Visualization of Protein Compartmentation within the Plasma Membrane of Living Yeast Cells

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Different distribution patterns of the arginine/H⁺ symporter Can1p, the H⁺ plasma membrane ATPase Pma1p, and the hexose transport facilitator Hxt1p within the plasma membrane of living *Saccharomyces cerevisiae* cells were visualized using fluorescence protein tagging of these proteins. Although Hxt1p-GFP was evenly distributed through the whole cell surface, Can1p-GFP and Pma1p-GFP were confined to characteristic subregions in the plasma membrane. Pma1p is a well-documented raft protein. Evidence is presented that Can1p, but not Hxt1p, is exclusively associated with lipid rafts, too. Double labeling experiments with Can1p-GFP- and Pma1p-RFP-containing cells demonstrate that these proteins occupy two different nonoverlapping membrane microdomains. The size of Can1p-rich (Pma1p-poor) areas was estimated to 300 nm. These domains were shown to be stable in growing cells for >30 min. To our knowledge, this is the first observation of a cell polarization-independent lateral compartmentation in the plasma membrane of a living cell.

INTRODUCTION

Lipid rafts (detergent-resistant membranes, detergent-insoluble glycolipid-enriched microdomains, or glycolipid-enriched membranes) are dynamic assemblies enriched in sterols and sphingolipids occurring laterally distributed in the plasma membrane of most, if not all, eukaryotes (for reviews, see Simons and Ikonen, 1997; Brown and London, 1998). In mammalian cells cholesterol, sphingomyelin, and glycosphingolipids are the basic constituents of detergent-resistant membranes (Rietveld and Simons, 1998), whereas in yeast these are ergosterol, inositolphosphoceramides, and its mannosylated derivatives (Kübler *et al.*, 1996; Bagnat *et al.*, 2000). Existence of similar plasma membrane domains in plant cells can also be expected, although it has not been reported so far.

A distinctive feature of lipid rafts is their insolubility in mild nonionic detergents (typically Triton X-100) at 4°C (Brown and Rose, 1992; Rietveld and Simons, 1998). Consequently, they are found floating in low-density fractions of solubilizates of mild detergent-treated membranes. Rafts obtained by this procedure selectively recruit specific membrane proteins, whereas others are excluded. The recognition of selective protein retention within the detergent-resistant domains led to a concept of lateral subcompartmentation within the plasma membrane (Simons and Ikonen, 1997; Brown and London, 1998).

It was proposed that rafts form a platform for lipid (Simons and Ikonen, 2000) and protein sorting and trafficking (Simons and van Meer, 1988; Galbiati *et al.*, 2001; Ikonen,

2001) and cell signaling (Field *et al.*, 1997; Stauffer and Meyer, 1997; Simons and Toomre, 2000; Dykstra *et al.*, 2001). Lipid rafts provide the cells also with a mechanism for functional and spatial control of exocytosis (Chamberlain *et al.*, 2001) and are involved in immune cell activation (for reviews, see Dykstra *et al.*, 2001; Galbiati *et al.*, 2001; Katagiri *et al.*, 2001). Accumulating evidence documents that compositionally distinct lipid microdomains are assembled and may coexist within a given membrane. They can differ both in their resistance toward different detergents (Roper *et al.*, 2000) and in the types of associated proteins. Glycosylphosphatidylinositol (GPI)-anchored proteins are typical residents of rafts both in mammalian (Simons and van Meer, 1988; Brown and Rose, 1992) and in yeast plasma membranes (Bagnat *et al.*, 2000). However, even GPI-anchored proteins sharing endocytic and recycling vesicles may be segregated to distinct lipid rafts in the plasma membrane. This was demonstrated for example for human folate receptors α and β and human placental alkaline phosphatase functionally expressed in Chinese hamster ovary cells (Wang *et al.*, 2002).

Besides GPI-anchored proteins, several membrane-spanning proteins, like transporters, have been recognized to associate with specific lipid microdomains. In yeast, plasma membrane ATPase Pma1p (Bagnat *et al.*, 2000; Lee *et al.*, 2002) and uracil permease Fur4p (Hearn *et al.*, 2003) were found to be enriched in rafts. It was shown that the targeting of these proteins to the plasma membrane required their association with rafts. Lack of one of the raft lipid components, sphingolipids, or ergosterol in *lcb1-100* or *erg 12-1* mutants, respectively, checkmated the proteins' delivery to the surface (Bagnat *et al.*, 2000; Lee *et al.*, 2002; Hearn *et al.*, 2003). Large oligomeric complexes of Pma1p are formed in the secretory pathway as early as in the endoplasmic reticulum. It was shown that the oligomerization is linked to

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Table 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	MAT α <i>ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> , 1988
SEY6210a	MAT α <i>ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801 suc2-Δ9</i>	Stolz, unpublished data
BY4742	MAT α <i>his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 trp1-Δ901 bar1-1</i>	Brachmann <i>et al.</i> , 1998
<i>erg 6</i>	BY4742 except <i>erg6Δ::kanMX4</i>	EUROSCARF
<i>erg24</i>	BY4742 except <i>erg24Δ::kanMX4</i>	EUROSCARF
<i>lcb1-100</i> (RH3804)	MAT α <i>lcb1-100 leu2 trp1 ura3 lys2 bar1</i>	H. Riezman
PMA1-GFP	SEY6210a except <i>PMA1::GFP::kanMX4</i>	This study
CAN1-GFP	SEY6210a except <i>CAN1::GFP::kanMX4</i>	This study
PMA1-RFP	SEY6210 except <i>PMA1::tdimer2(12)::kanMX4</i>	This study
CAN1-GFP/PMA1-RFP	SEY6210 except <i>PMA1::tdimer2(12)::kanMX4</i> <i>CAN1::GFP::kanMX4</i>	This study
SEY6210/pHXT1GFP	SEY6210 pHXT1GFP	This study
SEY6210/pCAN1BioHis	SEY6210 pCAN1BioHis	This study
BY4742/pCAN1GFP	BY4742 pCAN1GFP	This study
<i>erg24</i> /pCAN1GFP	<i>erg24</i> pCAN1GFP	This study
<i>erg 6</i> /pCAN1GFP	<i>erg 6</i> pCAN1GFP	This study
<i>lcb1-100</i> /pCAN1GFP	<i>lcb1-100</i> pCAN1GFP	This study

membrane lipid composition. In ceramide-depleted cells, Pma1p stayed monomeric. It was permitted to exit endoplasmic reticulum but was subsequently rerouted to the vacuole (Lee *et al.*, 2002).

The size of individual lipid rafts has been estimated to be ~25–70 nm (Varma and Mayor, 1998; Pralle *et al.*, 2000). It is not, therefore, possible to resolve rafts in the light microscope. Immunofluorescence studies of raft proteins result in a fairly uniform staining of plasma membranes of living cells (Mayor and Maxfield, 1995; Jacobson and Dietrich, 1999); punctuate patterns are not observable. Patched raft-marker proteins and lipids are light-microscopically visible only after antibody and cross-linking treatment (Harder *et al.*, 1998). The interpretation of these images is not unequivocal.

We studied the lateral localization of three membrane proteins residing in the plasma membrane of *Saccharomyces cerevisiae*: Can1p (H⁺-arginine symporter), Pma1p (plasma membrane H⁺-ATPase), and Hxt1p (hexose uptake facilitator). In this study, we report that the fully active fusion protein Can1p-GFP exhibits a fairly regular punctuated distribution in the plasma membrane. A patched pattern is also observed in Pma1p-green fluorescent protein (GFP) fluorescence, whereas the Hxt1p-GFP protein is evenly distributed in the plasma membrane. The raft association of Can1p has been demonstrated by biochemical criteria.

It was of further interest whether the H⁺-ATPase, which generates a proton gradient across the plasma membrane, and the Can1p, the H⁺-symporter using the proton gradient, are localized in the same membrane compartment. The immediate neighborhood of proton motive force (pmf)-producing and pmf-consuming proteins could facilitate their tight cooperation and thus favor the concept of the frequently discussed role of localized proton gradients in chemiosmotic coupling (Heberle *et al.*, 1994). However, the evidence reported in this study shows that the two proteins in question are fully separated in the plasma membrane.

MATERIALS AND METHODS

Strains and Growth Conditions

Yeast strains used in this study are listed in Table 1. For DNA cloning and propagation of plasmids, *Escherichia coli* strain DH5 α or *E. coli* SURE strain (Stratagene, La Jolla, CA) were used.

Bacterial strains were incubated in 2 $\times$ TY medium (1% trypton, 1.6% yeast extract, 0.5% NaCl). Ampicillin-resistant transformants were grown in the presence of 100 mg/ml ampicillin. *S. cerevisiae* strains were incubated in a rich medium YPD (2% peptone, 1% yeast extract, 2% glucose) or in a synthetic minimal medium (0.67% Difco yeast nitrogen base without amino acids [YNB]), 2% glucose, and amino acids as needed). Yeast cells transformed with pVTU100U-derived plasmids were selected and maintained on uracil-free medium. Geneticin-resistant yeast colonies were selected on YPD/G418 medium (YPD + 200 mg/l geneticin G418). In all experiments, studying Can1p, the appropriate strain was incubated in arginine-free medium to OD₆₀₀ of 1.0. Diploid strains were sporulated in liquid sporulation medium (2% potassium acetate, 0.02% glucose) at 25°C for 2–3 d.

Plasmids

pCAN1GFP: *CAN1* without its stop-codon was generated by polymerase chain reaction (PCR) on pWHY67 α (Hoffmann 1985) and subcloned using *XhoI/XbaI* sites into 2 μ vector pVTU100-GFP carrying *ADH1* promoter, S65G allele of *GFP*, and *URA3* marker (a kind gift from Jürgen Stolz, University of Regensburg, Regensburg, Germany). pHXT1GFP: *HXT1* without its stop-codon was PCR amplified from SEY6210 genomic DNA and subcloned using *XhoI/XbaI* sites into 2 μ vector pVTU100-GFP. Episomal plasmid pCAN1BioHis was described in Opekarova *et al.*, 2002.

Construction of Strains with Chromosome-integrated Fluorescence Tags

An integrative cassette containing *GFP* and *kanMX4* was created by PCR on template DNA pGFP-kanMX (kindly provided by A. Gillingham (Medical Research Council, Cambridge, United Kingdom). For the tagging of *CAN1* (*PMA1*), *CAN1ki5* and *CAN1ki3* (*PMA1ki5* and *PMA1ki3*) primers were used in PCR amplification. Purified PCR fragments were transformed into SEY6210a yielding the strains CAN1-GFP and PMA1-GFP. The correct integration of GFP-cassette before the stop codon of the targeted gene was confirmed by PCR amplification.

The strain expressing *PMA1-RFP* was prepared in a similar way: plasmid pV1 (kindly provided by A. Gillingham: Medical Research Council) was used as a PCR template for generating an integrative cassette and transformed into strain SEY6210. The resulting strain PMA1-RFP contained a tandem of dimer2 (reengineered DsRed) connected by 12 amino acids [tdimer2(12)] at 3' terminus of genomic *PMA1* (Campbell *et al.*, 2002). The tdimer2(12) was constructed and kindly provided by R. Tsien (Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA).

Strains CAN1-GFP and PMA1-RFP were mated, their diploid cells sporulated, and tetrads dissected. A haploid strain CAN1-GFP/PMA1-RFP carrying both *CAN1-GFP* and *PMA1-RFP* was selected.

Isolation of Lipid Rafts

Lipid rafts were isolated according to Bagnat *et al.* (2000), with the following modification: crude membranes, instead of the whole cell extract, were used for the detergent extraction analysis. An amount of cells equivalent to 100 OD₆₀₀ units of an overnight culture was broken by vortexing with glass beads in TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA)

supplemented with leupeptin (4 μ M) and pepstatin (2 μ M). After low-speed centrifugation, crude membranes were collected by centrifugation (20 min at 23,000 rpm, Beckman Ti70.1 rotor at 4°C). Aliquots of crude membranes (200 μ g of total protein) were resuspended in 270 μ l of TNE buffer. Triton X-100 was added to the final concentration of 1%, and the mixture was incubated for 30 min on ice. Then, Optiprep (Nycomed, Vienna, Austria) was added to a final concentration of 40% (wt/vol). The samples transferred to centrifuge tubes were overlaid with 1.32 ml of 30% Optiprep in TXNE (TNE plus 0.1% Triton X-100) followed by 220 μ l of TXNE, and centrifuged for 2 h at 50,000 rpm in a Beckman SW60 rotor at 4°C. Six equal fractions were collected from the top of each gradient, proteins were precipitated with trichloroacetic acid (final concentration 10%), collected by centrifugation at 4°C, and the pellets were dissolved in 10 μ l of 1 M Tris base and 25 μ l of dissociation buffer (0.1 M Tris-HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.02% bromophenol blue). Samples were incubated at 37°C for 10 min and analyzed by SDS-PAGE and immunoblotting.

Immunoblot Analysis

Biotinylated pCan1pBioHis was immunoanalyzed by streptavidine-horseradish peroxidase conjugate (diluted 1:5000; Amersham Biosciences, Piscataway, NJ). Rabbit anti-Pma1p (1:10,000; a gift of R. Serrano, Polytechnic University of Valencia, Valencia, Spain), anti-Hxt1p (1:100; a gift of E. Boles, University of Frankfurt, Frankfurt, Germany), and anti-Gas1p (1:500; a gift of L. Popolo, University of Milano, Milan, Italy) primary antibodies were used in a combination with peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Biosciences) and a chemiluminescence detection system (Amersham Biosciences).

Arginine Uptake Assay

The arginine uptake measurements were performed as described by Opekárová *et al.* (1998). A mixture of 14 C-labeled and cold arginine (7.5 mCi/mM) was added to the suspension of growing cells (OD₆₀₀ of 1.0) to a final concentration of 28 μ M. Aliquots of 100 μ l were withdrawn at 1-min intervals, diluted in 2 ml of water, filtered on cellulose nitrate filters (0.8- μ m pore size; Schleicher & Schuell, Keene, NH) and washed with water. The radioactivity was determined by scintillation counting.

Light Microscopy

Living cells were resuspended in a fresh synthetic medium and immobilized by 0.8% agarose (23°C) before microscopic observation of GFP and red fluorescent protein (RFP) fluorescence. Immunofluorescence mapping of Pma1p was performed according to Hasek and Streiblova (1996). Briefly, cells were grown to exponential phase on rich medium, fixed by 3.7% formaldehyde for 40 min, and cell walls were digested with zymolyase 20T (100 μ g/ml in 0.1 M potassium phosphate-citrate buffer, pH 5.9, 10 μ g/ml pepstatin). The spheroplasted cells were permeabilized with 1% Triton X-100 for 1 min. The cells were incubated subsequently with rabbit anti-Pma1p (1:50; 1 h), and with rhodamine-conjugated goat anti-rabbit (1:200; 45 min; Calbiochem, San Diego, CA) antibodies at room temperature.

Specimens were viewed using LSM510-Meta confocal microscope (Carl Zeiss, Jena, Germany) with 100 $\times$ PlanApoChromat objective (numerical aperture 1.4). Fluorescence signals of GFP (excitation 488 nm, Ar laser), RFP, and rhodamine (both excitation 543 nm, HeNe laser) were detected using band-pass 505- to 530-nm, long-pass 585-nm, and long-pass 560-nm emission filters, respectively. In double labeling experiments, a sequential scanning was used to avoid any cross talk of fluorescence channels. For maximum intensity projections, three-dimensional sets of <700-nm-thick optical sections 300 nm apart were collected.

RESULTS

Visualization of Different Proteins Occupying Different Lateral Domains within the Plasma Membrane

The arginine/H⁺-symporter Can1p, the plasma membrane H⁺-ATPase Pma1p, and the glucose facilitator Hxt1p were visualized *in vivo* by using GFP tagging. Strains carrying chromosomal C-terminal *CAN1*-GFP or *PMA1*-GFP fusions were constructed. The Can1p-GFP was fully functional as proved by the sensitivity of the *CAN1*-GFP strain to canavanine (a toxic arginine analog) and the Can1p-GFP-mediated uptake of radioactive arginine (our unpublished data). Because the *PMA1* knockout is lethal (Serrano *et al.*, 1986), the functionality of Pma1p-GFP was obvious from the viability of the haploid transformants. To localize Hxt1p, wild-type cells were transformed with an episomal plasmid pHXT1GFP encoding a C-terminal *HXT1*-GFP fusion. The

Figure 1. Localization of different proteins within the plasma membrane. Fluorescence patterns of Can1p-GFP (A and B), Pma1p-GFP (C and D), and Hxt1p-GFP (E and F) in living *S. cerevisiae* cells are shown. An individual transverse optical section (A, C, and E) and superposition of four consecutive surface optical sections (B, D, and F) are presented for each protein. Bar, 5 μ m.

functionality of Hxt1p-GFP has been proven in *hxt* null strain RE700 Δ hxt1-7, which does not grow on glucose (Reifenberger *et al.*, 1995). Hxt1p-GFP was able to restore the growth (our unpublished data).

As shown in Figure 1, each of the three studied proteins occupied a different compartment within the plasma membrane and displayed a characteristic pattern. Distinct clus-

Figure 2. Immunofluorescence mapping of Pma1p. An individual optical section is shown. Note that equal concentrations of the protein are detectable in the plasma membrane of buds and mother cells. Bar, 5 μ m.

ters of Can1p-GFP were observed all over the surface of the transformed cells (Figure 1, A and B). On a transverse confocal section, Pma1p-GFP showed also a nonhomogenous distribution (Figure 1C), but as evident from the surface view (Figure 1D), the region occupied by Pma1p-GFP was larger and the patterning different from that of Can1p-GFP. Also, compared with Can1p-GFP, the intensity of Pma1p-GFP fluorescence was stronger, which is in agreement with the abundance of the H^+ -ATPase (10–20% of membrane protein) in the plasma membrane (Serrano, 1991). In contrast to the two proteins mentioned above, the mapping of Hxt1p-GFP fluorescence (Figure 1, E and F) revealed a fairly homogeneous signal within the plasma membrane.

No significant accumulation of the fusion proteins in the cell interior was observed, which illustrated that the protein trafficking and targeting to the plasma membrane was not affected. A weak intracellular signal located mainly in the endoplasmic reticulum could be recognized in some cells expressing Pma1p-GFP and Hxt1p-GFP.

An interesting phenomenon was observed in the Pma1p-GFP pattern: the intensity of the fluorescence detected in buds was apparently weaker than that of mother cells. The bigger the bud, the smaller was the difference between the bud and the mother cell fluorescence intensity. A similar, but less pronounced trend could be recognized in the Hxt1p-GFP fluorescence. As an essential protein, Pma1p was expected to distribute promptly to the newly synthesized membrane of the buds. Therefore, it seemed likely that the lower fluorescence intensity observed in the buds was an artifact related to GFP properties rather than to the lack of the traced protein in the newly synthesized plasma membrane of the bud. The GFP fluorophore is formed by cyclization of three amino acids of its primary sequence. This posttranslational modification requires ~30 min to take place (Heim *et al.*, 1995). If the trafficking of a newly synthesized Pma1p to the proliferating membrane of the bud is faster, the delayed “lighting up” of the GFP tag will result in a lower intensity of the fluorescence signal. To confirm this explanation, Pma1p was visualized by indirect immunofluorescence in the wild-type strain. Characteristic Pma1p fluorescence patterns, but no difference between buds and mother cells, were detected (Figure 2). The weaker fluorescence signal of Pma1p-GFP and of Hxt1p-GFP in buds was, therefore, caused by the delay in the formation of the GFP fluorophore.

Figure 3. Detergent solubilizations. The solubilization of Can1pBio, Pma1p, Hxt1p, and Gas1p by 1% Triton X-100 was tested in Optiprep gradients (see MATERIALS AND METHODS). Fractions 1–6 (top–bottom of the gradient) were analyzed by immunoblotting.

Can1p Is Localized in Lipid Rafts

The association of Pma1p with lipid rafts is well documented (Bagnat *et al.*, 2000, 2001; Lee *et al.*, 2002). The punctuate pattern of the Can1p-GFP fluorescence, which we observed at the plasma membrane raised the question whether this protein also was associated with lipid raft-based microdomains.

To determine whether Can1p is associated with lipid rafts, the solubility of Can1p in 1% Triton X-100 at 4°C was tested. First, intact cells expressing a chromosomal *CAN1-GFP* fusion were treated with the detergent for 30 min. It was reported that nonraft proteins could be extracted this way (Bagnat *et al.*, 2001). No changes in the distribution of Can1p-GFP were observed after the Triton X-100 extraction (our unpublished data). In the next step, the protein solubility after detergent treatment of crude membranes was analyzed on Optiprep density gradients. For the sake of Can1p identification, both the strain expressing Can1p tagged with a biotinylation domain (Can1pBio) from a multicopy plasmid (Opekarova *et al.*, 2002) and the strain expressing chromosomal *CAN1-GFP* (constructed in this study) were used. The majority of the Can1pBio floated in the two top fractions, as did standard lipid raft proteins such as Pma1p and the GPI-anchored Gas1p (Figure 3; Bagnat *et al.*, 2000). The ratio between detergent-resistant (top fractions) and soluble proteins (bottom) was even higher for Can1p than for Pma1p and Gas1p. In contrast, the main portion of Hxt1p was confined to bottom fractions of the density gradient, though some Hxt1p was detected also in the top fractions (Figure 3). The presence of all these proteins in the top fractions was further confirmed by an additional Triton X-100 treatment performed with proteins from the top fraction and a subsequent second gradient centrifugation (our unpublished data).

Ergosterol and sphingolipids are required for correct intracellular targeting of raft-associated proteins (Bagnat *et al.*, 2000; Lee *et al.*, 2002; Hearn *et al.*, 2003). To further verify the Can1p association with lipid rafts, we measured Can1p-mediated transport of arginine into the cholesterol-defective mutants *erg24* and *erg6*. Compared with the wild-type, the arginine uptake in the mutant strains was reduced by a factor of 3 (Figure 4A). The localization of the specific arginine permease in the strains mentioned above was performed in transformants that produced the Can1p-GFP from

Figure 4. Mistargeting of Can1p-GFP in ergosterol/sphingolipids-deficient mutants. Can1p-mediated uptake of arginine was measured (A) in BY4742 strain (squares) and its ergosterol synthesis-defective mutants *erg6* (diamonds) and *erg24* (triangles). A plasmid encoding for Can1p-GFP was expressed in BY4742 (B), *erg24* (C), *erg6* (D), and *lcb1-100* (E) strains. Only in the wild type, the protein targeting to the plasma membrane was not impaired. Bars, 5 μm .

plasmid pCAN1GFP. As shown in Figure 4C, most of the Can1p-GFP in *erg24* cells remained in perinuclear or peripheral endoplasmic reticulum and did not reach the plasma membrane (note "dashed" pattern typical for peripheral endoplasmic reticulum). Figure 4D shows impaired targeting of Can1p-GFP to plasma membrane in *erg6* cells, where Can1p is obviously retained in membranes of the secretory pathway. Decreased content of sphingolipids in *lcb1-100* mutant (Zanolari *et al.*, 2000) substantially affects Can1p trafficking. Vacuolar degradation of a significant amount of Can1pGFP was observed already at permissive temperature (Figure 4E), which is in agreement with the reported reduced content of sphingosine intermediates and complex sphingolipids in the mutant (Hearn *et al.*, 2003). In contrast to the strains bearing the mutation in the lipid synthesis, Can1p-GFP produced from the same plasmid in corresponding wild-type strains exhibited the characteristic punctuated fluorescence pattern (Figure 4B), which was observed also with Can1p-GFP expressed from the chromosomal GFP integration (compare with Figure 1A). Together, the observations mentioned above document that Can1p is a typical raft-associated protein.

Pma1p- and Can1p-containing Domains of the Plasma Membrane Are Spatially Separated

As demonstrated by GFP fluorescence patterns, the two raft-associated proteins Pma1p and Can1p are segregated into different lateral compartments within the plasma membrane (Figure 1, B and D). The question was addressed, therefore, whether, and if so, to what extent these compartments overlap. Does any indication of a structural coupling of H^+ -ATPase and the arginine/ H^+ -symporter exist?

To answer this question, we constructed a strain carrying chromosomal *PMA1* tagged with RFP (Campbell *et al.*, 2002; see MATERIALS AND METHODS for details) and mated it with CAN1-GFP strain. The resulting diploid cells were sporulated, and a haploid strain carrying both *CAN1-GFP* and *PMA1-RFP* was used to visualize the two proteins in vivo simultaneously. As evident from Figure 5, the fluorescence patterns of Can1p-GFP and Pma1p-RFP did not colocalize.

Besides the Pma1p-rich areas (Figure 5, D and F; compare also with Pma1p-GFP patterns in Figure 1, C and D), dark spots lacking the protein were regularly present. Can1p-GFP fluorescence was confined exclusively to these spots. The fluorescence intensity profiles measured in directions indicated by arrows in Figure 5, E and F, clearly show Pma1p-RFP minima where Can1p-GFP maxima are located and vice versa (Figure 5, G and H). We, therefore, conclude that Pma1p and Can1p localize in different, nonoverlapping sub-compartments of the plasma membrane.

An additional feature was apparent from Figure 5: the fluorescence intensity of Pma1-RFP was different in different cells, and the RFP fluorescence signal was missing in buds. As discussed for Pma1p-GFP, we ascribe this absence of fluorescence in the proliferating membranes to a slow maturation of the RFP fluorophore. A maturation period of up to 2 h has been reported for the RFP version used in this study (Campbell *et al.*, 2002). Figure 5 shows that not only the buds but also young cells already separated from the mother cell still exhibit low RFP fluorescence. Once visible, the patterns of Pma1p-GFP and Pma1p-RFP are compatible (compare Figures 1, C and D, and 5, C and D).

Figure 5. Simultaneous localization of Can1p-GFP and Pma1p-RFP. Fluorescence patterns of Can1p-GFP (A and B; green in E and F) and Pma1p-RFP (C and D; red in E and F) in living *S. cerevisiae* cells (strain CAN1-GFP/PMA1-RFP) are shown. Individual transversal (A, C, and E) and tangential (B, D, and F) optical sections are presented. The fluorescence intensity profiles (G and H) measured in directions indicated by arrows in E and F clearly show that the places of accumulation of Can1p-GFP correspond to the local minima in Pma1p-RFP fluorescence pattern (A–F, arrowheads). A differential interference contrast image (I) is also shown. In G and H, fluorescence signals were normalized to the same maximum intensity. Low-pass filter was applied on the graphs to reduce the noise. Bar, 5 μ m.

Protein Compartments within the Plasma Membrane Are Stable in Time

The dynamic behavior of Can1p- and Pma1p-enriched plasma membrane domains was studied. Living cells ex-

pressing either *CAN1-GFP* or *PMA1-GFP* were grown under the microscope at room temperature and scanned each 10 min. In both cases, only minor changes in the protein distributions were observed during the time representing a

Figure 6. Dynamics of the protein domains in the plasma membrane. One tangential optical section of a cell expressing Can1p-GFP (A) and Pma1p-GFP (C) was scanned during cell growth. Transversal optical sections and differential interference contrast images of the same cells are also shown (B and D). Numbers indicate the minutes elapsing since the start of the examination. Fluorescence maxima in individual images were normalized to the same gray level. Bars, 5 μm .

substantial part of the cell cycle (Figure 6; note the growing bud in Figure 6D). As the Can1p-GFP fluorescence was bleached rapidly (probably due to a small number of fluorophores present in the membrane), only three different time scans are presented (Figure 6A; note the increasing noise level). The number, shape, and position of individual Can1p-GFP clusters (or the dark holes in the Pma1p-GFP pattern) were well preserved in mother cells. More pronounced changes in the pattern with time were observable in buds (Figure 6C, 70–90 min).

We further estimated the size of the Can1p-GFP clusters. The least diameter of 207 clusters measured in 20 cells yielded a mean value of 300 ± 60 nm. This value could be overestimated due to the blur introduced by microscopic imaging. We, therefore, verified the value obtained above in a control measurement. Because Can1p and Pma1p fluores-

cence patterns are mutually exclusive (Figure 5), the size of Can1p-GFP clusters could be estimated from the size of dark spots in Pma1p-GFP fluorescence pattern. The least diameter of 174 dark spots in 20 cells was measured, yielding a mean value of 270 ± 70 nm, which is in a good agreement with the value estimated from Can1p-GFP fluorescence pattern. Standard deviations were used to assess the error of the mean values.

DISCUSSION

We report herein a visualization of three different lateral compartments occupied by three different proteins in the plasma membrane of living *S. cerevisiae* cells. The arginine/ H^+ symporter Can1p, the H^+ plasma membrane AT-

Pase Pma1p, and the hexose transport facilitator Hxt1p tagged with fluorescent proteins show different fluorescence patterns in the membrane (Figure 1). Several arguments support the interpretation that these patterns reflect the true distributions of Can1p, Pma1p, and Hxt1p. First, the fusion proteins complement the functions of their nontagged versions. The weak tendency of GFP-tagged proteins to dimerize has been reported for highly concentrated proteins (Ward, 1998). Although this could be a potential source of an artifact, the different patterns observed for the three chosen proteins argue against that possibility. Obviously, no GFP oligomerization is visible in the case of Hxt1p. In addition, Pma1p-GFP and Pma1p-RFP patterns are compatible (as well as Can1p-GFP and Can1p-RFP; our unpublished data); the tdimer2(12) version of RFP was engineered to prevent the oligomerization of DsRed molecules (Campbell *et al.*, 2002). Last, but not least, the Pma1p-GFP distribution was confirmed by GFP-independent indirect immunofluorescence visualization of Pma1p in wild-type strain (compare Figures 1C and 2). Based on all the observations mentioned above, we assume that the GFP (RFP) tagging of the three studied proteins did not cause formation of artificial structures in the plasma membrane.

A possible explanation for the existence of Can1p-GFP clusters (and the corresponding dark regions in the Pma1p-GFP fluorescence pattern) could be the secretory vesicles mediated trafficking of the proteins to the plasma membrane. Because the vesicle membrane selectively enriched in Can1p-GFP fuses with the plasma membrane, the protein is spread to the neighboring membrane areas, where it eventually would be too diluted to be detectable. In this case, we would observe only the places of recent fusions as Can1p-GFP enriched areas. However, the Can1p-GFP clusters are rather stable in time, so continuous fusions of new vesicles at the selected places would be necessary to preserve the individual cluster. In agreement with this hypothesis, the Can1p-GFP clusters of an estimated diameter of 300 nm are larger than the area of the membrane of a single secretory vesicle, which was estimated to be ~80 nm (Lee *et al.*, 2002). We observed a high level of photobleaching during the repeated scans of Can1p-GFP fluorescence (Figure 6A; note the increasing noise level). This is contradictory to the assumption, that the new vesicles are bringing new (fluorescent) protein molecules into the cluster between the scans. From the results presented, we have to conclude that the observed Can1p-GFP clusters, as well as the dark holes in the Pma1p-GFP distribution, are more likely stable structures in the yeast plasma membrane than steady state pools of newly synthesized proteins. Although the Pma1p-GFP domain is stable for >1 h, preliminary fluorescence recovery after photobleaching (FRAP) experiments indicate that the individual Pma1p-GFP molecules move within this compartment (our unpublished data).

We demonstrated that the Can1p-clusters corresponded to membrane microdomains based on lipid rafts. Both Can1Bio and Can1p-GFP behaved like typical raft proteins (Figures 3 and 4). Because individual rafts are thought to be much smaller (Varma and Mayor, 1998; Jacobson and Dietrich, 1999; Pralle *et al.*, 2000) than the Can1p-GFP domains observed herein, we suppose that the Can1p-GFP fluorescence pattern corresponds to raft clusters. If we accept the raft size between 25 and 70 nm estimated for mammalian cells (Varma and Mayor, 1998; Pralle *et al.*, 2000), each cluster would contain from 10 to hundreds of rafts.

Potential functions of lipid rafts and/or their clusters during signal transduction have been discussed previously (Si-

mons and Toomre, 2000). Recently, evidence has been given for reorganization of the plasma membrane components resulting in clustering of subsets of lipid rafts during yeast mating (Bagnat and Simons, 2002). But, as demonstrated in this study on the example of pmf-generating Pma1p and pmf-dissipating Can1p, the tight spatial coupling is not obligatory for a cooperation of membrane proteins. The fact that Can1p and Pma1p occupy disjoint plasma membrane subcompartments (Figure 5) raises many questions. For instance, what are the local pH variations on the plasma membrane of a living cell? As we document herein, light microscopically visible Can1p-rich areas exist, through which the H⁺ influx occurs. On the other hand, the Pma1p-mediated proton efflux is confined to different areas. Thus, circular currents should be present around the cell surface. Can1p is not the only H⁺-symporter in the yeast plasma membrane. Are all the symporters located in the same membrane compartment? Could they be functional if located, for example, in the Pma1p compartment, where protons are pumped in the opposite direction? As shown herein, there exist at least three different compartments in the yeast plasma membrane: Can1p-rich compartment consisting of lipid raft clusters, lipid raft-based compartment of Pma1p, and the compartment occupied by Hxt1p. How many other compartments do exist? How many different membrane proteins share one compartment?

Different secretory vesicles could possibly supply different membrane compartments. For example, selective export vesicles have been reported for Pma1p (Roberg *et al.*, 1999; Shimoni *et al.*, 2000). On the other hand, a yeast mutant deficient in phosphatidyl ethanolamine is affected almost exclusively in the uptake of substrates via H⁺ symporters (Robl *et al.*, 2001). In this mutant, specifically Can1p is not targeted to the cell surface (Opekarova *et al.*, 2002).

Proteins spanning one leaflet of the membrane by a single hydrophobic domain are considered typical raft residents. However, an increasing number of multihelix transmembrane proteins has recently been reported to associate with cholesterol/ergosterol and glycolipid-enriched microdomains. Thus, the mammalian glucose transporter GLUT1 (but not GLUT3; Sakyo and Kitagawa, 2002), as well as the sodium glucose cotransporter SGLT1 (Runembert *et al.*, 2002) are raft proteins. Both are affected in their activities when the lipid environment is perturbed. A voltage-gated K⁺ channel (Kv2.1) targets to lipid rafts, whereas a related channel (Kv4.2), which is differently located within neuronal cells, does not (Martens *et al.*, 2000). The properties of the Kv2.1 channel were changed, when the protein was not located in raft environment (Martens *et al.*, 2000). Besides the proton symporter Can1p, two other plasma membrane proteins, the uracil/H⁺ symporter Fur4p (Hearn *et al.*, 2003) and the ABC transporter Ste6p (Bagnat and Simons, 2002) were shown to associate with rafts in yeast.

The high spatial and temporal stability of the distribution of Can1p-rich (Pma1p-poor) domains (Figure 6) is an interesting observation. This extraordinary fixation of membrane microdomains within a fluid lipid matrix obviously requires some sort of stabilization, which might involve, for example, an interconnection with components of cytoskeleton. Evidence for an interaction between specific membrane domains and the cytoskeleton in animal cells has repeatedly been reported (Kusumi and Sako, 1996; Rodgers and Zavzavadjian, 2001). Actin, spectrin and vimentin have been implicated in retarding the movement of membrane proteins in various mammalian cells (Sheetz *et al.*, 1980; Tomishige *et al.*, 1998; Oliferenko *et al.*, 1999; Runembert *et al.*, 2002). In yeast

cells, this question has not been investigated. Due to its localization, cortical actin (Botstein *et al.*, 1997) might very well be suited for stabilizing Can1p subcompartments of the plasma membrane. Also a potential role of the yeast extracellular matrix, the cell wall, and its protein components has to be considered.

Note added at proof. We were not aware of the publication of Young *et al.*, 2002 (Mol. Cell. Biol. 22, 927–934). These authors observed patch structures of the SUR7 gene product that were stable in time. These structures are very similar to the Can1p patches described here.

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