Gel Domains in the Plasma Membrane of Saccharomyces cerevisiae

HIGHLY ORDERED, ERGOSTEROL-FREE, AND SPHINGOLIPID-ENRICHED LIPID RAFTS

Received for publication, June 28, 2010, and in revised form, November 30, 2010. Published, JBC Papers in Press, December 2, 2010, DOI 10.1074/jbc.M110.154435

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The plasma membrane of Saccharomyces cerevisiae was studied using the probes trans-parinaric acid and diphenyl-hexatriene. Diphenylhexatriene anisotropy is a good reporter of global membrane order. The fluorescence lifetimes of trans-parinaric acid are particularly sensitive to the presence and nature of ordered domains, but thus far they have not been measured in yeast cells. A long lifetime typical of the gel phase (>30 ns) was found in wild-type (WT) cells from two different genetic backgrounds, at 24 and 30 °C, providing the first direct evidence for the presence of gel domains in living cells. To understand their nature and location, the study of WT cells was extended to spheroplasts, the isolated plasma membrane, and liposomes from total lipid and plasma membrane lipid extracts (with or without ergosterol extraction by cyclodextrin). It is concluded that the plasma membrane is mostly constituted by ordered domains and that the gel domains found in living cells are predominantly at the plasma membrane and are formed by lipids. To understand their composition, strains with mutations in sphingolipid and ergosterol metabolism and in the glycosylphosphatidylinositol anchor remodeling pathway were also studied. The results strongly indicate that the gel domains are not ergosterol-enriched lipid rafts; they are mainly composed of sphingolipids, possibly inositol phosphorylceramide, and contain glycosylphosphatidylinositol-anchored proteins, suggesting an important role in membrane traffic and signaling, and interactions with the cell wall. The abundance of the sphingolipid-enriched gel domains was inversely related to the cellular membrane system global order, suggesting their involvement in the regulation of membrane properties.

It is becoming clear (1–4) that the biophysical properties of the lipid moiety of the plasma membrane of eukaryotic cells and their modulation by lipid-protein and protein-protein interactions are involved and stimulate/respond to all sorts of signals and alterations of physiological conditions. For example, during cellular adaptation to hydrogen peroxide (H₂O₂) in Saccharomyces cerevisiae, we found that, together with alterations in the plasma membrane permeability to H₂O₂ (5, 6), there are altered patterns in protein expression and in the lipid composition of the plasma membrane (7) and, concomitantly, its biophysical properties (8). However, the physiological presence and role of stable domains formed on the sole basis of specific lipid-lipid interactions are still debatable (9).

Yeast cells have been used to address important questions regarding membrane microdomain organization and function. In this respect, they exhibit several advantages, such as a much simpler lipidome than mammalian cells and a considerably lower number of genes involved in lipid metabolic pathways (10, 11). Two different types of domains, thought to be the equivalent of liquid ordered domains in higher eukaryotes, have been observed in the plasma membrane of S. cerevisiae as follows: membrane domains enriched in sterols called MCC² for “membrane compartments occupied by Can1p” and domains called MCP for “membrane compartments occupied by Pma1p,” which are possibly sphingolipid-rich domains (12, 13). Recently, another type of compartment has been proposed (14). S. cerevisiae is thus highly suited to study the biophysical properties of different membrane compartments. To address that subject, the plasma membrane of S. cerevisiae was labeled with either of the following: 1) 1,6-diphenyl-1,3,6-hexatriene (DPH), one of the most commonly used membrane probes and known to distribute evenly among most membrane domains, thus giving an indication of the average or global order of the lipid bilayer (15); or 2) trans-parinaric acid (t-PnA), which is one of the few probes that partitions preferentially into ordered domains, where it displays increased fluorescence quantum yield (16), thus being especially sensitive to changes in the amount and composition of those ordered domains (17). For both probes, the chromophore is buried in the hydrophobic core of the lipid bilayer, providing direct information on acyl chain packing. Thus, the results can be interpreted without taking into consideration biophysical complexities on the lipid-water interface/lipid headgroup region or interactions with extra membrane molecules or domains of membrane proteins. The

* This work was supported by Grants PPCDT/BIA-MIC/59925/2004 and PTDC/QUI-BIQ/104311/2008 from Fundação para a Ciência e a Tecnologia, Portugal. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Tables S1 and S2.

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2 The abbreviations used are: MCC, membrane compartment occupied by Can1p; MCP, membrane compartment occupied by Pma1p; DPH, 1,6-diphenyl-1,3,6-hexatriene; t-PnA, trans-parinaric acid; GPI, glycosylphosphatidylinositol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; TMA, trimethylammonium; MLV, multilamellar vesicle; IPC, inositol phosphorylceramide.
probe t-PnA presents the additional benefit of displaying a long lifetime component in its fluorescence intensity decay that is characteristic of the type of ordered domains being detected, namely gel ordered (if clearly above 30 ns) or liquid ordered (below 30 ns) domains (18–21). This probe has been used to characterize membrane domains in different types of mammalian cells (22, 23) but has never been used in yeast cells.

The results obtained in this study show that the yeast plasma membrane contains a significant fraction of gel-like highly ordered sphingolipid-enriched microdomains, with a composition independent of sterol content, and possibly involving glycosylphosphatidylinositol (GPI)-anchored proteins. Those domains cannot be detected by changes in the global order of the membrane, because the latter is inversely related to the abundance of the former.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains**—The *S. cerevisiae* strains used in this work are indicated in Table 1 and were obtained from EUROSCARF (Frankfurt, Germany) and also were the kind gifts from Prof. A. Conzelmann, University of Fribourg, Switzerland, and Prof. H. Riezman, University of Geneva, Switzerland. Deletion of the genes in the mutant strains was checked by PCR amplification using the appropriate primers.

Yeast extract, bactopeptone, yeast nitrogen base, and agar were from Difco. Yeast lytic enzyme (*Arthrobacter luteus*) was purchased from ICN Biomedicals (Aurora, OH). t-PnA and DPH were purchased from Invitrogen, and Ludox (colloidal silica diluted to 50 weight % in water) and ergosterol were purchased from Sigma. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and phytoceramide from *Arthrobacter luteus* were obtained from Avanti Polar Lipids (Alabaster, AL). Lipids were extracted using the Folch method (24). The pure plasma membranes were resuspended in a concentrated ethanol stock solution to the cells to a final concentration of 2 μM and incubated for 20 and 5 min, respectively, at room temperature (24 °C) or at 30 °C.

**Spheroplast Preparation**—Harvested cells were washed once with sterile water and washed once with buffer B (25 mM Tris-HCl, pH 7.5). The pellet was then suspended in buffer D to an A600 = 0.5. Hydrolysis was made by adding Yeast Lytic Enzyme (250 μl of a 1.25 mg/ml solution for each 10 ml of culture) to the culture and incubating for 90 min at 35 °C. Cell wall digestion by zymolase was checked by measuring the decrease of absorbance at 600 nm of cell suspensions. After a centrifugation (5 min at 300 × g), the pellet containing spheroplasts was resuspended with buffer C, and addition of the probes was as described above.

**Total Lipid Extraction**—*S. cerevisiae* cells were inoculated at an A600 of 0.075 and were harvested at A600 = 0.3. They were then centrifuged at 7200 × g for 5 min, washed once with 0.4 M sucrose in buffer C (25 mM imidazole HCl, pH 7.0), resuspended in 0.4 M sucrose in buffer C with protease inhibitor PMSF, and lysed by vortexing three times with glass beads for 2 min, alternated with 2 min on ice. Immediately after, lipids were extracted using the Folch method (24).

**Plasma Membrane Isolation and Lipid Extraction**—The isolation of the plasma membrane was carried out according to Ref. 25 with some minor modifications (7). Briefly, cells (300 A600) were harvested by centrifugation at 5000 × g for 5 min and washed once with 0.4 M sucrose in buffer C (25 mM imidazole HCl, pH 7.0). Cells were resuspended in 0.4 M sucrose in buffer C, containing a mixture of protease inhibitors (100 mM PMSF, 1 mg/ml leupeptin, 0.15 mg/ml benzamidine, and 0.1 mg/ml pepstatin), and lysed by vortexing with glass beads. After a low speed centrifugation at 530 × g for 20 min, the supernatant was recovered and centrifuged at 22,000 × g for 30 min to obtain a pellet containing a crude membrane extract. The crude membrane extract was resuspended in 2 ml of buffer C with the protease inhibitor mixture, applied on top of a discontinuous sucrose gradient (constituted by 12 ml of three layers of 2.25, 1.65, and 1.1 M sucrose in buffer C), and centrifuged in a Beckman SW 28 rotor at 22,000 rpm (90,000 × g) for 18 h. Purified plasma membranes were obtained from the interface between the 2.25 and 1.65 M sucrose layers. The pure plasma membranes were resuspended in buffer C and centrifuged at 30,000 × g for 40 min. The final pellet was resuspended either in buffer C or in buffer D (buffer C plus glycercol (1:1, w/v)) when stored at -80 °C. Plasma membrane lipids were extracted from purified plasma membranes in buffer C using the Folch method (24).

**TABLE 1**

| Yeast strains used in this study | Genetic background/genotype | Source |
|-------------------------------|-----------------------------|--------|
| BY4741 (wild-type)            | MATa his3Δ1 leu2Δ20 met15Δ20 ura3Δ0 | EUROSCARF |
| erg6Δ                         | BY4741; MATa his3Δ1 leu2Δ20 met15Δ20 ura3Δ0 | EUROSCARF |
| per1Δ                         | BY4741; MATa his3Δ1 leu2Δ20 met15Δ20 ura3Δ0 | EUROSCARF |
| scc7Δ                         | BY4741; MATa his3Δ1 leu2Δ20 met15Δ20 ura3Δ0 | EUROSCARF |
| RH3435 (wild type)            | MATa his4 ura3 lys2 leu2 can1 bar1 | H. Riezman |
| RH3616 (erg2Δerg6Δ)           | MATa erg2Δ::URA3 erg6Δ ura3 leu2 can1 bar1 | H. Riezman |

Sterol-independent Lipid Rafts in Yeast Plasma Membrane

JOURNAL OF BIOLOGICAL CHEMISTRY

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VOLUME 286 • NUMBER 7 • FEBRUARY 18, 2011
Liposome Preparation—Lipid stock solutions were made in chloroform except for phytoceramide, which was dissolved in chloroform/methanol (2:1, v/v). Multilamellar vesicles (MLV) of binary and ternary mixtures of POPC with ergosterol and/or phytoceramide or of lipid extracts from plasma membrane fractions and whole cells were prepared in buffer A with either t-PnA or DPH, as described previously (15, 21). Total lipid concentration in MLV suspensions was 0.2 mM in ergosterol-containing systems to minimize the ratio was 1:200 in ergosterol-containing systems to minimize sterol auto-fluorescence to negligible levels. In the case of ergosterol-containing samples, in all steps of the experimental procedure the solutions and suspensions were bubbled with N₂(g) and at all times the samples were protected from light. The absence of oxidation products of ergosterol was confirmed by inspection of the ultraviolet-visible absorption spectra.

Liposomes Reconstituted from Plasma Membrane Lipids without Ergosterol—The removal of ergosterol through incubation with methyl-β-cyclodextrin followed the procedure described in Ref. 26, with minor modifications. Briefly, the liposome suspensions were incubated with 20 mM methyl-β-cyclodextrin for 30 min at room temperature with shaking. They were then centrifuged at 15,000 × g for 45 min. The precipitated liposomes were resuspended in buffer A and labeled with t-PnA as described above for untreated liposomes.

Fluorescence Measurements and Data Analysis—Fluorescence measurements were carried out on a Horiba Jobin Yvon FL-1057 Tau 3 spectrofluorometer. The experiments were carried out at 24 or 30 °C in a temperature-controlled sample compartment with magnetic stirring.

For steady-state measurements, the excitation and emission wavelengths were 358 and 430 nm for DPH and 320 and 404 nm for t-PnA. The steady-state anisotropy (r) was calculated according to Equation 1,

\[ r = \frac{I_{VH} - G \times I_{VH}}{I_{VV} + 2G \times I_{VH}} \]  

(Eq. 1)

in which G is the instrumental correction factor (15). Subscripts V and H represent the vertical and horizontal orientations of the polarizers, and the order of the subscripts corresponds to the excitation and emission. An adequate blank was subtracted from each intensity reading.

For time-resolved measurements by the single photon counting technique, nanoLED N-320 (Horiba Jobin Yvon) was used for the excitation of t-PnA, and emission wavelength was 404 nm. Ludox was used as the scatterer to obtain the instrumental response function. The program TRFA data processor version 1.4 (Minsk, Belarus) was used for the analysis of the experimental fluorescence decays. A global analysis method was applied to completely separate probe emission from the auto-fluorescence of the cells. The decays were analyzed by fitting a sum of exponentials as shown in Equation 2,

\[ I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i) \]  

(Eq. 2)

where \( \alpha_i \) and \( \tau_i \) are the normalized amplitude and lifetime of component i, respectively. The mean fluorescence lifetime was obtained through Equation 3,

\[ \langle \tau \rangle = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \]  

(Eq. 3)

The quality of fit was judged by a reduced \( \chi^2 \) value close to 1 and random distribution of weighted residuals and residuals autocorrelation (see supplemental Fig. S1, A and B for examples).

Labeling and fluorescence measurements did not affect cell viability determined using trypan blue (data not shown). The readings of DPH fluorescence anisotropy were stable at least 5–20 min after probe addition (data not shown).

RESULTS

Plasma Membrane of S. cerevisiae Contains Highly Ordered Lipid Domains—The plasma membrane of S. cerevisiae WT cells was labeled with t-PnA, and the fluorescence decay of the probe was obtained. As can be seen in Fig. 1A, a long lifetime component of 41 ns was present (see also supplemental Fig. S1A). This long component is a fingerprint of a gel phase, i.e. a solid ordered phase. This gel lipid phase is not commonly found in living cells, especially eukaryotic cells, under physiological conditions (27–30). Although the amplitude of this lifetime component was small (Fig. 1B), the contribution to the total fluorescence intensity decay was very significant, due to the long lifetime value, and the average fluorescence lifetime was also among the highest found in cells labeled with t-PnA (Fig. 1C). To confirm that the long component was due to the probe being stably incorporated in the plasma membrane, the fluorescence decay of t-PnA labeling WT cells was obtained for different incubation times (2, 5, and 15 min). Although a very small decrease of the mean fluorescence lifetime could be noted (supplemental Fig. S2), no significant differences were found in the long component lifetime (supplemental Fig. S2C). At short incubation times, the probe was still incorporating in the plasma membrane, as shown by the time-dependent steady-state fluorescence intensity (supplemental Fig. S2B), which reached a plateau at ~5–10 min after probe addition. This is the minimum incorporation time found for single bilayer systems, such as unilamellar liposomes or platelets (23, 31). Thus, the presence of the long lifetime component of t-PnA at short incubation times (supplemental Fig. S2C) indicates that this long lifetime reflects a property of the plasma membrane and not of intracellular membranes or structures (see also text accompanying supplemental Fig. S2). To rule out any temperature effect, controls were also performed where all the procedures and measurements were carried out at 30 °C, and the results are shown in supplemental Fig. S3. The long component is still present (supplemental Fig. S3, panel A), and is slightly shorter (37 ns) than at 24 °C. This was expected because, for a solid ordered domain, the maximum degree of order or rigidity of the lipid bilayer decreases with temperature. However, the amplitude (supplemental Fig. S3, panel B) is the same at both temperatures, i.e. the relative abundance of the domains remains the same at 24 and 30 °C.
However, fast redistribution of probe through intracellular membranes accompanied by continued incorporation of probe in the plasma membrane could not be ruled out. Thus, the plasma membrane fraction from WT cells was isolated, and the fluorescence properties of t-PnA labeling these membranes were characterized. It was previously shown that in mammalian (Chinese hamster ovary) cells the plasma membrane isolated by ultracentrifugation retains to a very significant extent the domain organization of the plasma membrane in intact cells (32). As can be seen in Fig. 1A, the long lifetime component is present in the plasma membrane fraction of WT cells and thus it can be safely assigned to plasma membrane domains. The lifetime is slightly longer than in the plasma membrane of living cells, which may be due to the fact that these are much more dynamic than the isolated plasma membrane fraction. In contrast, the amplitude of the long component (Fig. 1B) and mean lifetime (Fig. 1C) of t-PnA in the isolated plasma membrane are much higher than in intact cells, suggesting that the shorter components are due to t-PnA incorporated in intracellular membranes, as will be discussed below.

Notwithstanding, the fact that the long component was found in an intact cell, with a thick and rigid cell wall, could raise the question of an artifact due to the presence of that cellular structure. This was ruled out by the presence of the same t-PnA fluorescence lifetime component of 41 ns in spheroplasts, i.e. WT cells where the cell wall was removed (Fig. 1A).

Finally, to test if yeast lipids alone are able to form sufficiently rigid domains to yield a fluorescence lifetime component of t-PnA longer than 30 ns, total lipid extracts were obtained from WT cells and reconstituted into liposomes, and these were labeled with t-PnA. The fluorescence properties of the probe in those liposomes were determined (Fig. 1A). As can be clearly seen, the long component of the fluorescence decay was still present, and in fact, it was even longer (~48 ns) than the one obtained in the plasma membrane of intact cells. Thus, yeast lipids in the absence of proteins have the ability to form domains that are even more rigid.

The higher rigidity observed in the lipid extracts could also be due to a reorganization of the lipids upon mixing of intracellular and plasma membrane lipids. To test this the isolated plasma membrane, reconstituted into liposomes, and labeled with t-PnA. The long lifetime component was again detected (Fig. 1A), and the value was identical to the one measured in total lipid extracts. This identity reinforces the following: 1) the plasma membrane lipids are the ones responsible for the formation of the very ordered domains, and 2) the presence of proteins somehow limits the rigidity/compactness of those domains (see supplemental text on the relation between the value of the long lifetime component and rigidity of the environment of the fluorescent probe).

**Relationship between Ordered Domains and Changes in Sterol and Sphingolipid Composition, the Two Major Lipid Classes Involved in Lipid Raft Formation**—The yeast plasma membrane had never been studied with t-PnA, and a priori, the properties of sterol-enriched domains, known as lipid rafts, could be different in yeast and in mammalian plasma membranes.

**FIGURE 1.** Plasma membrane of *S. cerevisiae* contains highly ordered (gel-like) lipid domains. The long component lifetime (A) and normalized amplitude (B) and the mean fluorescence lifetime (C) of t-PnA were obtained from the fluorescence intensity decay of the probe at 24 °C as described under “Experimental Procedures.” The analysis was performed for WT (BY4147) intact cells and spheroplasts (SP) in mid-exponential phase, in the isolated plasma membrane fraction (PM), and in liposomes reconstituted from the lipids extracted from the isolated plasma membrane of WT cells (PM lipids). The long component is also shown for liposomes reconstituted from total lipid extracts (A). The values are the mean ± S.D. of at least four independent experiments. *, p < 0.001 versus WT cells; **, p < 0.01 versus WT cells; ***, p < 0.05 versus WT cells.
membrane. Therefore, it was also hypothesized that the long fluorescence lifetime could be due to incorporation of the probe into ergosterol-enriched ordered domains. However, the 41-ns component was also present in t-PnA fluorescence decay when the probe was labeling the plasma membrane of erg6Δ cells (Fig. 2A), which have deficient lipid rafts (33). These cells synthesize zymosterol and cholesta-5,7,24-trienol instead of ergosterol (see supplemental Table S1). It is thus concluded that gel-like domains are present in the plasma membrane of WT cells and spheroplasts and of erg6Δ cells. Moreover, they should have similar compositions because their rigidity is the same, as explained in the supplemental text.

erg6Δ cells, unlike WT cells, do not possess one major sterol. Therefore, experiments were also carried out with erg2Δerg6Δ cells, which accumulate only the non-raft-promoting zymosterol (see also supplemental Table S1) (34). Because these mutant cells were obtained from a different genetic background than the BY4741 cells, the corresponding WT cells (RH3435) were also analyzed. A long lifetime component of 42 ns was detected in RH3435 cells, which is not significantly different from the one found in the BY4741 WT strain (Fig. 2A). The amplitude and mean fluorescence lifetime were also identical in both WT strains (Fig. 2, B and C). This result shows that the very ordered domains detected by t-PnA are not specific of one genetic background but are a general feature in the organization of S. cerevisiae plasma membrane. Regarding erg2Δerg6Δ cells, a long lifetime component (>30 ns) in t-PnA fluorescence decay was also detected (Fig. 2A). Because erg2Δerg6Δ cells have no sterols with ordering effect, the ordered domains have to be formed by other classes of lipids and should not correspond to a liquid ordered phase. It should be noted that while in erg6Δ cells, the sphingolipid composition remains very similar to WT cells, and in erg2Δerg6Δ cells, in addition to a very different sterol profile, there are profound changes in sphingolipid composition (supplemental Table S2).

If the domains detected by the long component of t-PnA are sterol-depleted, they should be mainly composed by sphingolipids, because these are the other membrane lipids usually associated with membrane ordered domains. Therefore, a major change in sphingolipid composition should lead to an alteration in the value of t-PnA long lifetime component. Thus, scs7Δ cells, which lack α-hydroxylation of sphingolipid-associated fatty acids and have a sphingolipid profile very different from WT cells (supplemental Table S2) (35), were also studied. Those cells have a sterol composition that is identical to the respective WT cells (supplemental Table S1). It was found that the long component of t-PnA was significantly higher in scs7Δ cells when compared with WT cells (Fig. 2A). This constitutes strong evidence that the domains detected by t-PnA are mainly formed by sphingolipids.

On another hand, the amplitude of the long lifetime component is proportional to the amount of the ordered domains relative to the whole membrane (16, 18, 22, 36, 37). From Figs. 1B and 2B, it is thus clear that the relative abundance of the highly ordered domains was significantly different in the plasma membrane of WT cells and spheroplasts and of erg6Δ and scs7Δ cells. These differences are also reflected on the values obtained for the average fluorescence lifetime of t-PnA (Figs. 1C and 2C). Therefore, changes in cell physiology lead
to small alterations in the abundance of the gel-like domains in yeast plasma membrane.

*S. cerevisiae* Plasma Membrane Behavior as a Whole Can Be Differentiated from the Highly Ordered Domains—To understand how the whole membrane system may respond to cellular alterations (absence of ergosterol or absence of cell wall and concomitant decreased fraction of highly ordered domains), the membranes of *S. cerevisiae* were labeled with DPH, a probe that is sensitive to its global properties, rather than to a particular kind of domain (see also supplemental Fig. S4). DPH is a rod-shaped probe that incorporates in the membrane with its long axis parallel to the acyl chain palisade, and its steady-state fluorescence anisotropy is a well-established parameter to report on the alterations undergone in the global order of the membrane (15, 23, 31, 38). As can be seen in Fig. 3, the steady-state anisotropy of DPH was significantly increased in *erg6Δ* cells, *scs7Δ* cells, and spheroplasts from WT cells, when compared with intact WT cells, i.e. the global order was higher in the cells that contain less highly ordered gel-like domains. The outcome of the cellular adaptation to the absence of ergosterol in the composition of the plasma membrane is such that there are less sphingolipid domains and a higher global order of the membrane.

Ergosterol is among the set of special sterol molecules able to induce the increase of the membrane order and the concomitant formation of lipid rafts. Because *erg6Δ* cells accumulate zymosterol, which does not have the ordered domain-forming ability of ergosterol, (39) and cholesa-5,7,24-trienol, a lower order of the membrane bilayer was anticipated in *erg6Δ* cells when compared with WT cells. However, the average order of the plasma membrane in *erg6Δ* cells was increased when compared with WT cells (Fig. 3), as observed previously (8).

In spheroplasts, however, ergosterol was present, and at the same time, the highly ordered domains were less abundant. Spheroplasts displayed the highest average order (Fig. 3), and thus it seems that the lower abundance of the highly ordered domains increases the average order of the membranes. Even in *erg2Δerg6Δ* cells, which have a much lower order of the gel-like domains and a very high abundance of the nonordering zymosterol, the fluorescence anisotropy of DPH was similar to WT cells (supplemental Fig. S5). Altogether, these results point toward a compensating effect from lipids with ordering ability other than sterols. As will be discussed below, *erg2Δerg6Δ* cells have a very increased content of mannosylated sphingolipids as compared with its WT strain (supplemental Table S2).

Moreover, the abundance of the long component in *scs7Δ* cells was smaller than in WT cells (Fig. 2B), leading to a mean fluorescence lifetime of t-PnA that is similar in both WT and mutant cells (Fig. 2C). As concluded above, a lower abundance of highly ordered domains increases the average order of the membrane. Therefore, it can be anticipated that in *scs7Δ* cells the global membrane order was higher than in WT cells. The fact that the fluorescence anisotropy of DPH labeling *scs7Δ* cells was higher than in WT cells (Fig. 3) confirms this prediction.

Highly Ordered Domains in *S. cerevisiae* Plasma Membrane Are Sphingolipid-enriched Gel-like Domains—To further clarify the composition and nature of the highly ordered domains responsible for the presence of a 41-ns lifetime component in the fluorescence decay of t-PnA labeling the plasma membrane of yeast cells, studies in membrane model systems comprising fungal lipid mixtures with well defined compositions were also conducted (Fig. 4). Although the fluorescence decay of t-PnA has been previously studied in synthetic saturated phosphatidylcholine and in mammalian-sphingolipid-containing membranes (17, 21), no studies have been performed in the presence of fungal sphingolipids. In Fig. 4A, the value of the longest lifetime component detected in t-PnA fluorescence decay in mixtures of POPC/ergosterol and in mixtures of POPC/yeast phytoceramide is shown. POPC is one of the most abundant phospholipids in the plasma membrane of *S. cerevisiae* WT cells (35), whereas ergosterol is the major sterol. Phytoceramide is the backbone of all the major complex sphingolipids in yeast plasma membrane (35, 40). In addition, a thorough study of the lipid composition of yeast subcellular membranes using ESI-MS/MS clearly shows that the plasma membrane of *S. cerevisiae* contains a significant quantity of phytoceramide (41). It can be clearly seen in Fig. 4A that even for a very high concentration of ergosterol, corresponding to the lipid bilayer being totally in the liquid ordered phase (42, 43), the longest lifetime component obtained for the fluorescence decay of t-PnA was between 12 and 22 ns. Conversely, in POPC/phytoceramide mixtures, a small amount of phytoceramide was enough to cause a remarkable increase of the longest component of t-PnA fluorescence decay. For mole fractions of phytoceramide above 20%, the value of that component was stabilized at ~41 ns. The amplitude of the long component (inset to the right of Fig. 4A) increased with phytoceramide mole fraction, i.e. with the mole fraction of the gel phase, particularly between 5 and 30 mol % phytoceramide, highlighting the sensitivity of the probe to the presence of gel
phase when this phase does not constitute the majority of the membrane, as should be the case for the membranes of living cells. Thus, these results show that yeast phytoceramide, but not ergosterol, forms a gel phase that gives t-PnA its characteristic long lifetime component. The amplitude of the longest component of the probe decay in POPC/ergosterol was smaller and does not show a well defined trend. This is because it is not the very long component that can be specifically attributed to the gel phase.

We have confirmed that the POPC/phytoceramide binary system undergoes at least one gel/fluid phase transition and obtained a melting temperature for such domains of ~42 °C (Fig. 4B). To do so, we have measured the steady-state fluorescence anisotropy of t-PnA as a function of temperature. Although this parameter is a weighted sum of all the environments felt by the probe, it is a very straightforward measure of the presence of ordered domains, and it allows us to distinguish a gel/fluid transition (a sharp decrease of anisotropy) versus the progressive melting of liquid ordered domains and temperature-induced disordering of the lipid bilayer (a slow decrease of the anisotropy upon raising temperature). In addition, the huge difference between the long lifetime component of ergosterol-enriched and phytoceramide-enriched membrane model systems highlights the sensitivity of t-PnA to the nature of the ordered domains formed by fungal lipids. DPH anisotropy, on another hand, shows variation profiles that are much more similar (supplemental Fig. S4) among the POPC/ergosterol and POPC/phytoceramide mixtures. The fact that DPH anisotropy increased with both ergosterol and phytoceramide is another indication that a reduction of the amplitude of the long lifetime component in yeast cells leads to a significant ordering of the remainder of the membrane, because the diminished abundance of sphingolipid-enriched domains per se, should lead to a decrease of DPH anisotropy.

To confirm that phytoceramide-enriched gel domains are still formed in the presence of ergosterol, ternary mixtures of POPC/ergosterol/phytoceramide were also prepared and labeled with t-PnA. Phytoceramide was used at a constant 1:5 phytoceramide/(POPC + ergosterol) ratio, which corresponds to a mole fraction of 17 mol %, because it is on the order of the sphingolipid concentrations found in yeast when considering sphingolipids, ergosterol, and phospholipids (11,

**FIGURE 4. Lipids from the plasma membrane of S. cerevisiae and phytoceramide form gel domains at physiological temperatures.** A, phytoceramide, but not ergosterol, when mixed with POPC promotes the formation of a gel phase and the appearance of a long component >30 ns in t-PnA fluorescence decay: long component lifetime $\tau_t$ of t-PnA fluorescence intensity decay incorporated in MLVs composed of mixtures of POPC/phytoceramide (gray circles) and POPC/ergosterol (open circles) at 24 °C (see inset for normalized amplitudes). B, steady-state fluorescence anisotropy of t-PnA incorporated in MLVs of POPC/phytoceramide (80:20 mol/mol) as a function of temperature. C, ergosterol at high concentrations abolishes the phytoceramide-enriched gel phase: long component of t-PnA fluorescence decay at 24 °C incorporated in MLVs composed of 1:5 (mol/mol) mixtures of phytoceramide/(POPC + ergosterol) in varying proportions. The dashed line indicates the 30-ns value above which gel domains are known to be present. D, plasma membrane lipids of S. cerevisiae undergo a gel/fluid transition at high temperatures: steady-state fluorescence anisotropy of t-PnA labeling liposomes reconstituted from isolated plasma membrane lipid extracts of mid-exponential WT (BY4147) cells as a function of temperature. E, steady-state fluorescence anisotropy of t-PnA (black bars) versus DPH (white bars) for WT cells in mid-exponential phase and liposomes reconstituted from isolated plasma membrane lipid extracts (PM lipids) and from total lipid extracts of WT (BY4147) cells. A and C, X stands for mole fraction; the lines are merely to guide the eye; in $\tau_t$, $i = 3$ or 4, depending on the mixture. B and D, straight lines are linear fits to the data points. They are used to determine the initial and the final temperatures of the gel/fluid transition, pointed by the arrows, from the intercept of the line with the steepest slope with the lines at lower and at higher temperatures, respectively. All panels: the values are the mean ± S.D. of at least four independent experiments. *, $p < 0.001$ versus t-PnA; **, $p < 0.001$ versus WT cells; ***, $p < 0.05$ versus t-PnA; #, $p < 0.05$ versus WT cells.
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Also, the long component of t-PnA fluorescence decay (Fig. 4A), average lifetime, and anisotropy (data not shown) attains the highest value at ~20 mol % phytoceramide, but still the system is close enough to the gel/fluid coexistence boundary so that the fluorescence of t-PnA will be sensitive to even a partial solubilizing effect of ergosterol. In addition, studying this system allows us to compare its behavior with an analogous system composed of the mammalian lipids POPC/ceramide/cholesterol previously studied by us (21) and to assess which sterol has a stronger ability to solubilize a gel phase. From Fig. 4C, it can be clearly observed that a sphingolipid-enriched gel phase persists until ergosterol concentrations reach ~30 mol % (in relation to POPC), because the long lifetime component of t-PnA remains longer than 30 ns. This concentration is similar to the ergosterol/phospholipid ratio found in the plasma membrane of the S. cerevisiae BY4741 WT strain used in this work and also other WT strains (7, 41). In addition, the sphingolipids are highly enriched at the plasma membrane, i.e. they are present at a molar ratio higher than the one used in this experiment. Thus, although ergosterol has some ability to solubilize phytoceramide-enriched gel domains, the results suggest that at least a small fraction of those domains should persist at physiological concentrations of those lipids. Similarly, in the ternary mixture of mammalian lipids POPC/ceramide/cholesterol, for 17 mol % of ceramide the gel phase was completely solubilized for ~30 mol % cholesterol (21). Therefore, the ability of the main mammalian sterol to abolish gel phases was similar to that of ergosterol.

If the lipids from the yeast plasma membrane are able to form a gel phase at room temperature, then it should be possible to detect a gel/fluid transition at this temperature or higher in liposomes made from such lipids. Thus, liposomes from plasma membrane lipids were prepared and labeled with t-PnA, and the fluorescence anisotropy of the probe was measured as a function of temperature (Fig. 4D). It is clear that a gel/fluid transition that starts at temperatures higher than ~42 °C was present. The shape of the curve on that temperature range was remarkably similar to a recent study with DPH of the transition temperature of inositol phosphorylceramide (IPC) purified from S. cerevisiae (45). In the same work, it is shown that the melting temperature of IPC is higher than that of a major mammalian sphingolipid (45). Because we also obtained a very high gel/fluid transition temperature for liposomes reconstituted from S. cerevisiae plasma membrane lipids (Fig. 4D), and ergosterol and cholesterol showed a similar ability to abolish gel domains, the fact that thus far all studies with t-PnA in mammalian cells always yielded a long component clearly shorter than 30 ns, i.e. a gel phase was not detected, is probably a consequence of the lower melting temperature of their main sphingolipids, as compared with S. cerevisiae.

Additional evidence that t-PnA is detecting gel domains in both cells and liposomes reconstituted from plasma membrane or total lipids stems from the large difference observed between the fluorescence anisotropy of t-PnA and DPH. In a pure gel, a pure liquid ordered or a pure liquid disordered phase, both probes have similar fluorescence anisotropy values (21, 36). In the case of liquid disordered/liquid ordered domains, the anisotropy of t-PnA is only slightly larger than that of DPH because the quantum yield of t-PnA is moderately higher in this phase. However, in the presence of a gel phase, and as described in the Introduction, the quantum yield of t-PnA increases abruptly and its preference for gel phases is also highly marked. This is the only case where the fluorescence anisotropy of t-PnA can display a value typical of a gel phase (close to 0.3 or higher), whereas the anisotropy of DPH is clearly that of a mixture between ordered and disordered phases (close to 0.2 or lower). This marked difference of behavior has been previously used by us to characterize the presence of multiple phases in complex mixtures of mammalian lipids (20, 21, 36). In Fig. 4E, the anisotropy at 24 °C of t-PnA and DPH in WT cells, in liposomes reconstituted from plasma membrane lipids, and from total lipids is compared. In every case, there was a large difference between the anisotropy of the two probes, and thus in all of them a gel phase should be present. The comparison was also made for erg2Δerg6Δ cells in supplemental Fig. S5. Even for erg2Δerg6Δ cells, where the value of the long lifetime component (32 ns) is on the border of the fingerprint of the gel phase, the huge difference among DPH and t-PnA anisotropy confirms that it is due to a gel phase. In addition, and as stated before, the long component is present in WT cells at 30 °C (supplemental Fig. S3), and moreover, the anisotropy of t-PnA at 30 °C in WT and ssc7Δ cells is also very high,3 indicating that at optimal growth temperature the gel domains are present.

A final confirmation that ergosterol is not required for the formation of the highly ordered domains detected by t-PnA was obtained from an experiment in which ergosterol was removed from liposomes made of plasma membrane lipids through incubation with methyl-β-cyclodextrin (Fig. 5). Re-

3 A. F. Fernandes, L. Cyrne, R. F. M. de Almeida, F. Antunes, and H. S. Marinho, unpublished observations.
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moving ergosterol did not affect the presence of the long component but rather leads to a slight increase of its lifetime, possibly due to an added segregation of the lipids with the ability to form a gel phase that otherwise would be in liquid ordered domains stabilized by the raft-promoting ergosterol.

**GPI-anchored Proteins Can Be Accommodated in Highly Rigid Sphingolipid-enriched Domains**—One of the questions that is pertinent to ask refers to which kind of proteins are able to incorporate in the gel-like domains detected by a t-PnA lifetime component >40 ns. In fact, for most transmembrane proteins, the energetic penalty associated with the disruption of strong lipid-lipid interactions will prevent a significant incorporation on those domains (9). However, GPI-anchored proteins possess a lipid anchor, which in yeast mature into two types of lipid moieties. One type is phytoceramidem-based, i.e. presents the same backbone as sphingolipids, and the acyl chain is also the one commonly found in yeast sphingolipids, i.e. the C26:0 chain (hydroxylated or not).

The other type is glycerolphospholipid-based, with a very long chain saturated fatty acid at the sn-2 position. Because the sn-1 position is usually acylated with a saturated fatty acid, and the phytoceramide long chain base is also saturated, both types of anchors are expected to accommodate in sphingolipid-enriched gel-like domains (46). To investigate this possibility, per1Δ cells, which lack one enzyme of the GPI anchor remodelling pathway (47), were also studied. The GPI anchor in per1Δ cells is not reacylated and remains with an unsaturated acyl chain at the sn-2 position. In addition, the ceramide-type anchor is not synthesized (47). The unsaturated lipid anchor would, in principle, be much less efficiently accommodated in sphingolipid-enriched gel domains. The per1Δ cells were labeled with t-PnA, and the value of the long lifetime component is shown in Fig. 2A. The long lifetime component was increased in comparison with all cell types, with the exception of scs7Δ cells. In addition, the abundance of the domains was not significantly different from WT cells (Fig. 2B), and the net result was a longer mean fluorescence lifetime (Fig. 2C). If the major difference in per1Δ cells is that GPI-anchored proteins are not incorporated in gel domains, then these domains may become more compact, because even though the GPI anchor is saturated, the presence of the sugar residues in the headgroup of the attached protein may limit the extent of packing.

Finally, if the abundance of the domains is inversely related to the global order of the membrane, as proposed above, then no significant changes would be expected for the DPH anisotropy in per1Δ cells in comparison with the WT cells. This was indeed observed when per1Δ cells were labeled with DPH (Fig. 3).

**DISCUSSION**

This study presents evidence supporting the recently developed notion that *S. cerevisiae* plasma membrane contains sphingolipid-enriched domains, with either a very low or no sterol content. Furthermore, these domains can be biophysically described as gel-like or solid ordered domains, as opposed to the “typical” lipid rafts, which are described as liquid ordered domains. In addition, the longest values were observed in liposomes reconstituted from plasma membrane lipids and total lipid extracts, i.e. in the absence of proteins. This suggests that the rigidity of the domains is limited by the presence of proteins and that their formation is based on the tendency of certain lipids to laterally segregate.

The probe t-PnA has been used previously to detect ordered domains in mammalian cells. The long component found had values shorter than 21 ns, which correspond to cholesterol-enriched liquid ordered domains (37). To the best of our knowledge, this is the first time that this probe is used to label yeast plasma membrane, and also the first time that a long component typical of the gel phase (>30 ns) is found in living cells. Its presence was detected in two different WT strains (Fig. 2) and in one of these cases at both 24 and 30 °C (supplemental Fig. S3), which is the optimal growth temperature of the strain, indicating that this may be a general result for this type of organism.

Based on the biophysical evidence presented in this study, it is likely that the sphingolipid-enriched domains detected by t-PnA are related to the plasma membrane domain MCP, which comprises a large portion of the membrane. MCP and MCC cover ~80% of the plasma membrane. In this work, it was found that the plasma membrane isolated from *S. cerevisiae* is overall highly ordered (anisotropy of DPH higher than 0.2) and contains a significant fraction of liquid ordered and gel domains (anisotropy of t-PnA higher than 0.3) (Fig. 4E).

Mannosylated species, which comprise the majority of the sphingolipids of the *S. cerevisiae* plasma membrane (48), due to their large headgroup, are expected to be less tightly packed than IPC or phytoceramide. In addition, they are, in principle, more suitable to stabilize liquid ordered domains through interaction with sterols by protecting their hydrophobic ring system from water, because the headgroup of these molecules is merely a hydroxyl moiety. In erg6Δ cells, there is only a very slight increase in mannosyl inositol phosphoceramide levels when compared with WT cells (2), although its biosynthetic rate is significantly slowed down (49). In erg6Δ cells, there is no alteration of the rigidity of sphingolipid-enriched domains, only a slightly smaller fraction (Fig. 2, A and B, respectively). Similarly, sphingolipid synthesis/composition of the elongase system mutant elo3Δ, which contains C22:0/24:0 acyl chain sphingolipids, instead of C26:0, is not affected by the activity of Erg6p (50). However, in erg2Δerg6Δ cells, most sphingolipids are mannosylated, at the expense of IPC, and the gel domains are considerably less tightly packed than in the other cell types studied, because the long component of t-PnA decreases from >40 to 32 ns. Considering that the gel/fluid phase transition detected in the plasma membrane lipid extracts (Fig. 4D) is similar to the one recently obtained for *S. cerevisiae* IPC (45), it is reasonable to expect that the nonmannosylated sphingolipids are the major components of the solid ordered domains of WT cells. It will be interesting in the future to study the biophysical properties and liquid ordered versus gel phase stability of IPC versus mannosylated IPC, in the absence and presence of several yeast sterols, to clarify the differences in sphingolipid compositions between the liquid ordered and gel domains in yeast plasma membrane. An additional physiological significance comes
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SCHEME 1. Depiction of the proposed model explaining the inverse relationship between the abundance of highly ordered sphingolipid-enriched domains and the global order of the membrane. Top represents the lipid components of the plasma membrane of WT or per1Δ cells, and the bottom may represent the plasma membrane of WT spheroplasts or the cell membrane system of erg6Δ or scs7Δ intact cells. Sphingolipids are indicated with their polar heads in gray and glycerophospholipids in black. Top, high abundance of sphingolipid-enriched domains in the plasma membrane, with concomitant sphingolipid depletion in the remainder of the plasma membrane and/or other membranes. Bottom, decreased gel domain abundance implies that the sphingolipids are more scattered through the disordered domains of the plasma membrane and/or intracellular membranes, leading to an increased global order of the cell membrane system.

from the fact that changes in sphingolipid composition seem to compensate for lethality produced by changes in sterol composition and abundance (51).

In all single mutant strains analyzed in this study, an inverse relation between sphingolipid-enriched domains abundance and global order of the membranes was observed (Fig. 3 versus Fig. 2B). However, measurement of membrane order with the probe trimethylammonium-DPH (TMA-DPH), which labels exclusively the plasma membrane surface due to its TMA moiety (52), indicates that there is a decrease of its order in erg6Δ cells, and no alteration in scs7Δ cells, when compared with WT cells (2). A different behavior of DPH and TMA-DPH is better explained by a distinct change in the domain structure of intracellular and plasma membranes. In the mutants described above, the reduced abundance of sphingolipid domains in the plasma membrane may be explained by an accumulation of these lipids in intracellular membranes, which become more ordered but are still below the concentration threshold necessary to segregate into gel domains. In the case of erg6Δ cells, the anisotropy of TMA-DPH is probably smaller due both to the absence of ergosterol and reduced sphingolipid domains in the plasma membrane. In the case of scs7Δ cells, this latter effect is compensated by their increased rigidity due to the absence of the hydroxyl group in sphingolipid-associated fatty acids, and the order of the plasma membrane as detected by TMA-DPH is virtually the same as in WT cells.

In spheroplasts from WT cells, the average order of the bilayer is higher than in intact WT cells (Fig. 3), but the abundance of sphingolipid domains is smaller (Fig. 1B). A possible explanation for these observations may be a cell wall–induced stabilization of the sphingolipid-enriched domains in the plasma membrane. The removal of the cell wall destabilizes those domains, and they may be partially mixed in the bulk membrane or internalized. As a consequence, the bulk membrane is sphingolipid–enriched when compared with intact cells, and this in turn leads to an increased average order of the whole membrane (Scheme 1). It may be speculated that this could be a feedback-like mechanism, by which the cell uses the high transition temperature sphingolipids accumulated in highly ordered domains to impart a higher mechanical resistance to the plasma membrane as a response to cell wall removal or damage. Alternatively, this could be a way of triggering signaling mechanisms. In fact, it cannot be ruled out that the removal of the cell wall is activating stress response pathways that may also alter the plasma membrane. Cell wall stabilization of sphingolipid-enriched domains may involve GPI-anchored proteins, because the sorting of these proteins is sphingolipid-dependent (53, 54), and many of them are targeted to the cell wall (55). However, this hypothesis seems unlikely because per1Δ cells show the same abundance in sphingolipid domains as the WT cells. Noteworthy is the existence of immobile membrane domains that are stabilized by interaction with the cell wall and that contain transmembrane proteins intimately involved in sphingolipid metabolism, the Sur7p family cortical patches (56).

The study of per1Δ cells suggests that the sphingolipid-enriched domains contain a significant amount of GPI-anchored proteins. Thus, it is possible that these domains are involved in GPI-anchored protein traffic and signaling, because their low abundance combined with special packing properties should lead to a high and stable membrane compartmentalization of any molecules that accumulate within those domains. Several findings concerning GPI-anchored proteins may relate to this. Sphingolipids, but not ergosterol, are indispensable both for GPI-anchored protein transport from the endoplasmic reticulum to the Golgi and for the stabilization of their association with membranes (53, 54, 57). GPI-anchored proteins are transported from the endoplasmic reticulum to the Golgi in particular types of vesicles that are ceramide-enriched, which probably contain a significant fraction of gel-like domains as a consequence of their composition. These domains may be delivered to the plasma membrane. It has been suggested that Gas1p, a GPI-anchored protein, would partition into such domains that are not “the typical lipid-raft, i.e. a sphingolipid–rich but ergosterol-poor domain” (50). The formation of this particular type of domain in yeast, but not in mammals, can also help to explain differences encountered in the transport of GPI-anchored proteins in the two groups of organisms.

The presence of the very long chain fatty acid C26:0 in the sphingolipids of S. cerevisiae should be related to certain essential functions, because mutant cells with sphingolipids containing shorter acyl chains are less resistant, e.g. to thermal stress (40). The first biological function associated with those sphingolipids was signal transduction (44). More recently, they have been implicated in cellular growth, endocytosis, and in the vesicular transport of GPI-anchored proteins from the endoplasmic reticulum to the Golgi complex (58). There is no direct evidence that sphingolipid functions are dependent on their segregation into a particular type of lipid domains such as gel domains. However, there are important pieces of evidence suggesting that the biophysical nature or structural properties of the sphingolipid domains are very important. Candidate genes for the regulation of sphingolipid-enriched gel domains should include those involved in cer-
amine synthesis, such as \textit{LCB1}, \textit{LAG1}, and \textit{LAC1}. In fact, \textit{lcbl} \(\Delta\) cells are unviable unless they carry the semi-dominant \textit{SLC1}–1 mutation, which carries a Q44L substitution in the \textit{Slc1} protein, a 1-acyl-sn-glycerol-3-phosphate acyltransferase. This mutation allows cells to survive by catalyzing the incorporation of C26:0 fatty acids into the sn-2 position of glycerolipids, which then mimic yeast ceramides and serve as substrates for the enzymes that add the polar headgroups found in yeast sphingolipids (59). The lethal double mutation \textit{lag1} \(\Delta\) \textit{lac1} \(\Delta\) is rescued by overexpression of the \textit{YDC1} gene. This gene codes for a reverse ceramidase that catalyzes the synthesis of unusual glycerophospholipids of the phosphatidylinositol family, which are sometimes mannosylated and also contain very-long-chain fatty acids that are exclusively found in sphingolipids (60). C26:0-fatty acylglycerolipids can replace sphingolipid functions (61). These unusual glycerophospholipids can, in principle, partially mimic the biophysical properties of the WT sphingolipids, including the tendency to segregate into gel-like domains. A role for the very long chain fatty acid containing phosphoinositol in stabilizing highly curved membrane domains in nuclear membranes has also been suggested (62). In the proposed model, the clustering of this type of lipid would occur in a sterol-independent manner.

The domains detected in this study may be involved in cellular processes related to plasma membrane dynamics. In fact, the plasma membrane of \textit{S. cerevisiae} cells adapted to \(\text{H}_2\text{O}_2\) has reduced levels of the hydroxylated very long chain fatty acid 2-OH-C26:0 present in the sphingolipids when compared with control cells (7) and simultaneously has a lower permeability to \(\text{H}_2\text{O}_2\) and several alterations of the membrane order, including an increased fluorescence anisotropy of DPH (8) and a higher quantum yield of t-PnA (7).

Recently, cholesterol-independent and sphingolipid-enriched microdomains containing signaling molecules have been identified in the plasma membrane of mammalian cells (3). In addition, two mammalian raft receptors have been localized to distinct plasma membrane domains (63). Such domains may well be the mammalian counterparts of the yeast domains identified in this study. It was also found that dihydrosphingomyelin has an increased ability to form gel phase domains when mixed with cholesterol and mammalian phospholipids when compared with sphingomyelin with the same acyl chain (64). Dihydrosphingomyelin has a sphingoid backbone that is closer to yeast sphingolipids than sphingomyelin. In addition, the rigidity of the ordered domains was affected by a small alteration of the long chain base near the headgroup region, as was observed in this study for \textit{scs7} \(\Delta\) cells (Fig. 2A).

The mechanism of formation, dynamics, and physiological relevance of the gel-like sphingolipid-enriched domains in yeast remains open. However, this study paves the way for future developments. Furthermore, because these domains have a less elusive nature and are more stable than the liquid ordered-like lipid rafts, they offer the possibility to clarify many open questions in this rapidly expanding field.

Acknowledgments—We thank Prof. Andreas Conzelmann for the kind gift of per1 \(\Delta\) cells and for helpful discussions. We also thank Prof. Howard Riezman for the kind gift of erg2 \(\Delta\)erg6 \(\Delta\) cells.

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