Yeast Lipids Can Phase-separate into Micrometer-scale Membrane Domains*

Christian Klose†, Christer S. Ejsing†, Ana J. García-Sáez†, Hermann-Josef Kaiser†, Julio L. Sampaio†, Michal A. Surma‡, Andrej Shevchenko‡, Petra Schwille‡, and Kai Simons†‡

From the †Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany, the ‡Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark, and the †Institute for Biophysics, Biotechnology Center (BIOTEC), Technische Universität Dresden, Tatzberg 47-51, 01307 Dresden, Germany

The lipid raft concept proposes that biological membranes have the potential to form functional domains based on a selective interaction between sphingolipids and sterols. These domains seem to be involved in signal transduction and vesicular sorting of proteins and lipids. Although there is biochemical evidence for lipid raft-dependent protein and lipid sorting in the yeast Saccharomyces cerevisiae, direct evidence for an interaction between yeast sphingolipids and the yeast sterol ergosterol, resulting in membrane domain formation, is lacking. Here we show that model membranes formed from yeast total lipid extracts possess an inherent self-organization potential resulting in liquid-disordered-liquid-ordered phase coexistence at physiologically relevant temperature. Analyses of lipid extracts from mutants defective in sphingolipid metabolism as well as DRMs (18–21). In analogy to mammalian cells, yeast DRMs are enriched in the sphingolipids inositolphosphoceramide (IPC), mannosyl-inositol phosphoceramide (MIPC), mannosyl-dinositolphosphoceramide (M(IP)2C), and ergosterol (19). Consequently, proper biosynthesis of sphingolipids and/or ergosterol is a prerequisite of plasma membrane delivery of ramethyldicarbocyanine perchlorate; GP, generalized polarization; DRM, detergent-resistant membrane; LD, liquid-disordered; Lo, liquid-ordered; Rh-DOPC, rhodamine B-dioleoyl-phosphatidyl-ethanolamine; DPH, diphenyl-1,3,5-hexatriene; SL, sphingolipids; Ch, channel; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene.

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The membranes that surround the various organelles of eukaryotic cells have distinct lipid compositions. For example, the concentration of sphingolipids and sterols increases along the secretory pathway, being lowest in the endoplasmic reticulum and highest at the plasma membrane (1–3). The major sorting station for vesicular transport of proteins and lipids within the cell is the trans-Golgi network (4). Here, clusters of sphingolipids and sterols as well as proteins have been proposed to be involved in the formation of secretory vesicles (SVs) (5, 6). These clusters, called lipid rafts, were proposed to form by the preferential interaction between lipids containing saturated acyl chains, especially (glyco-) sphingolipids and sterols, and by intermolecular hydrogen bonds between (glyco-) sphingolipids. As compared with bulk cellular membranes, lipid rafts are characterized by a higher acyl chain order and tight packing of lipids (7).

Protein-free model membranes have been widely used to study the self-associative properties of sphingolipids and sterols, which are believed to be responsible for lipid raft formation in vivo (8, 9). In model membranes with a lipid composition similar to that of detergent-resistant membranes (DRMs) from mammalian cells, the preferential interaction between sphingolipids and sterols is manifested as the coexistence of two fluid membrane phases, which can be observed microscopically in giant unilamellar vesicles (GUVs) (10–13). More specifically, model membranes produced from equimolar mixtures of sphingomyelin (SM), phosphatidylcholine (PC), and cholesterol show domains in the liquid-disordered (Ld) state that are enriched in PC coexisting with a liquid-ordered (Lo) phase rich in SM and cholesterol, the latter being a defining component of the Lo phase (14, 15). The Lo phase is characterized by a higher acyl chain (conformational) order than the Ld phase. Both phases exhibit translational disorder, i.e. the lipid molecules are able to diffuse freely in the plane of the membrane (16, 17).

The yeast Saccharomyces cerevisiae has been used as an experimental system to study lipid raft-dependent processes (18, 19). In the present study, the issue whether yeast sphingolipids and ergosterol give rise to fluid/fluid phase coexistence in model membranes is addressed. There is a wealth of information available in the literature supporting the idea of lipid raft-dependent protein and lipid sorting in yeast. Many membrane proteins that are transported along the secretory pathway to the plasma membrane are associated with DRMs (18–21). In analogy to mammalian cells, yeast DRMs are enriched in the sphingolipids inositolphosphoceramide (IPC), mannosyl-inositolphosphoceramide (MIPC), mannosyl-dinositolphosphoceramide (M(IP)2C), and ergosterol (19). Consequently, proper biosynthesis of sphingolipids and/or ergosterol is a prerequisite of plasma membrane delivery of.
several integral membrane proteins (22–25). Moreover, genes involved in sphingolipid and sterol biosynthesis have been shown to interact genetically, further substantiating the view that they are also interacting functionally (26). Finally, a lipidomic analysis of purified post-Golgi vesicles recently provided direct evidence for the selective sorting of sphingolipids and ergosterol into SVs (27). However, experimental evidence for a selective interaction between yeast sphingolipids and ergosterol resulting in phase separation is lacking. Therefore, total lipid extracts and purified yeast lipids were reconstituted in model membranes and investigated with respect to their phase separation propensity. Both spectroscopic and microscopic methods provided evidence for a selective interaction between yeast sphingolipids and ergosterol. This interaction results in phase separation into membrane domains with Lo- and Ld-like properties. Accordingly, yeast sphingolipids and ergosterol fulfill an important criterion of the lipid raft concept.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—The yeast strains used in this study were in the BY4741 background (csg2::kanMX4, sur2::kanMX4, or elo3::kanMX4 in MATa his3Δ leu2Δ met15Δ ura3Δ; EUROSCARF). Yeast strains were grown in complete synthetic medium containing 2% glucose and supplemented with 100 μM inositol at 25 °C.

Reagents—C18-sphingomyelin, palmitoyl-oleyl phosphatidylcholine, cholesterol, and lissamine-rhodamine B-dioleoyl-phosphatidyl-ethanolamine (Rh-DOPE) were from Avanti Polar Lipids. Ergosterol and diphenyl-1,3,5-hexatriene (DPH) were from Sigma. Yeast phosphatidylinositol (PI) was from Larodan Fine Chemicals. The species composition of yeast PI was confirmed by thin layer chromatography and mass spectrometry. IPC-containing fractions were also analyzed by mass spectrometry. IPC-containing fractions were pooled, and the solvent was evaporated. IPC was dissolved in chloroform/methanol/water 65:25:4 (v/v/v) to give a total SL extract. For the purification of IPC, liquid column chromatography with Silica Gel 60 (Sigma) as solid phase was performed. The total SL extract (~5 mg of lipid) was loaded onto the column and eluted with 60 ml of chloroform/methanol/water 65:25:4 (v/v/v) followed by 60 ml of chloroform/methanol/water 65:35:8 (v/v/v). Fractions were collected and analyzed by mass spectrometry. IPC-containing fractions were pooled, and the solvent was evaporated. IPC was dissolved in chloroform/methanol 1:2 (v/v). The purity of the preparation was confirmed by thin layer chromatography and mass spectrometry. About 2 mg of IPC were obtained as determined by phosphate analysis.

Formation of GUVs and Confocal Fluorescence Microscopy—GUVs were formed by electro-formation in custom-made Teflon chambers with platinum electrodes having a distance of 5 mm (31). Lipids and fluorescent membrane dyes were mixed in organic solvent. A total of 100 nmol of lipids were loaded onto the platinum electrodes and dried under vacuum. The electrodes were placed into GUV formation chambers filled with 350 μl of 300 mM sucrose solution. Electro-formation was carried out with an alternating field at 1.2 V and 10 Hz at 68 °C. GUVs were detached from the electrodes at 1.2 V and 2 Hz at 68 °C. Samples were cooled slowly to room temperature and added to observation chambers (Lab-Tek chambered coverglass #1 German borosilicate, Nunc) filled with PBS. The observation chambers were blocked before with 2 mg/ml BSA or poly-L-lysine (Sigma). GUVs were observed with a confocal laser-scanning microscope (Zeiss LSM 405/594) with a C-Apochromat 40×/1.2 W corr objective at room temperature.

Two-focus Scanning Fluorescence Correlation Spectroscopy (FCS)—Diffusion measurements by two-focus scanning FCS on GUVs were performed at room temperature (22 °C) on a laser scanning microscope Meta 510 system (Carl Zeiss, Jena, Germany) using a 40× NA 1.2 UV-VIS-IR C-Apochromat water immersion objective as described previously (31, 32). The laser power was 25 microwatts. BODIPY-cholesterol (a dye that partitions equally into Lo and Ld domains) at a concentration of 0.01 mol % was used as fluorescent probe for both the Lo and the Ld phase (29). This ensures that the differences in diffusion measured in the two domains are caused by differences in membrane properties and not by the structure of the fluorescent probe itself. In two-focus scanning FCS, two parallel lines are repeatedly scanned in a perpendicular way through a verti-
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cal membrane. The intersections with the membrane give rise to two intensity traces from which the autocorrelation and the spatial cross-correlation curves can be calculated. Auto- and cross-correlation curves were fitted to a two-dimensional elliptical Gaussian model with software written in MATLAB (MathWorks) (32).

C-laurdan Spectroscopy and Microscopy—Membrane order was determined by C-laurdan spectroscopy with large unilamellar vesicles (LUVs) at 23 °C as described by Kaiser et al. (33). LUVs were prepared as described (34). In brief, organic solvent was evaporated, and the lipids were rehydrated in 200 μl of liposome formation buffer (50 mM Hepes, 150 mM NaCl, 0.2 mM EDTA, pH 7.25). The samples were incubated at 68 °C, 1000 rpm for 30 min followed by five freeze-thaw cycles (freeze in liquid nitrogen, thaw at 25 °C and 1000 rpm). Liposomes were formed by extrusion at 68 °C using gas-tight syringes (Hamilton), 100-nm Whatman® Nucleopore track-etch membranes (Schleicher and Schuell), Whatman drain discs, and the Avanti mini-extruder (Avanti Polar Lipids). 2 pmol of C-laurdan was added. Liposomes were incubated 15 min at 23 °C before spectra acquisition with a FluoroMax-3 fluorometer (Jobin Yvon Horiba). The instrumental setting was: increment = 1 nm; excitation slit = 4 nm; emission slit = 3 nm. No polarizers were used. Measurements were performed in a Quartz cuvette. The sample was excited with a wavelength λ = 385 nm, and the emission spectrum was recorded from 400 to 550 nm. The background spectrum (obtained with the blank sample) was subtracted from the emission spectra, and the generalized polarization (GP) value was calculated according to

$$GP = \frac{I_{400-460} - I_{470-530}}{I_{400-460} + I_{470-530}}$$  \hspace{1cm} (Eq. 1)

where $I_{400-460}$ is the sum of the fluorescence intensities from λ = 400 – 460 nm and $I_{470-530}$ is the sum of the fluorescence intensities from λ = 470 – 530 nm.

For C-laurdan microscopy, GUVs were formed, and imaging was carried out as described above. To GUVs corresponding to 15 nmol of lipid, 0.1 nmol of C-laurdan was added. GP images were acquired on a Bio-Rad two-photon setup with a Mira 2000 two-photon laser and a 543 nm laser line using a 60 water immersion objective (NA 1.2). C-laurdan was excited at 800 nm, and the emission was captured using 425/50 (Ch1-low) and 452/70 (Ch1-high) filters. Image processing and analyses were carried out using MATLAB R2006B (MathWorks). GP images were computed according to the following

$$GP = \frac{l_{ch1} - G*i_{ch2}}{l_{ch1} + G*i_{ch2}}$$  \hspace{1cm} (Eq. 2)

where the G-factor served to calibrate the channels. GP images were displayed as two-fold binned heat maps as indicated next to the images (Fig. 6A) (33).

DPH Anisotropy Measurements—The main phase transition temperature of IPC was determined by DPH fluorescence anisotropy on unilamellar vesicles as described previously (14). 15 nmol of lipid were dried under vacuum and rehydrated with 70 μl of liposome formation buffer (50 mM Hepes, 150 mM NaCl, 0.2 mM EDTA, pH 7.25) at 68 °C for 60 min followed by five freeze-thaw cycles in liquid nitrogen and at room temperature. DPH was added to a final concentration of 10 μM, and the vesicles were heated slowly to 60 °C and incubated for 30 min in a quartz cuvette. Then, the vesicles were cooled slowly to 4 °C and again heated to 74 °C with a heating rate of 0.4 °C/min. Temperature was controlled with a Thermoe-Haake thermostat. DPH fluorescence anisotropy was measured with a FluoroMax-3 fluorescence spectrometer (Jobin Yvon Horiba). The samples were excited with 345 nm light, and the fluorescence at 427 nm was detected with an integration time of 0.2 s. The main phase transition temperature was defined by the midpoint of a sigmoid fit to the anisotropy versus temperature curve (35).

RESULTS

GUVs from Yeast Total Lipid Extracts Show Micrometer-scale Phase Separation—Membranes with a complex lipid composition have been shown to possess an inherent organizational heterogeneity caused by the preferential interaction between certain lipids. This heterogeneity may be manifested in micrometer-scale phase separation within the membrane bilayer. Dietrich et al. (11) showed that model membranes formed from purified brush border membranes exhibit fluid/ fluid phase coexistence below 45 °C. Furthermore, chemically induced giant plasma membrane vesicles derived from rat basophilic leukemia (RBL) mast cells can separate into two coexisting fluid phases at temperatures between 10 and 25 °C (36). Finally, plasma membranes have been shown to have the positional capacity for a raft-based and sterol-dependent membrane phase separation at physiologically relevant temperature (37). If there are lipid species within the yeast lipidome that preferentially interact with one another to facilitate the formation of membrane rafts, one would predict that model membranes of yeast lipid extracts should show properties similar to that of membranes made of lipids from mammalian cells.

To test this prediction, GUVs were formed from total lipid extracts of yeast cells. As revealed by confocal fluorescence microscopy at 23 °C, the GUVs were homogenously labeled with BODIPY-cholesterol, which distributes simi- larly between the Lo and the Ld phases (Fig. 1A). However, a significant proportion of vesicles (~10%) showed domains enriched in DiD, a marker for the Ld phase, coexisting with domains that excluded this dye. The partitioning of the dyes between the two membrane domains implied the coexistence of two fluid-like membrane phases. To confirm this assumption, the translational diffusion of BODIPY-cholesterol in both membrane domains was determined by two-focus scanning FCS (Fig. 1, B and C) (32). In the DiD-enriched domain, BODIPY-cholesterol diffused with $D = 3.1 \mu$m$^2$/s (±0.2, S.E.). In contrast, BODIPY-cholesterol diffused with $D = 0.13 \mu$m$^2$/s (±0.02, S.E.) in the DiD-depleted domain. Both values are in agreement with diffusion coefficients reported for the Ld and the Lo phase, respectively (38). Therefore, the yeast lipidome contains species that facilitate Ld-Lo phase coexistence.

Defects in Sphingolipid Metabolism Reduce Membrane Order and Prevent Phase Separation—According to the lipid raft concept, the selective interaction between sphingolipids and sterols result in the formation of biologically active membrane domains (5, 8). To ascertain whether yeast sphingolipids are

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Note: The text is a continuation from the previous page, discussing the experimental methods and results related to phase separation in yeast lipids, including spectroscopy and microscopy techniques. The context involves the study of membrane order and phase separation in yeast, highlighting the role of sphingolipids and sterols in membrane organization.
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A fatty acid elongase, results in depletion of C26 very long chain fatty acids as well as reduced levels of sphingolipids (28, 39). SLUR2 encodes the hydroxylase responsible for the conversion of dihydrosphingosine to phytosphingosine. Its deletion results in sphingolipids lacking the C4 hydroxylation of the sphingoid base moiety (40).

The mutant total lipid extracts were used for the formation of LUVs and subsequent C-laurdan spectroscopy. C-laurdan is a fluorescent probe that exhibits a fluorescence emission spectrum that is sensitive to lipid packing (30, 33). From the emission spectrum, one can calculate a GP value that can theoretically assume values between −1 and 1; a high GP value reflects ordered membranes, whereas a low GP value is indicative of more disordered membranes (33). LUVs from wild type total lipid extract have a GP = 0.133 (Fig. 2A). Notably, LUVs produced from sur2Δ total lipid extracts exhibit a significantly lower GP value (0.101), suggestive of a lower membrane order. The membrane order is even further decreased in LUVs made from elo3Δ extracts, as reflected by a GP value of 0.043. The reduced membrane order in these mutants was not caused by the accumulation of free long chain bases (28) because the addition of exogenous long chain bases did not affect membrane properties of liposomes formed from wild type lipid extracts (not shown). To examine whether defects in sphingolipid metabolism also affect the propensity of the membranes to exhibit phase separation, GUVs were formed from mutant total lipid extracts. Interestingly, neither GUVs from sur2Δ extracts nor GUVs from elo3Δ extracts showed phase separation (Fig. 2B).

Taken together, lipid extracts from mutant yeast strains with defective sphingolipid biosynthesis involved in phase separation, we characterized model membranes made from lipid extracts of the sphingolipid metabolism mutants sur2Δ and elo3Δ. Deletion of ELO3, the gene encoding a concomitantly perturbed sphingolipid composition confer a decreased order of model membranes, which is accompanied by the inability to promote micrometer-scale phase sep-
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Characterization of Model Membranes with Defined Compositions of Yeast Lipids—Next, we undertook a systematic investigation of the properties of yeast lipids and their impact on membrane order. To this end, we made binary and ternary lipid mixtures of IPC, yeast PI, and ergosterol similar to the well characterized raft mixture SM/PC/cholesterol. This result confirms previous reports showing the property of ergosterol to have a condensing effect on glycerophospholipids (43–46). (ii) Yeast PI increases the order of membranes containing IPC, irrespective of whether cholesterol or ergosterol is used as the sterol in these mixtures. Notably, this effect is most significant for IPC/yeast PI/ergosterol (compare with IPC/PC/ergosterol). (iii) IPC-containing membranes have a lower order than membranes containing SM. However, their order is considerably higher than that of pure PC vesicles, which are bona fide Ld membranes and give rise to negative GP values (33).

GUVs Containing IPC/PI/Ergosterol Show Lo-Ld Phase Separation—The GP value measured for IPC/yeast PI/ergosterol suggests that this mixture has the capacity to promote phase separation. Therefore, we tested whether this mixture would phase-separate in GUVs. GUVs composed of IPC/yeast PI/ergosterol 1:1:1 (molar ratio) were formed and visualized by confocal fluorescence microscopy. Importantly, the GUVs showed prominent micrometer-scale phase separation. Domains that exclude the Ld phase marker DiD coexist with domains that are labeled by DiD (Fig. 5A). To ensure that the domains are present in the fluid Lo- and Ld-like states, respectively, two-focus scanning FCS was performed. The calculated diffusion coefficients confirmed that the DiD-labeled domains are in the Ld-like phase, whereas the DiD-excluding domains are present in the Lo-like phase (Fig. 5B and C). However, the diffusion coefficient measured for the Ld-like phase is lower than previously reported values for the Ld phase in GUVs containing SM/DOPC/cholesterol (38). Slower diffusion is indicative of a higher membrane order. To get another measure for order, C-laurdan microscopy was applied to GUVs containing IPC/yeast PI/ergosterol (Fig. 6). The GUVs were labeled with Rh-DOPC, a marker for the Ld-like phase, and with C-laurdan. Again, GUVs were phase-separating in domains that exclude or

![FIGURE 3. Phase transition temperature of IPC. Phase transition temperatures (T_m) were determined by DPH fluorescence anisotropy measurements as described under “Experimental Procedures.” The T_m of IPC was determined to be 53.4 °C (± 0.2; S.E.; red). As a control, the T_m of C18-SM was determined (T_m = 44.0 °C ± 0.1; S.E.; black). (n = 3).](image)

![FIGURE 4. IPC and ergosterol form ordered membranes. Membrane order was measured by C-laurdan spectroscopy of LUVs consisting of binary and ternary equimolar lipid mixtures. Error bars indicate S.E. (n ≥ 3), SM = C18-SM; chol = cholesterol; erg = ergosterol; PI = yeast PI; PC = palmitoyl-oleyl phosphatidylcholine.](image)
properties of model membranes composed of yeast lipids and compared them with the well characterized model membrane system containing mammalian SM, PC, and cholesterol.

Phase Transition Temperature of IPC—As revealed by a decrease in DPH anisotropy, membranes formed from IPC, a major representative of the yeast sphingolipids, undergo a transition from gel phase to Ld phase at 53.4 °C. This value is higher than the \( T_m \) of C18-SM (\( T_m = 44^\circ \mathrm{C} \)), which was used for comparison throughout this study because its properties in model membranes have been studied in detail (14, 38). A major structural parameter known to have an impact on the phase transition temperature of lipid bilayers is the ability of a lipid to form intermolecular hydrogen bonds with surrounding lipids. Intermolecular hydrogen bonds are mainly formed between the carbonyl and the amide group of SM and the 3-hydroxyl group and the phosphoryl oxygens (47). In comparison with SM, yeast IPC contains additional hydroxyl groups at the 2 position of the amide-linked fatty acid moiety (\( \alpha \)-hydroxylation) and at the 4 position of the sphingoid base (i.e. phytosphingosine, see structures in Fig. 7) (28). These structural attributes increase the probability of hydrogen bond formation between adjacent lipid molecules, thereby stabilizing the gel phase and presumably resulting in increased phase transition temperature (48). Moreover, yeast sphingolipids contain hydroxyl-rich inositol phosphate headgroups that have a positive effect on the phase transition temperature of lipids. For example, glycosphingolipids have higher phase transition temperatures than non-glycosylated sphingolipids (48). It is therefore reasonable to assume that the inositol headgroup of IPC with five hydroxyl groups is involved in extensive hydrogen bonding (48, 49). This view is supported by a recent study reporting a higher \( T_m \) for an inositol phosphate-containing sphingolipid than for an acyl chain-matched SM (50).

Another structural feature of IPC is its hydrocarbon chain asymmetry. As reported for C24-sphingomyelin, hydrocarbon chain asymmetry might result in partially or even mixed interdigitated bilayers in the gel phase (51–53). Interdigitated bilayers are characterized by a complex thermotropic behavior caused by changes in the degree of interdigitation upon heating above the phase transition temperature (51). One might speculate that the rather broad transition from gel to fluid phase observed for IPC is caused by similar transitions between interdigitation states as observed for C24-SM.

### DISCUSSION

Biophysical studies of lipids have so far focused on a limited number of lipids. Of the specific yeast lipids, only ergosterol has been investigated in detail. The question that we addressed herein was whether yeast sphingolipids and ergosterol are capable of forming membrane domains. We characterized the biophysical base properties of model membranes composed of yeast lipids and compared them with the well characterized model membrane system containing mammalian SM, PC, and cholesterol.
hydrogen bonds between ceramide backbones of SMs are likely to facilitate their interaction. Moreover, despite the fact that the amide group of IPC and the 3-hydroxyl of ergosterol might interact with each other, mainly caused by van der Waals attractive forces between the saturated very long fatty acid chains of IPC and ergosterol and hydrogen bonds between the headgroup nitrogen of SM and the 3-hydroxyl of cholesterol (55, 56). Alternatively, charge pairing is unlikely to occur and could potentially increase the condensation state of the bilayer (56). This view is supported by the finding that cholesterol does not increase the distance between the phosphates of adjacent C18-SM molecules and hence is not decreasing the probability of headgroup interactions (56).

Interestingly, the order of IPC/cholesterol bilayers is significantly lower than that of IPC/ergosterol bilayers. However, there is no difference between C18-SM/cholesterol and C18-SM/ergosterol bilayers (Fig. 4). This result implies that ergosterol has some structural properties distinct from cholesterol that are required for the condensation of IPC-containing bilayers. Ergosterol differs from cholesterol in additional double bonds in the B ring and the hydrocarbon side chain and an additional methylation at C24, the latter two making the side chain stiffer and bulkier, respectively (59). These structural attributes were proposed to restrict the motions of acyl chains more efficiently and thus lead to stronger ordering by ergosterol as compared with cholesterol (43–46). As judged from the higher order of IPC/ergosterol membranes, this effect is more pronounced for IPC, whereas the sterol structure does not seem to be critical for the ordering of C18-SM-containing bilayers (Fig. 4). One might speculate that the typical ergosterol structure, i.e. the stiff and bulky side chain, is critical for ordering lipids with very long chain fatty acids. In support of this view, a functional relation between ergosterol side chain structure and fatty acid length has been suggested based on the finding that the mutant erg6 genetically interacts with elo3Δ (60). Future experiments will reveal whether the most abundant yeast SL M(IP2)C has similar properties with respect to its interaction with ergosterol and phase separation.

IPC-Ergosterol Interactions—Because the preferential interaction between sphingolipids and sterols is an essential tenet of the lipid raft concept, the next step was to investigate the interaction of IPC with ergosterol (5, 7). Several experimental data indicate a strong tendency of SM and cholesterol to interact with each other, mainly caused by van der Waals attractive forces between the saturated acyl chain of SM and the rigid cholesterol ring backbone (52, 54). In addition, experimental and computational data suggest that hydrogen bonds between SM and cholesterol might facilitate their interaction, presumably via the amide group of SM and the 3-hydroxyl of cholesterol (55, 56). Alternatively, charge pairing between the headgroup nitrogen of SM and the 3-hydroxyl of cholesterol was proposed to be involved in the interaction of IPC with SM (55, 57). Because the order of IPC/cholesterol membranes is comparable with SM/cholesterol membranes (Fig. 4), the IPC-cholesterol interactions seem to be similar to the interactions described for SM and cholesterol. Although charge pairing is unlikely to occur because the IPC headgroup is negatively charged, van der Waals interactions between the saturated very long fatty acid chain of IPC and ergosterol and hydrogen bonds between the amide group of IPC and the 3-hydroxyl of ergosterol might facilitate their interaction. Moreover, despite the fact that hydrogen bonds between ceramide backbones of SMs are known to be reduced in the presence of cholesterol, IPC-IPC interactions via the headgroup hydroxyl groups might still occur and could potentially increase the condensation state of the bilayer (56). This view is supported by the finding that cholesterol does not increase the distance between the phosphates of adjacent C18-SM molecules and hence is not decreasing the probability of headgroup interactions (56).

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Micrometer-scale Phase Separation in Membranes Containing Yeast Lipids—Model membranes containing SM, PC, and cholesterol exhibit micrometer-scale phase separation into Lo and Ld domains (11, 38). We wanted to determine whether yeast sphingolipids and ergosterol exhibited a similar property. As revealed by C-laurdan spectroscopy (Fig. 4), the membrane order of ternary mixtures containing IPC is similar to that of other phase-separated membrane systems (33). Membranes containing IPC, ergosterol, and PI, the major glycerophospholipid in yeast (28), showed a higher order than bilayers containing PC. This mixture also showed prominent micrometer-scale phase separation, resembling SM/PC/cholesterol-containing GUVs (Figs. 5 and 6) (11, 38). The phase separation property of IPC/yeast PI/ergosterol membranes depends on the presence of IPC because GUVs containing yeast PI/ergosterol (2:1 molar ratio) did not show any phase separation (not shown).

What drives the domain formation in membranes containing IPC, yeast PI, and ergosterol? First, the preferential interaction of IPC with ergosterol via hydrogen bonds and van der Waals interactions between the very long saturated fatty acid and the rigid sterol ring backbone could be important. IPC might be preferred over yeast PI because the most abundant yeast PI species comprise an unsaturated fatty acid, which is probably unfavorable for an interaction with ergosterol (28, 46). Second,
the very long chain fatty acid of IPC might lead to a hydrophobic mismatch between the IPC-rich Lo-like domain and the adjacent Ld-like domain (61). Third, asymmetric sphingolipids with very long chain fatty acids interdigitate into the opposing leaflet of the bilayer, even in the presence of a sterol, whereas the more symmetric yeast PI acyl chains are not expected to interdigitate (52, 62). This difference in acyl chain organization between an IPC-rich Lo-like phase and a PI-rich Ld-like phase might be an additional determinant for phase separation (53). Together, the differences in domain properties give rise to a high line tension at the domain boundaries, eventually leading to micrometer-scale phase separation to minimize domain boundaries.

The interactions between yeast sphingolipids (represented by IPC) and ergosterol in model membranes of simple composition can also give rise to phase separation in GUVs produced from a yeast total lipid extract (Fig. 1). As revealed by two-focus scanning FCS, the coexisting domains have Lo- and Ld-like properties. Furthermore, membrane order and phase separation depend on a proper sphingolipid composition because model membranes derived from total lipid extracts of mutants with defective sphingolipid metabolism have a reduced order and fail to phase-separate microscopically (52, 62). The finding that model membranes formed from total lipid extracts of mutants with defective sphingolipid metabolism have a reduced order and fail to phase-separate microscopically has interesting implications for lipid raft-dependent protein transport in yeast. The mutants used here, sur2Δ and elo3Δ, have been shown to be defective for plasma membrane transport of integral membrane proteins (23, 24). It has been suggested that an aberrant membrane structure might be a reason for the observed effects (24). The altered properties of model membranes from sur2Δ and elo3Δ extracts support this notion (Fig. 2). Hence, the self-associative properties of yeast sphingolipids and ergosterol might facilitate both lipid and protein sorting into SVs.

Taken together, we provide direct evidence for membrane domain formation mediated by yeast sphingolipids and ergosterol. Yeast lipids thereby fulfill a key tenet of the lipid raft concept (5, 7, 8, 64). With this result, a comprehensive picture of raft-based lipid and protein sorting in yeast is emerging; sphingolipids and ergosterol are selectively sorted into secretory vesicles together with cargo protein destined to the plasma membrane (27). This process eventually results in the enrichment of these lipids at the plasma membrane (2, 63). During transport, yeast sphingolipids and ergosterol become detergent-resistant, and DRM association is required for proper localization of several trans-membrane proteins to the cell surface (18–21, 24, 25). These proteins lose DRM association in mutants with defective sphingolipid and/or ergosterol biosynthesis. Moreover, genes involved in sphingolipid and ergosterol biosynthesis interact genetically, indicating that these lipids also interact functionally (26, 60). The finding that yeast sphingolipids and ergosterol interact in membranes to form distinct fluid domains now provides a mechanistic framework for these indirect observations.

4 C. Klose, D. Lingwood, H.-J. Kaiser, M. A. Surma, and K. Simons, unpublished data.
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