Introduction

Several lines of evidence document that the plasma membrane of eukaryotic cells contains lipid microdomains, so-called rafts, that are enriched in sterols and sphingolipids, and in specific raft proteins (Bagnat and Simons, 2002; Anderson and Jacobson, 2002; Edidin, 2003; Zurzolo et al., 2003). Owing to their insolubility in mild nonionic detergents at 4°C (London and Brown, 2000), these microdomains are defined as detergent-resistant membranes (DRMs). After density-gradient centrifugation, rafts are found floating in low-density fractions of membrane solubilizates. Whether the results of density gradients reflect the true organization of lipids in living cells or whether the rafts and their association with defined proteins arise during the detergent extraction is still under debate (Heerklotz, 2002). One of the main shortcomings of the evidence for lipid rafts in vivo is the fact that fluorescence microscopy generally shows an even distribution of green fluorescent protein (GFP) fusions along the plasma membrane of mammalian cells (for a review, see Munro, 2003).

In fungal cells, sterol-rich plasma-membrane domains can be visualized, for example, at the growing tips of Schizosaccharomyces pombe. In these mega-raft-like domains, some specific proteins like Bgs4p (Wachtler et al., 2003) or specific H+ symporters (J. Stolz, personal communication) are concentrated. Uneven distributions of GFP-tagged membrane proteins have also been observed in living cells of Saccharomyces cerevisiae under conditions of polarized growth (distributions of Fus1p, Fus2p, Prm1p, Ste6p and Fig1p) in mating-induced shmoo formation (Bagnat and Simons, 2002). Valdez and Pelham (Valdez and Pelham, 2003) showed that the accumulation of Snc1p in shmoo tips results from its polarized exocytosis and slow diffusion in the plasma membrane in the presence of non-polarized endocytosis. Non-homogeneous distribution of integral plasma membrane proteins was also documented in vegetative cells (Pma1p and Can1p; Sur7p) (Malinska et al., 2003; Young et al., 2002). By contrast, the common statement (Young et al., 2002; Heil-Chapdalaine et al., 2000) that fluorescent fusion proteins are not seen and therefore not present in early buds is generally incorrect. The lack of the fluorescence in these newly synthesized membranes is due to the long maturation times of GFP and red fluorescent protein (RFP) chromophores (see Campbell et al., 2002; Malinska et al., 2003).

Two different patterns of uneven membrane-protein distributions in the plasma membrane of yeast have been observed so far. The arginine/H+ symporter Can1p fused to GFP forms 300 nm patches at the cell surface of living cells, whereas the proton ATPase Pma1GFP fusion gives rise to a more irregular patterning containing 300 nm non-fluorescing ‘holes’. As shown by double fluorescence experiments, these ‘holes’ are exactly filled with Can1p-rich patches. Both Pma1p and Can1p were shown to associate with DRMs (Bagnat et al., 2000; Lee et al., 2002; Malinska et al., 2003). The data of Malinska et al. (Malinska et al., 2003) lead to the conclusion that at least two different raft-based membrane compartments (RMCs) co-exist in the plasma membrane of living yeast cells.

Summary

Recently, lipid-raft-based subdomains within the plasma membrane of living Saccharomyces cerevisiae cells were visualized using green fluorescent protein fusions, and non-overlapping subdomains containing either Pma1p or Can1p were distinguished. In this study, the long-term stability of the subdomains was investigated. Experiments with latrunculin A and nocodazole ruled out the involvement of cytoskeletal components in the stabilization of the subdomains. Also a putative role of the cell wall was excluded, because protoplasting of the cells changed neither the pattern nor the stability of the subdomains. By contrast, the expected inner dynamics of the membrane subdomains was documented by FRAP experiments. Finally, two other proteins were localized within the frame of the Can1p/Pma1p plasma-membrane partition. We show that Fur4p (another H+ symporter) and Sur7p (a protein of unknown function) occupy the Can1p subdomain.

Key words: Lipid rafts, CAN1, FUR4, SUR7, PMA1
For the sake of simplicity, we call the Pma1p-rich domain as RMC P and the 300 nm patches housing Can1p as RMC C.

A surprising observation concerning the two previously characterized types of RMC is their topological stability. The patterns detected in the plasma membranes of mother cells were stable for more than 30 minutes under growing conditions. The high spatial and temporal stability of these membrane domains raised the question of the mechanism of their stabilization. In this study, we applied toxins interfering with actin filament and microtubule formation to see whether these cytoskeletal elements are responsible for the stable RMC pattern. The possible stabilization effect of the cell wall was tested on S. cerevisiae protoplasts.

In addition, we focused on determining whether there are more than two different RMCs hosting different transmembrane proteins and co-existing in the plasma membranes of vegetatively growing cells. In addition to Pma1p and Can1p, the uracil/His+ symporter Fur4p was recently shown to associate with rafts (Hearn et al., 2003; Dupre and Hagnenauer-Tsapis, 2003). Another yeast plasma-membrane protein, Sur7p (a membrane protein of unknown function), was reported to exhibit a patchy cortical distribution (Young et al., 2002). We therefore asked the question of whether these proteins localize to one of the compartments specified as RMC P and RMC C, or whether they define additional lateral membrane compartments.

Materials and Methods

Strains and growth conditions

DNA cloning and propagation of plasmids was performed in Escherichia coli strain DH5α. Bacteria were incubated in 2× TY medium (1% trypton, 1.6% yeast extract, 0.5% NaCl). Ampicillin-resistant transformants were grown in the presence of 100 mg l−1 ampicillin. S. cerevisiae strains used in this study are listed in Table 1. Yeast cells were cultured in YPD medium (2% peptone, 1% yeast extract, 2% glucose) or in a synthetic minimal medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, and amino acids as needed). Geneticin-resistant yeast colonies were selected on YPD/G418 medium (YPD + 200 mg l−1 geneticin G418). In all experiments with Can1p, the appropriate strain was incubated in arginine-free medium to an optical density at 600 nm (OD600) of 0.7-1.0. For the chromosomal expression of FUR4, cells were cultured in synthetic minimal medium with a reduced concentration of uracil (17.8 µM) to an OD600 of 0.7. Sporulation was performed in liquid sporulation medium (2% potassium acetate, 0.02% glucose) at 25°C for 2-3 days.

Construction of plasmids

Construction of pCAN1GFP has been described previously (Malinska et al., 2003). Other plasmids were constructed as follows. (1) YCP33Fur4GFP: FUR4 plus 560 bp from its upstream sequence was amplified using the polymerase chain reaction (PCR) from SEY6210 genomic DNA (primers KM24, KM30) and subcloned using XhoI/BamHI sites into centromeric vector YCp33GFP carrying the S65G allele of GFP and the URA3 marker. (2) YCp111Can1mRFP: CAN1 plus 460 bp from its upstream sequence was PCR amplified from SEY6210 genomic DNA (primers KM41, 3Can1XbaI) and subcloned using XhoI/XbaI sites into the centromeric vector YCp111mRFP carrying monomeric red fluorescent protein (mRFP) and the LEU2 marker. (3) pYK75pMulkMX: mRFP was amplified by PCR (primers km31, km32) from the template pRE7b-mRFP (kindly provided by R. Tsien, HHMI, University of California at San Diego, La Jolla, CA) and subcloned in pV1 (Malinska et al., 2003), from which the tdimer2(12) sequence was excised (BsrBI/NheI restriction sites).

Construction of strains with chromosome-integrated fluorescence tags

An integrative cassette containing mRFP(GFP) and kanMX4 was created by PCR on template DNA pmRFPkanMX or pgFPkanMX, respectively. For the tagging of PMA1 (SUR7, FUR4), PMA1ki5 and PMA1ki3 (SUR7ki5 and SUR7ki3; FUR4ki5 and FUR4ki3) primers were used in PCR amplification. Purified PCR fragments were transformed into SEY6210, yielding the strain PMA1mRFP (KM127), and into SEY6210a, yielding the strains SUR7mRFP (KM153) and FUR4GFP (KM79). The correct integration of the DNA cassette before the stop codon of the targeted gene was confirmed by PCR amplification.

Strains CAN1GFP and SUR7mRFP were crossed, their diploid cells sporulated and tetrads dissected. A haploid strain CAN1GFP/SUR7mRFP (KM160) expressing both CAN1GFP and SUR7mRFP was selected. The diploid strain PMA1GFP/SUR7mRFP (KM155) expressing both PMA1GFP and SUR7mRFP was created by crossing KM153 and KM12 strains.

Microtubule and actin depolymerization

Mid-log-phase cells were grown with 50 µg ml−1 nocodazole (5 mg ml−1 stock in dimethyl sulfoxide; Sigma-Aldrich) for 30 minutes in order to depolymerize microtubules. After the treatment, an aliquot was taken for the microscopic inspection; the rest of the sample was used for the indirect immunofluorescence localization of tubulin. For the actin destabilization, latrunculin-A (Sigma-Aldrich) was added in a final concentration of 200 µM to the mid-log-phase cell culture. Cells were incubated for 1 hour at 30°C, an aliquot was analysed by confocal microscopy and the rest was used for subsequent actin staining.

Cell-wall digestion

Mid-log-phase cultures were washed three times with water, resuspended in KPCS buffer (0.1 M KH2PO4, 0.1 M sodium citrate, 1.2 M sorbitol, pH 6.25) and incubated with 0.5 mg ml−1 zymolyase 20 T (Seikagaku) for 2 hours at 30°C. β-Glucuronidase (G0876; Sigma Aldrich) was added to a concentration of 1000 U ml−1 and cultures were incubated for an additional 15 hours at 30°C.

Isolation of lipid rafts

Lipid rafts were isolated according to Bagnat et al. (Bagnat et al., 2000) with the modifications described by Malinska et al. (Malinska et al., 2003). Briefly, the crude membranes were isolated, resuspended in TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) and solubilized with 1% Triton X-100 for 30 minutes on ice. Then, Optiprep (Nycomed) density gradients were created and centrifuged for 2 hours at 200,000 g in a Beckman SW60 rotor at 4°C. Six equal fractions were collected from the top of the gradient and proteins were precipitated with trichloroacetic acid. Dissolved samples were incubated at 37°C for 10 minutes and analysed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and immunoblotting.

Immunoblot analysis

For the immunodetection of Sur7GFP, a rabbit anti-GFP antibody was used (1:2000; a gift from F. Grieco, ISPA, Lecce, Italy). Pma1p was detected using rabbit anti-Pma1p antibody (1:10,000; a gift from R. Serrano, Polytechnic University of Valencia, Spain). Primary
antibodies were detected using peroxidase-conjugated anti-rabbit IgG antibody (NA934V; Amersham Biosciences) and a chemiluminescent detection system (ECL, Amersham Biosciences).

**Light microscopy**

Before microscopic observation of GFP and mRFP fluorescence, living cells were resuspended in a fresh synthetic medium and immobilized by 0.8% agarose (23°C). For the protoplasts, 0.8% agarose in 1 M sorbitol was used. For actin staining, cells were fixed by 3.7% formaldehyde for 15 minutes at room temperature and washed with PEM buffer (0.1 M PIPES, 5 mM EGTA, 5 mM MgCl2, pH 6.9). Pelleted cells were resuspended in 10 µl PEM buffer, mixed with 2 µl Phalloidin-TRITC (8 µg ml⁻¹; Sigma-Aldrich) and incubated for 1 hour at room temperature in the dark. The cells were washed twice with PEM and observed immediately.

For immunodetection of tubulin (Hasek and Streiblova, 1996), cells were fixed by 3.7% formaldehyde for 2 hours and cell walls were digested with zymolyase 20 T (100 µg ml⁻¹ in 0.1 M potassium-phosphate/citrate buffer, pH 5.9; 10 µg ml⁻¹ peptain). The spheroplasted cells were permeabilized with 1% Triton X-100 for 1 minute. The cells were incubated with α-tubulin-specific rat antibody YOL1/34 (1:50; Amersham) for 1 hour and subsequently with Alexa-Fluor-546-conjugated goat anti-mouse antibody (A11003; Molecular Probes; 1:200) for 45 minutes at room temperature.

Specimens were viewed using LSM510-Meta confocal microscope (Zeiss) with a 100× PlanApochromat objective (NA=1.4). Fluorescence signals of GFP (excitation 488 nm using an Ar laser), mRFP, Alexa Fluor 546 and TRITC (excited at 543 nm using a HeNe laser) were detected using band-pass 505-550 nm and long-pass 585 nm emission filters, respectively. In double-labelling experiments, sequential scanning was used to avoid any cross-talk of fluorescence channels.

For the FRAP (fluorescence recovery after photobleaching) measurement, cells expressing Pma1GFP (strain KM12) were immobilized by agarose and observed. A single round spot 0.5 µm in diameter was bleached within Pma1GFP fluorescence pattern and the recovery of the fluorescence intensity was measured for 20 minutes, 3 frames per minute. A similar spot on an adjacent (unbleached) cell was used as a reference for the correction of photobleaching caused by repeated scanning of the sample. The experimental data were fitted to a simple exponential recovery curve using SigmaPlot 5 (Jandel Scientific).

**Table 1. Yeast strains used in this study**

| Strain | Name | Genotype | Source or reference |
|--------|------|----------|---------------------|
| SEY6210 | SEY6210 | MATα ura3-52 leu2-3,112 his3-Δ100 trpl-Δ901 lys2-801 suc2-Δ9 | Robinson et al., 1988 |
| SEY6210a | SEY6210a | MATα ura3-52 leu2-3,112 his3-Δ100 trpl-Δ901 | J. Stolz, unpublished* |
| BY4742 | BY4742 | MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 | Brachmann et al., 1998 |
| BY4742+pCAN1GFP | KM30 | BY4742 pCAN1GFP | Malinska et al., 2003 |
| PMA1-GFP | KM12 | SEY6210a except PMA1::GFP::kanMX4 | Malinska et al., 2003 |
| CAN1-GFP | KM10 | SEY6210a except CAN1::GFP::kanMX4 | Malinska et al., 2003 |
| SUR7GFP | YJC2054 | SEY6210a except CAN1::GFP::kanMX4 | Young et al., 2002 |
| FUR4GFP | KM79 | SEY6210a except FUR4::GFP::kanMX4 | This study |
| PMA1mRFP | KM127 | SEY6210 except PMA1::mRFP::kanMX4 | This study |
| SUR7mRFP | KM153 | SEY6210a except SUR7::mRFP::kanMX4 | This study |
| PMA1GFP/SUR7mRFP | KM155 | SUR7::mRFP::kanMX4 | This study |
| CAN1GFP/SUR7mRFP | KM160 | SEY6210 except CAN1::GFP::kanMX4 | This study |
| SEY6210c+YCP33FUR4GFP | KM162 | SEY6210+YCP33FUR4GFP | This study |
| SUR7mRFP+YCP33FUR4GFP | KM158 | KM153+YCP33FUR4GFP | This study |
| PMA1mRFP+YCP33FUR4GFP | KM159 | KM127+YCP33FUR4GFP | This study |
| SEY6210c+YCP33FUR4GFP+YCP111CAN1mRFP | KM149 | SEY6210+YCP33FUR4GFP+YCP111CAN1GFP | This study |

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**Results**

Can1p-rich plasma-membrane subdomains are preserved in the absence of actin and/or microtubules

First, we tested the role of actin filaments and cortical actin patches in the maintenance of Can1p-rich membrane domains (RMC C). The strain BY4742 expressing CAN1-GFP from episomal plasmid pCAN1GFP (KM30; Table 1) was treated with latrunculin A in order to depolymerize F-actin. Neither the Can1p distribution (Fig. 1A-C,F-H) nor the temporal stability of Can1p patches (our unpublished work) was altered in the treated cells. The efficiency of actin depolymerization was confirmed by subsequent actin staining with TRITC-phalloidin (Fig. 1D,I). In the biogenesis of new Can1p microdomains, we observed that actin participates in trafficking of Can1p vesicles. The depolymerization of actin substantially decreases the amount of Can1p delivered to the plasma membrane, but it does not influence the formation of Can1p patches (our unpublished work). Importantly, in an experiment performed with cells expressing PMA1::GFP, the Pma1GFP pattern (complementary to the Can1p pattern) and its stability in time were also preserved after latrunculin-A treatment (our unpublished work).

Possible actin-mediated stabilization of RMC C was further excluded by the simultaneous localization of actin and Can1GFP (Fig. 2): no correlation was observed between patches of cortical actin and the Can1GFP plasma membrane distribution. We therefore conclude that the actin cytoskeleton is not directly involved in the maintenance of RMCs.

Next, the role of microtubules in RMC stabilization was tested. The strain KM30 was incubated in a medium containing nocodazole, a microtubule-stabilizing agent. Both the characteristic Can1p plasma-membrane pattern (Fig. 3A-C,F-H) and its stability (our unpublished work) were preserved after this treatment, the efficiency of which was verified by indirect immunofluorescence detection of α-tubulin (Fig. 3D,I). As for actin, the corresponding experiments carried out with KM12 cells confirmed the stability of RMC P pattern in the absence of microtubules (our unpublished work).
Altogether, the results presented above ruled out the possibility that the cytoskeleton components (actin and microtubules) are involved in the formation and/or stabilization of C and P plasma-membrane subdomains.

**Plasma-membrane proteins remain compartmentalized in yeast protoplasts**

Distribution of Can1p, PmA1p and Sur7p in the plasma membrane of protoplasts was addressed. Early logarithmic cells expressing CAN1GFP (strain KM30) were protoplasted with zymolyase, an enzyme that hydrolysé β-1,3-glucan, stabilized in 1.2 M sorbitol and observed (Fig. 4). The fluorescence signal in protoplasts was weaker than in the control cells (treated with sorbitol without zymolyase) but the protoplasts retained the characteristic patchy distribution of Can1p in the plasma membrane (Fig. 4D,F,H).

The parallel experiments with PMA1GFP- and SUR7GFP-expressing cells confirmed this finding (Fig. 5). For the latter protein, a patchy distribution within the plasma membrane and its dependence on an intact cell wall had been reported (Young et al., 2002). However in our experiments, neither the prolonged incubation with zymolyase nor the subsequent treatment with glucuronidase (Fig. 5, right-hand column) resulted in any changes in the plasma-membrane distribution of these proteins. Moreover, the protein distribution remained preserved within the plasma membrane sheets of the burst protoplasts (Fig. 5C,I, arrowheads). Taken together, we assume that the plasma-membrane protein compartmentation is not subject to an interaction between the cell wall and the plasma membrane.

**Pma1p moves freely within its plasma-membrane compartment**

The observed enormous stability of the fluorescence patterns gave rise to a question about the dynamics of the studied proteins within the membrane. Using FRAP, we checked the lateral diffusion of Pma1GFP within its membrane compartment (RMC P) in KM12 cells. As is apparent from Fig. 6, most of the Pma1GFP molecules were mobile. The fitted curve converged on 0.77±0.08, which corresponded to the mobile fraction of the protein. The half-time of the fluorescence recovery was 130±15 seconds. Because of the small size of individual RMC C spots, we did not measure the protein (Can1p) mobility within these areas. Negligible recovery of the fluorescence intensity was observed if the whole RMC C spot was bleached (our unpublished work).

**Can1p, Fur4p and Sur7p colocalize in the plasma membrane**

Within the frame of RMC P and RMC C, the localizations of a raft-associated H⁺ symporter, Fur4p (Dupre and Haguenauer-Tsapis, 2003), and a membrane protein accumulating in distinct patches within the plasma membrane, Sur7p (Young et al., 2002), were investigated. First, we showed that Can1GFP
Plasma membrane protein segregation and Fur4GFP exhibit similar patchy membrane patterns (Fig. 7). Also, under conditions of a weak overexpression from the centromeric plasmid, the patchy pattern of Fur4p remained unchanged (Fig. 7D,E). Both Can1p and Fur4p patterns are comparable to the plasma-membrane distribution of Sur7p described by Young et al. (Young et al., 2002). We tested whether all these proteins occupy the same plasma-membrane compartment. The strains producing GFP and mRFP fusion proteins with Fur4p and Can1p (strain KM149), Can1p and Sur7p (KM160), and Fur4p and Sur7p (KM158) were constructed (Table 1). The mutual localization of the fusion proteins was analysed in each strain (Fig. 8). The simultaneous detection of weakly expressed Can1p and Fur4p was tricky, but very weak fluorescence intensities were detected in both green and red channels (Fig. 8A,D). However, the membrane patchy pattern of both Can1p and Fur4p was clearly visible.

As apparent from merged images, the distribution of all three proteins overlapped to a great extent (notice the yellow spots in the merged fluorescence in Fig. 8G-I). In all cases, the positive correlation of green and red fluorescence signals was documented by the intensity profiles (Fig. 8J-L). Even a comparison of the weak Fur4p and Can1p signals exhibited a non-random coincidence of local fluorescence maxima. Compared with Can1p or Fur4p, Sur7p distribution regularly

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Fig. 3. Microtubules are not required to maintain the Can1p distribution. Cells expressing CAN1GFP (strain KM30; A-E) were treated with nocodazole in order to disassemble the microtubules (F-J). Differential interference contrast (DIC) images (A,F), fluorescence signals from the corresponding transversal confocal sections (B,G) and surface confocal sections (C,H) are presented. The treatment efficiency was tested by indirect immunofluorescence detection of tubulin in fixed cells (fluorescence in D,I; DIC in E,J). Notice the absence of the fluorescence signal from I. The identical scanning parameters were set during the acquisition of D and I. Bar, 10 µm.

Fig. 4. Can1p plasma-membrane distribution is not related to the cell wall. Cells expressing CAN1GFP (strain KM30) were protoplasted with zymolyase. Differential interference contrast images (A,B,G,I), fluorescence signals from the corresponding transversal confocal sections (C,D), and surface confocal sections (E,F,H) before (A,C,E) and after (B,D,F-I) the treatment are presented. Bar, 10 µm.
showed very low background outside the patches (Fig. 8E,F). All protein combinations showed the patches with various intensity ratios. The appearance of patches exhibiting red or green fluorescence only was rare, with the exception of newly synthesized membranes (buds and young daughter cells), which did not show any red fluorescence (see also Malinska et al., 2003).

In order to confirm the localization of Fur4p and Sur7p in

| no treatment | + zymolyase | + zymolyase + glucuronidase |
|--------------|-------------|---------------------------|
| Pma1p        | B           | C                         |
| Sur7p        | D           | E                         |
|              | F           | G                         |
|              | H           | I                         |
|              | J           | K                         |
|              | L           | M                         |
|              | N           | O                         |
|              | P           | Q                         |
|              | R           |                           |

**Fig. 5.** Pma1p and Sur7p plasma-membrane distributions in various protoplasts. Cells expressing Pma1GFP (top left) or Sur7p (bottom left) were protoplasted by a consecutive treatment with zymolyase (middle) and glucuronidase (right). Differential interference contrast images (A-C,J-L), fluorescence signals from the corresponding transversal confocal sections (D-F,M-O) and surface confocal sections (G-I,P-R) are presented. Arrowheads in C,I point to a burst cell. Bars, 10 μm.
Plasma membrane protein segregation

RMC C, both proteins were expressed with the Pma1p, the marker of RMC P (strains KM159 and KM155, respectively). Obviously, neither Fur4p nor Sur7p fluorescence signals localized with Pma1p (Fig. 9). As shown previously for Can1p (Malinska et al., 2003), the Fur4p and Sur7p patches were also confined exclusively to the local minima of Pma1p fluorescence pattern (Fig. 9B,F, arrowheads; Fig. 9M-P, diagrams).

Both Can1p and Pma1p plasma-membrane compartments were defined as raft-based membrane domains. The question now arises of whether Sur7p, which shares RMC C with Can1p and Fur4p, is also associated with lipid rafts. To determine this, the crude membranes of SUR7GFP cells were subjected to the standard procedure of Triton-X-100 treatment and density-gradient centrifugation. Most Sur7p floated in the two top fractions, as did Pma1p, the standard lipid-raft protein (Fig. 10). The data presented above allow the conclusion that the three raft proteins Fur4p, Sur7p and Can1p occupy the same lateral compartment (RMC C) within the plasma membrane of living \textit{S. cerevisiae} cells.

Discussion

Stability of raft-based membrane compartments

Previously, we have shown (Malinska et al., 2003) that two raft-based proteins (Can1p and Pma1p) are located in two distinct subdomains in the yeast plasma membrane and that the pattern of these subdomains is stable for 60 minutes and more in growing cells. With reference to the generally accepted model of fluid mosaic membrane, this observation seems to be rather surprising. Stabilization of plasma-membrane compartments in mammalian cells was proposed to be dependent on the actin-based membrane skeleton (Fujiwara et al., 2002). In yeast, this is obviously not the case because latrunculin A, which depolymerizes F-actin, affects neither the pattern of RMC P and C subdomains nor their stability in time (Fig. 1). In addition, as visualized with Can1GFP, the cortical actin does not colocalize with RMC C (Fig. 2). Also, the depolymerization of microtubules by nocodazole did not affect either the pattern or the stability of the raft-based plasma-membrane subdomains. These data agree with the observation of Young et al. (Young et al., 2002) that the patchy localization of Sur7p and its homologues in the plasma membrane is independent of cytosolic interactions. The possibility that raft-type proteins in yeast are stabilized via an interaction of their external domains with the cell wall had also been checked. We show that removing the cell wall had no effect on the RMC distributions. Our experiments did not confirm the observation of Young et al. (Young et al., 2002), who reported a disassembly of the Sur7p patches after the cell wall removal performed by consecutive treatment with zymolyase and crude glucuronidase (Fig. 5). The reason for this discrepancy is not understood. Based on our results, we conclude that there is no correlation between the integrity either of major cytoskeletal elements or the cell wall and the high stability of the RMC P and RMC C patterns in the yeast plasma membrane. At the moment, the explanation of the pattern preservation can be speculative only.

First of all, the spontaneous separation of liquid ordered and disordered phases in a lipid bilayer has to be considered. Individual small rafts (tens of nm in

Fig. 6. Dynamics of Pma1GFP in the plasma membrane. A round spot of 0.5 µm diameter was bleached within a surface optical section of a living cell (strain KM12) and the fluorescence recovery was measured. Fluorescence intensities were corrected for the photobleaching caused by the repeated scans of the sample and normalized to the intensity before the bleaching (level 1 in the graph). Mean values of the relative fluorescence intensities in nine experiments (dots) and the fitted curve are presented.

Fig. 7. Can1p and Fur4p show similar plasma-membrane distributions. Plasma-membrane distributions of Can1p (A,B) and Fur4p (C-E) are compared. Fluorescence signals from transverse confocal sections (A,C,D) and surface views (B,E) are shown. Notice that proteins expressed from the chromosome [strains KM10 (A,B) and KM79 (C)] or from the centromeric plasmid (strain KM162; D,E) show the same fluorescence patterns. Surface views in B,E were constructed as an average of fluorescence signals from eight consecutive confocal sections each (z-axis sampling: 0.3 µm). Bar, 5 µm.
**Fig. 8.** Fur4p, Sur7 and Can1p colocalize in the plasma membranes of living cells. Simultaneous localizations of Fur4GFP/Can1mRFP (A,D,G,J; strain KM149), Can1GFP/Sur7mRFP (B,E,H,K; strain KM160) and Fur4GFP/Sur7mRFP (C,F,I,L; strain KM158) were performed. GFP (top) and RFP (middle) fluorescence channels, and a merged image (bottom) are shown. Notice the absence of mRFP signal from buds and daughter cells. The fluorescence-intensity profiles along the cell surface (outside the dashed arrows in G-I) were plotted in J-L, respectively. The curves were smoothed using a mean filter to reduce the noise and normalized to the same maximum value. Bar, 10 µm.

**Fig. 9.** Fur4p and Sur7p do not localize with Pma1p in plasma membranes of living cells. Simultaneous localizations of Fur4GFP/Pma1mRFP (two left columns) and Pma1GFP/Sur7mRFP (two right columns) were performed. Fluorescence signals from transversal confocal sections (first and third columns from the left), and surface confocal sections (second and fourth columns) are shown. GFP (A-D) and RFP (E-H) fluorescence channels and a merged image (I-L) are shown. The fluorescence-intensity profiles along the cell surface (outside the dashed arrows in I,K) and through the surface section (between the lines in J,L) were plotted in M,O and N,P, respectively. The curves were smoothed by mean filter to reduce the noise and normalized to the same maximum value. Bar, 10 µm.
Plasma membrane protein segregation

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diameter) are supposed to be quite unstable, with a lifetime ranging from less than a millisecond to seconds (Subczynski and Kusumi, 2003). However, large cholesterol-containing membrane domains of several micrometers diameter persist up to 1 hour in pure lipid membranes (Baumgart et al., 2003). Within these homogeneous, self-organized domains, nothing hinders the movement of individual molecules. In this respect, our observation of Pma1p mobility inside RMC P is not surprising (Fig. 6).

The outstanding stability of RMCs in *S. cerevisiae* can be further related to the specific lipid composition of the yeast plasma membrane. A typical yeast sterol, ergosterol, was shown to form the most stable lipid-raft microdomains of all sterol derivatives tested (Xu et al., 2001). Moreover, the yeast plasma membrane contains a higher proportion of sterols (molar ratio of ergosterol:phospholipid) than the plasma membranes of mammalian cells (for a review, see Opekarova and Tanner, 2002). Hence, the rafts can cover a substantial part of the plasma membrane in yeast. The amount of Pma1p corresponds to about 20% of total yeast plasma-membrane proteins (Serrano, 1991). Even if Pma1p is the only protein of RMC P, this compartment should cover about one-fifth of the membrane surface. Based on the figures shown in this study, the RMC C fraction occupies roughly 10% of the membrane area. In other words, RMCs compose at least one-third of the plasma membrane. According to the relative number of raft proteins identified so far, this fraction could in fact be much higher. Among 24 yeast plasma-membrane proteins studied in relation to their raft association, only three appear to be non-raft proteins (Table 2). This allows the estimate that more than 80% of the plasma membrane of yeast might be constituted as lipid ordered phase. Indeed, there is no continuous area corresponding to lipids in disordered phase (the non-raft membrane) resolvable by fluorescence microscopy (Fig. 9). The complementary fluorescence patterns of RMC C and RMC P cover almost completely the whole cell surface.

**Segregation of membrane proteins into different RMCs**

We have found that membrane proteins Fur4p (a uracil/H+ symporter) and another DRM-associated protein, Sur7p (Fig. 10; a three transmembrane helix protein with unidentified function), are integrated into RMC C (Fig. 8). The colocalization of these proteins with Can1p is almost perfect, although a few patches with only GFP fusions and some with only mRFP fusions can be recognized. We have shown previously that the absence of the GFP (or mRFP) fluorescence signal in the newly synthesized membranes does not necessarily mean the absence of the protein (Malinska et al., 2003). Formation of the GFP fluorophore after the fusion protein has been synthesized takes 30 minutes, whereas it takes about 60 minutes for the mRFP fluorophore to become fluorescent (Campbell et al., 2002). Thus, the occurrence of most of the ‘only green patches’ in Fig. 8 can be easily explained in the terms of a delayed folding of mRFP molecule. The presence of ‘only red patches’, however, cannot but reflect

**Table 2. Raft and non-raft proteins of the plasma membrane in *S. cerevisiae***

| Protein | Triton X-100 resistance* | Patchy distribution† | Source or reference |
|---------|--------------------------|----------------------|---------------------|
| Pma1p   | +                        | +                    | Bagnat et al., 2000; Lee et al., 2002; Malinska et al., 2003 |
| Gas1p   | +                        | ?                    | Bagnat and Simons, 2002 |
| Can1p   | +                        | +                    | Malinska et al., 2003 |
| Fur4p   | +                        | +                    | Hearn et al., 2003; Dupre and Haguener-Tsapis, 2003; this study |
| Sur7p   | +                        | ?                    | Young et al., 2002; this study |
| Ynl194p | ?                        | +                    | Young et al., 2002 |
| Ydl222p | ?                        | +                    | Young et al., 2002 |
| Tat2p   | +                        | (+)                  | Umebayashi and Nakano, 2003 |
| Trk2p   | +                        | (+)                  | Zeng et al., 2004 |
| Hxt2p   | +                        | (+)                  | Bagnat and Simons, 2002 |
| Mid2p   | +/-                      | +                    | S. Strahl et al., unpublished² |
| Wsc1p   | +/-                      | ?                    | Lodder et al., 1999 |
| Fus1p   | +                        | (+)                  | Bagnat and Simons, 2002 |
| Fus2p   | +                        | ?                    | Bagnat and Simons, 2002 |
| Fig1p   | +                        | ?                    | Bagnat and Simons, 2002 |
| Sho1p   | +                        | ?                    | Bagnat and Simons, 2002 |
| Prm1p   | +/-                      | (+)                  | Bagnat and Simons, 2002; Heiman and Walter, 2000 |
| Ste6p   | +                        | ?                    | Bagnat and Simons, 2002 |
| Rvs161p | +                        | +                    | Balguerie et al., 2002 |
| Hxt1p   | -                        | -                    | Malinska et al., 2003 |
| Gap1p   | –                        | –                    | Bagnat and Simons, 2002; K. Malinska et al., unpublished |
| YPL176c-prot | –             | –                  | Bagnat and Simons, 2002 |
| Psr1p   | ?                        | –                    | Siniossoglou et al., 2000 |
| Psr2p   | ?                        | –                    | Siniossoglou et al., 2000 |

*+/– indicates that, after Triton X-100 solubilization, the ratio of solubilized to non-solubilized protein is approximately 1:1.
†(+) indicates a patchy pattern apparent from published figures but not discussed by authors.
²Heidelberger Institut für Pflanzenwissenschaften, Heidelberg, Germany.
the different (more or less overlapping, cell-cycle or growth-phase dependent) time windows, in which the three proteins appear in RMC C. The possibility that each protein occupies an exclusive subpopulation of RMC C spots can be excluded from the presented data. The decision of whether there is another partition within RMC C, in which an individual RMC C spot is subdivided into smaller areas occupied by different proteins, is beyond the resolution limit of fluorescence microscopy. We hypothesize that this is not the case. More likely, the principle of protein co-patching could be based on variability in membrane lipid composition or sorting during trafficking. From this point of view, RMC C represents a homogeneous steady-state compartment with dynamic protein exchange housing various proteins that do not necessarily directly interact with each other.

What structural feature of a membrane protein determines its sorting into different plasma-membrane compartments? At the moment, there is no answer to the question of what controls whether a protein distributes to RMC P or RMC C, but we do not even know what the eventual raft-targeting signals are in general. It has been shown that the overall length of transmembrane domains predisposes the protein to partition into membranes of different thickness (Munro, 1995). This feature is also supposed to influence the raft association. However, a low degree of homology is observed among the three proteins that we have shown to localize to the RMC C compartment. By contrast, a high degree of homology (56%) of two amino acid permeases (Can1p and Gap1p) does not ensure the same final pattern of their distribution in the plasma membrane. Can1p shares (at least in part) its plasma-membrane targeting sequence with Gap1p (Malkus et al., 2002), but the membrane distributions of the two proteins are different (Table 2). A C-terminal deletion of more than one-half of Ynl194p (a homologue of Sur7p) fused to β-galactosidase did not affect its localization in cortical patches. Because this construct did contain the first transmembrane domain, it seems possible that this domain represents or contains the RMC C targeting signal. No obvious homology was found between this transmembrane domain and corresponding sequences within Can1p or Fur4p, however.

Furthermore, many raft proteins are modified. O-Glycosylation is necessary for cell-surface delivery of Fus1p, a raft protein involved in cell fusion during yeast mating (Proszynski et al., 2004). Lipid modifications of glycosylphosphatidylinositol-anchored proteins in mammalian cells were shown to play a role in protein targeting to rafts. A high frequency of palmitoylation among raft proteins has been reported but not all palmitoylated proteins are in rafts (Melkonian et al., 1999). Sequences responsible directly or indirectly for targeting proteins to different RMCs are not known.

What could be the functional significance of the plasma-membrane compartmentation? An obvious answer emerges from studies of protein distribution in polarized, mating-induced S. cerevisiae cells. Only those proteins are concentrated in the shmoo tip (and only there), which are required for the fusion of haploid a and α cells (Bagnat and Simons, 2002). If a lipid-phase separation is based on the changing membrane curvature as demonstrated in giant vesicles (Baumgart et al., 2003), the increased concentration of ergosterol in the shmoo (Bagnat and Simons, 2002) might indeed be the cause for concentrating some proteins in this membrane compartment. However, any functional relevance of raft-based membrane compartments in vegetatively multiplying cells cannot be explained on the same principle. The compartmentation might be relevant for protein clustering and/or for intracellular protein trafficking, as discussed for mammalian cells (Simons and Ikonen, 1997). It is noteworthy that the proton-extruding plasma-membrane ATPase is located in subcompartment RMC P, which is separated from compartments inhabited by proton symporters like Can1p and Fur4p (and possibly others), and thus the areas of opposite proton currents are segregated. The local separation of ion currents has frequently been observed in biological objects, such as in pollen tubes (Weisenseel et al., 1973) or Chara cells (Lucas and Smith, 1973). It would be interesting to learn whether this phenomenon plays a role within the small dimensions of a yeast cell.

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