Visualization of protein compartmentation within the plasma membrane of living yeast cells

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Abstract
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. While 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 non-overlapping 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 more than 30 minutes. 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 (DRMs - detergent resistant membranes, DIGs - detergent insoluble glycolipid-enriched microdomains, or GEMs - glycolipid enriched membranes) are dynamic assemblies enriched in sterols and sphingolipids occurring laterally distributed in the plasma membrane of most, if not all, eukaryotes (see reviews Simons and Ikonen, 1997; Brown and London, 1998). In mammalian cells cholesterol, sphingomyelin and glycosphingolipids are the basic constituents of DRMs (Rietveld and Simons, 1998), whereas in yeast these are ergosterol, inositolphosphoceramide 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 while 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; Ikonen, 2001; Galbiati et al., 2001) and cell signaling (Stauffer and Meyer, 1997; Field et al., 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 (reviews Galbiati et al., 2001; Dykstra 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 towards different detergents (Roper et al., 2000) and in the types of associated proteins. 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 (FR-α and FR-β) and human placental alkaline phosphatase (PLAP) functionally expressed in CHO 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 ER. It was shown that the oligomerization is linked to membrane lipid composition. In ceramide depleted cells, Pma1p stayed monomeric. It was permitted to exit ER, but was subsequently re-routed to the vacuole (Lee et al., 2002).

The size of individual lipid rafts has been estimated to approximately 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 (Jacobson and Dietrich, 1999; Mayor and Maxfield, 1995); punctuate patterns are not observable. Patched raft-marker proteins and lipids are light-microscopically visible only after antibody and crosslinking 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 \textit{S. cerevisiae}: Can1p (H\textsuperscript+-arginine symporter), Pma1p (plasma membrane H\textsuperscript+-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-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\textsuperscript+-ATPase, which generates a proton gradient across the plasma membrane, and the Can1p, the H\textsuperscript+-symporter utilizing 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.
Material and Methods

Strains and growth conditions

Yeast strains used in this study are listed in Table 1. For DNA cloning and propagation of plasmids, *E. coli* strain DH5α or *E. coli* SURE strain (Stratagene) were used.

Bacterial strains were incubated in 2 x 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 w/o amino acids (YNB), 2% glucose, and amino acids as needed). Yeast cells transformed with pVT100U-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 OD_{600} of 1.0. Diploid strains were sporulated in liquid sporulation medium (2% potassium acetate, 0.02% glucose) at 25°C for 2-3 days.

Plasmids

pCAN1GFP: *CAN1* without its stop-codone was generated by PCR on pWHY67α (Hoffman, W., 1985) and subcloned using XhoI/XbaI sites into 2µ vector pVTU100-GFP carrying ADH1 promoter, S65G allele of GFP and URA3 marker (a kind gift from Jürgen Stolz, University of Regensburg, Germany). pHXT1GFP: *HXT1* without its stop-codone was PCR-amplified from SEY6210 genomic DNA and subcloned using XhoI/XbaI sites into 2µ 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, MRC, Cambridge, UK). 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 codone 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, MRC, Cambridge, UK) 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 (HHMI, University of California at 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 OD_{600} 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 µM) and pepstatin (2 µM). After low-speed centrifugation, crude membranes were collected by centrifugation (20 minutes at 23,000 rpm, Beckman Ti70.1 rotor at 4°C). Aliquots of crude membranes (200 µg of total protein) were
resuspended in 270 µl 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) was added to a final concentration of 40% (w/v). 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 µl 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 µl of 1 M Tris base and 25 µl of dissociation buffer (0.1 M Tris-HCl, pH 6.8, 4 mM EDTA, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.02% bromphenol blue). Samples were incubated at 37 °C for 10 min and analyzed by SDS-PAGE and immunoblotting.

**Immunoblot analysis**

Biotinylated pCan1pBioHis was immuno-analyzed by streptavidine-HRP conjugate (diluted 1:5000; Amersham Biosciences). Rabbit anti-Pma1p (1:10000; a gift of R. Serrano, Polytechnic University of Valencia, Spain), anti-Hxt1(1:100; a gift of E. Boles, University of Frankfurt, Germany), and anti-Gas1p (1:500; a gift of L. Popolo, University of Milano, Italy) primary antibodies were used in a combination with peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Biosciences) and a chemiluminiscent detection system (ECL, Amersham Biosciences).

**Arginine uptake assay**

The arginine uptake measurements were performed as described by Opekarova et al., (1998). A mixture of 14C-labelled 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 µM. Aliquots of 100 µl were withdrawn at 1 minute intervals, diluted in 2 ml water, filtered on cellulose nitrate filters
(pore-size 0.8µm; Schleicher & Schuell) 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) prior to microscopic observation of GFP and 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 minutes and cell walls were digested with Zymolyase 20 T (100 µg/ml in 0.1 M potassium phosphate-citrate buffer, pH 5.9, 10 µg/ml pepsstatin). The spheroplasted cells were permeabilized with 1% Triton X-100 for 1 minute. The cells were incubated subsequently with rabbit anti-Pma1p (1:50; 1 hour), and with rhodamine-conjugated goat anti-rabbit (1:200; 45 min; Calbiochem) antibodies at room temperature.

Specimens were viewed using LSM510-Meta confocal microscope (Zeiss) with 100 x PlanApochromat objective (N.A.=1.4). Fluorescence signals of GFP (exc. 488 nm, Ar laser), RFP and rhodamine (both exc. 543 nm, HeNe laser) were detected using band-pass 505-530 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 crosstalk 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* using GFP-tagging. Strains carrying chromosomal C-
terminal \textit{CAN1-GFP} or \textit{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 analogue), and the Can1p-GFP mediated uptake of radioactive arginine (our unpublished results). Since the \textit{PMA1} knock out is lethal (Serrano \textit{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 \textit{HXT1-GFP} fusion. The functionality of Hxt1p-GFP has been proven in hxt null strain RE700\text{Δ}hxt1-7, which does not grow on glucose (Reifenberger \textit{et al.}, 1995). Hxt1p-GFP was able to restore the growth (our unpublished results).

As shown in Fig.1, each of the three studied proteins occupied a different compartment within the plasma membrane and displayed a characteristic pattern. Distinct clusters of Can1p-GFP were observed all over the surface of the transformed cells (Fig.1A, B). On a transversal confocal section, Pma1p-GFP showed also a non-homogenous distribution (Fig. 1C) but, as evident from the surface view (Fig. 1D), the region occupied by Pma1p-GFP was larger, and the patterning different from that of Can1p-GFP. Also, as compared to 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 above, the mapping of Hxt1p-GFP fluorescence (Fig.1E, 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 post-translational modification requires about 30 minutes 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 (Fig. 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.

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 also this protein 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 minutes. It was reported that non-raft 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 results). 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 employed. The majority of the Can1pBio floated in the two top fractions, as did standard lipid raft proteins like Pma1p and the GPI-anchored Gas1p (Fig. 3; see also 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 (Fig.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 results).

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. As compared to the wild type, the arginine uptake in the mutant strains was reduced by a factor of 3 (Fig. 4A). The localization of the specific arginine permease in the strains above was performed in transformants that produced the Can1p-GFP from plasmid pCAN1GFP. As shown in Fig. 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). Fig. 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 (Fig. 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 (Fig. 4B), that was observed also with Can1p-GFP expressed from the chromosomal GFP integration (compare with Fig. 1A). Alltogether, the observations 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 (Fig. 1B 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, we constructed a strain carrying chromosomal *PMA1* tagged with RFP (red fluorescent protein; Campbell *et al.*, 2002; see 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 Fig. 5, the fluorescence patterns of Can1p-GFP and Pma1p-RFP did not co-localize.

Besides the Pma1p-rich areas (Fig. 5D, F; compare also with Pma1p-GFP patterns in Fig. 1C, 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 Fig. 5E and F clearly show Pma1p-RFP minima where Can1p-GFP maxima are located and vice versa (Fig. 5G, H). We, therefore, conclude that Pma1p and Can1p localize in different, non-overlapping subcompartments of the plasma membrane.

An additional feature was apparent from Fig. 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 two hours has been reported for the RFP version used in this study (Campbell et al., 2002). Fig. 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 Figs. 1C, D and 5C, D).

*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 expressing either CAN1-GFP, or PMA1-GFP were grown under the microscope at room temperature and scanned each 10 minutes. In both cases, only minor changes in the protein distributions were observed during the time representing a substantial part of the cell cycle (Fig. 6; note the growing bud in Fig. 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 (Fig. 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 (Fig. 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: Since Can1p and Pma1p fluorescence patterns are mutually exclusive (Fig. 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 here 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 ATPase Pma1p and the hexose transport facilitator Hxt1p tagged with fluorescent proteins show different fluorescence patterns in the membrane (Fig. 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 non-tagged 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 results); 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 Fig. 1C and Fig. 2). Based on all the observations above, we assume that the GFP (RFP) tagging either of the three studied proteins did not cause a 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. As 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
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 about 80 nm (Lee et al., 2002). We observed a high level of photobleaching during the repeated scans of Can1p-GFP fluorescence (Fig. 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 more than one hour, preliminary FRAP experiments indicate that the individual Pma1p-GFP molecules move within this compartment (our unpublished results).

We demonstrated that the Can1p-clusters corresponded to membrane microdomains based on lipid rafts. Both Can1Bio and Can1p-GFP behaved like typical raft proteins (Figs. 3, 4). Since individual rafts are thought to be much smaller (Pralle et al., 2000; Varma and Mayor, 1998; Jacobson and Dietrich, 1999) than the Can1p-GFP domains observed here, 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 ten to hundreds of rafts.

Potential functions of lipid rafts and/or their clusters during signal transduction have been discussed previously (Simons 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 (Fig. 5) raises many questions. For instance, what are the local pH variations on the plasma membrane of a living cell? As we document here, 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 here, there exist at least three different compartments in the yeast plasma membrane: Can1p-rich compartment consisting of lipid raft clusters, lipid rafts-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 multi-helix 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 (Fig. 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 Zavzavidjian, 2001). Actin, spectrin and vimentin have been implicated in retarding the movement of membrane proteins in various mammalian cells (Sheetz et al., 1980; Tomishigo et al., 1998; Oliferenko et al., 1999; Runembert et al., 2002). In yeast cells, this question has not been investigated so far. 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, have to be considered and will have to be checked.

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References


Legends to the Figures

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

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 µm.

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

Figure 4. Mis-targeting 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.

Figure 5. Simultaneous localization of Can1p-GFP and Pma1p-RFP. Fluorescence patterns of Can1p-GFP (A, B; green in E, F) and Pma1p-RFP (C, D; red in E, F) in living *S. cerevisiae* cells (strain CAN1-GFP/PMA1-RFP) are shown. Individual transversal (A, C, E)
and tangential (B, D, F) optical sections are presented. The fluorescence intensity profiles (G, 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 (see also arrowheads in A-F). A differential interference contrast image (I) is also shown. In G and H, fluorescence signals were normalized to the same maximum intensity. Lowpass filter was applied on the graphs to reduce the noise. Bar: 5 µm.

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, 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.
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