percentage of Pma1p in the floating fractions was smaller as compared to TX-100 solubilized membranes, and it was virtually the same in both PE-containing and PE-depleted membranes (Fig. 2D). Provided that the detergent/protein ratio was kept strictly constant as was the case in these experiments, the following conclusions can be made: (a) Can1p and Pma1p are associated with rafts both in the plasma membrane (TX-100 resistance of membranes from PE-containing cells) and in the membranes of the secretory pathway (TX-100 resistance of membranes from PE-depleted cells); (b) the lipid composition surrounding Can1p in the Golgi membranes is different from that in the plasma membrane (susceptibility of Can1p from internal membranes to NG treatment is higher than that of Can1p from the plasma membrane); (c) the lipid composition intimately surrounding Pma1p in the ER membranes is probably similar to that in the plasma membrane (the solubility of Pma1p in NG is similar irrespective of Pma1p localization in the plasma membrane or the ER membranes). In short, the results of the treatment of membranes by TX-100 shows that Can1p and Pma1p from both types of membranes are localized in rafts, but it cannot not be concluded whether these rafts are of the same type or not. However, the different solubility in another mild detergent, NG, points to different lipid surroundings of these two transporters and hence, the rafts hosting Can1p differ from those occupied by Pma1p. This conclusion is supported by the microscopic visualization of distinct non-overlapping domains in the plasma membrane occupied either by Can1p or Pma1p [9].

It should be noted that, in this study, the operational concentration of 1% TX-100 represents more than 60-fold of its critical micelle concentration (cmc ~0.014–0.016%), and that of 0.3% NG corresponds to less than 2-fold of the cmc (~0.198%). Both detergents are of nonionic nature and the treatments were carried out at 4 °C.

To further investigate the membrane compartmentalization of Can1p and Pma1p, we employed the detergent extraction protocol without sucrose gradient ultracentrifugation as described by Babiychuk and Draeger [31] This protocol is frequently used for the processing of multiple samples of mammalian cells at identical experimental conditions and, to our knowledge, has not been used for similar studies in yeast.

Equal amounts of membranes from PE-containing or PE-depleted cells were subjected to treatment with increasing concentrations of TX-100 and the insoluble pellets were collected by low-speed centrifugation (see Materials and methods). The proteins in the pellets were analyzed by Western blotting, and Can1p and Pma1p were detected by specific antibodies. As documented in Fig. 7, the extraction patterns of these two proteins differ substantially: Pma1p remains largely in the insoluble pellet irrespective of the TX-100 concentration; after treatment with 2% TX-100 more than 60% of the protein is still detected in the pellet. The extractability of Pma1p from PE-containing and PE-depleted membranes does not differ substantially indicating thus similar lipid surroundings of the protein in both types of the membranes. Can1p, on the other hand, is extracted more readily: The protein content in the detergent-treated membranes from PE-containing cells decreases with the increasing detergent concentration nearly linearly; at 2% TX-100 only about 20% of Can1p remain in the pellet. In contrast, Can1p from PE-depleted membranes is largely extracted at a TX-100 concentration as low as 0.5%. This procedure circumvents the possibility of putative coalescence of proteins residing in different rafts — an objection called in question when the classical method for raft purification is used [30]. Yet, the conclusions derived from this experiment confirm the conclusions made from the experiments employing two different detergents and protein separation on density gradients (see above). The selective extraction of the proteins by increasing amount of the same detergent shows that Can1p and Pma1p associate with membrane domains of distinct lipid composition. More so,

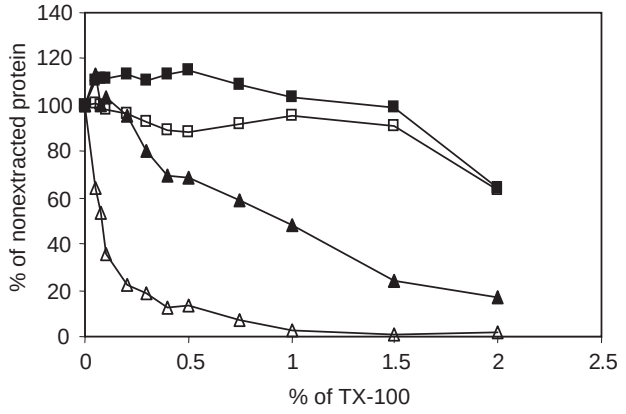

Fig. 7. Differential extraction of Pma1p and Can1p by TX-100 at 20 °C. Membranes prepared from PE-containing (full symbols) or PE-depleted (empty symbols) cells were incubated at 20 °C for 30 min at the indicated TX-100 concentrations. The suspensions were subjected to low-speed centrifugation. Each of the resulting pellets was analyzed for the content of Pma1p (■, □) or Can1p (▲, △) and their amounts evaluated by AIDA software. The values are means of two independent experiments.

the differential effect of PE depletion on the accessibility of Can1p to the detergent can be interpreted as that the raft association of this permease, besides being dependent on the presence of the typical raft lipids, sterols and sphingolipids, is, in addition, guided by the presence of a specific phospholipid.

The presented data are also consistent with an alternative model of lipid raft generation, the shell hypothesis [32], which is based on protein–lipid interactions. This means that each of these proteins penetrating the lipid bilayer contains, within the membrane spanning domains, high affinity binding sites for specific lipids. These tightly bound lipids form the lipid shell of the protein and would determine its susceptibility towards detergents.

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