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Phospholipid Synthesis and Lipid Composition of Subcellular Membranes in the Unicellular Eukaryote *Saccharomyces cerevisiae*

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Subcellular membranes of *Saccharomyces cerevisiae*, including mitochondria, microsomes, plasma membranes, secretory vesicles, vacuoles, nuclear membranes, peroxisomes, and lipid particles, were isolated by improved procedures and analyzed for their lipid composition and their capacity to synthesize phospholipids and to catalyze sterol Δ^{24}-methylation. The microsomal fraction is heterogeneous in terms of density and classical microsomal marker proteins and also with respect to the distribution of phospholipid-synthesizing enzymes. The specific activity of phosphatidylserine synthase was highest in a microsomal subfraction which was distinct from heavier microsomes harboring phosphatidylinositol synthase and the phospholipid *N*-methyltransferases. The exclusive location of phosphatidylserine decarboxylase in mitochondria was confirmed. CDP-diacylglycerol synthase activity was found both in mitochondria and in microsomal membranes. Highest specific activities of glycerol-3-phosphate acyltransferase and sterol Δ^{24}-methyltransferase were observed in the lipid particle fraction. Nuclear and plasma membranes, vacuoles, and peroxisomes contain only marginal activities of the lipid-synthesizing enzymes analyzed. The plasma membrane and secretory vesicles are enriched in ergosterol and in phosphatidylserine. Lipid particles are characterized by their high content of ergosteryl esters. The rigidity of the plasma membrane and of secretory vesicles, determined by measuring fluorescence anisotropy by using trimethylammonium diphenylhexatriene as a probe, can be attributed to the high content of ergosterol.

Most of the enzymes involved in cellular phospholipid biosynthesis are membrane associated. In mammalian cells, the majority of phospholipids is synthesized in the endoplasmic reticulum (14). Phospholipids specifically required for mitochondrial function (cardiolipin and its precursor phosphatidylglycerol) as well as phosphatidylethanolamine (via decarboxylation of phosphatidylserine) are synthesized in mitochondrial membranes (11).

In previous studies, several enzymes of phospholipid biosynthesis of the yeast *Saccharomyces cerevisiae* (10, 26), namely glycerol-3-phosphate acyltransferase, CDP-diacylglycerol synthase, phosphatidylserine synthase, and phosphatidylinositol synthase, were detected both in the microsomal fraction and in the outer mitochondrial membrane. These observations were based mainly on the separation of subcellular membranes by differential centrifugation and on commonly used marker enzymes for the respective fractions. Motivated by our interest in the mechanisms of lipid flow and membrane assembly in yeasts and by conflicting data concerning the subcellular targeting of phosphatidylserine synthase (38), we reinvestigated the subcellular distribution of lipid-synthesizing enzymes by employing recently developed or improved fractionation procedures for mitochondrial and microsomal membranes, the nuclear membrane (24), the plasma membrane (37), secretory vesicles (42), vacuoles (40), and peroxisomes. The results obtained led us to revise previous assumptions concerning the dual localization of several phospholipid-synthesizing enzymes in the endoplasmic reticulum and in the outer mitochondrial membrane. It appears that at least some of the enzymes investigated reside in distinct compartments which are not mitochondrial and do not contain classical marker enzymes specific for endoplasmic reticulum membranes.

Yeast subcellular membranes were also characterized with respect to their protein-to-lipid ratio, their content of ergosterol and ergosteryl esters, and their pattern of individual glycerophospholipids. Measurements of fluorescence anisotropy revealed significant differences between the various membrane fractions. High anisotropy could be correlated with a high ergosterol-to-protein ratio, whereas a high protein-to-phospholipid ratio led to low anisotropy in some, but not all, membranes tested.

MATERIALS AND METHODS

Yeast strains and culture conditions. *S. cerevisiae* wild-type strains D273-10B (ATCC 25657) and X-2180, *S. cerevisiae cho1* null mutant SDK03-1A (38), and a mutant defective in the secretory pathway (*S. cerevisiae sec1*, provided by R. Schekman) were used in our studies. Wild-type cells were cultivated in 2-liter flasks at 30°C in a rotary shaker with vigorous aeration. Routinely, semisynthetic growth media were used containing 2% lactate as the carbon source (12). For the preparation of plasma membrane, vacuoles, lipid particles, and nuclei, cells were grown on YPD medium (defined below) containing 3% glucose. For the induction of peroxisomes, strain D273-10B was grown in a medium containing 0.1% oleic acid, 0.2% Tween 80, and 0.05% galactose. In control experiments, analogous media lacking oleic acid and Tween 80 were used. SDK03-1A was grown in complex (YPD) medium containing 3% glucose, 1% yeast extract (Difco), and 2% peptone (Difco). Secretory vesicles were isolated from *S. cerevisiae sec1* grown overnight at 24°C in YPD medium, transferred to fresh YPD low glucose medium (0.2% glucose), and shifted to the restrictive tem-

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TABLE 1. Marker enzymes and immunological markers used for the characterization of subcellular fractions of *S. cerevisiae*

Subcellular fraction	Strains and growth conditions	Marker enzymes and antisera (reference)
Plasma membrane	Wild-type X-2180; YPD	Plasma membrane ATPase antiserum[a]
Secretory vesicles	*sec1* shifted to restrictive temperature (37°C) for 2 h prior to preparation; YPD low (0.2%) glucose	Invertase (18)
Vacuoles	Wild-type X-2180; YPD	α-D-Mannosidase (33)
Mitochondria, inner membrane	Wild-type D273-10B; 2% lactate medium (12)	Succinate dehydrogenase (1)
Mitochondria, outer membrane	Wild-type D273-10B; 2% lactate medium (12)	Porin antiserum (12)
Heavy microsomal fraction[b]	Wild-type D273-10B; 2% lactate medium (12)	GDP-mannosyl transferase (3)
Light microsomal fraction[c]	Wild-type D273-10B; 2% lactate medium (12)	NADPH cytochrome *c* reductase (34); 40-kDa protein antiserum (12)
Nucleus	Wild-type D273-10B; YPD	38-kDa protein antiserum[d] (24)
Peroxisomes	Wild-type D273-10B; induced in the presence of 0.5% oleic acid and 0.1% Tween 80 (22)	Catalase (2)

[a] Donated by Serrano, EMBL, Heidelberg, Federal Republic of Germany.
[b] 20,000 to 30,000 × *g* pellet.
[c] 30,000 to 100,000 × *g* pellet.
[d] Donated by Hurt, EMBL, Heidelberg, Federal Republic of Germany.

perature (37°C) for 2 h to induce the block in the secretory pathway.

Cell fractionation. (i) Preparation of mitochondria. Cells were grown for 16 h at 30°C to an optical density at 546 nm of 5 to 6 in semisynthetic media containing lactate as the carbon source, were harvested by centrifugation, and were converted to spheroplasts as previously described (12). The spheroplasts were homogenized in breaking buffer (0.6 M sorbitol, 5 mM MES [morpholineethanesulfonic acid], 1 mM KCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 6.0) or in MT buffer (0.6 M mannitol, 10 mM Tris Cl, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) by using a Dounce homogenizer (Brown) with a tight-fitting pistil (10 strokes). Unbroken cells and debris were removed by centrifugation at 3,000 × *g* for 5 min. The resulting supernatant (total homogenate) was centrifuged at 30,000 × *g* for 15 min, and the pellet was resuspended in breaking buffer (pH 6.0) or MT buffer (pH 7.4). After removing debris by centrifugation at 3,000 × *g* for 5 min, the organelle pellet containing mitochondria and heavy microsomes was isolated by recentrifugation of the supernatant at 30,000 × *g* for 15 min. The organelle pellet was suspended in the appropriate homogenization buffer and layered on top of a linear sucrose gradient, ranging from 30 to 60% (wt/wt) sucrose in 5 mM MES–1 mM KCl, pH 6.0, or 10 mM Tris Cl, pH 7.4, respectively. Gradient centrifugation was carried out in a SW-28 swing out rotor (Beckman) at 25,000 rpm for 3 h. The gradient was at equilibrium after this amount of time of centrifugation. Fractions of 2 ml were withdrawn with a needle from the bottom of the tube. Mitochondria were collected in fractions 8 to 14, corresponding to a sucrose concentration of about 40 to 50%.

(ii) Preparation of peroxisomes. Peroxisomes were isolated from *S. cerevisiae* D273-10B cultivated under inducing conditions (Table 1). Cells were harvested by centrifugation and washed once with distilled water. The preparation of a 30,000 × *g* membrane pellet was performed following the procedure described above by using breaking buffer, pH 6.0. Density gradient centrifugation was carried out in a linear sucrose gradient (30 to 60% sucrose, wt/wt) in breaking buffer, pH 6.0. The band containing peroxisomes was withdrawn with a syringe. Fractions were diluted with 4 volumes of the appropriate breaking buffer and recentrifuged at 34,500 × *g* for 30 min. Enriched peroxisomal membranes lacking matrix proteins were prepared by suspending isolated peroxisomes in buffer containing 10 mM Tricine, 1 mM EDTA, and 0.6 M sorbitol, pH 8.0, removing unbroken organelles by centrifugation at 8,000 × *g* for 10 min, and centrifuging the supernatant at 200,000 × *g* for 30 min. Peroxisomal membranes were suspended in 10 mM Tris Cl buffer, pH 7.4, for further analysis.

(iii) Preparation of other subcellular membranes. Yeast plasma membrane was isolated by the method of Serrano (37), and nuclei were prepared following the procedure of Hurt et al. (24). Secretory vesicles were obtained from the *S. cerevisiae* mutant *sec1* after transfer to the restrictive temperature (37°C) for 2 h (see "Yeast strains and culture conditions") by the method of Walworth and Novick (42). Vacuoles were isolated by the method of Uchida et al. (40) with the following modification to remove most of the adhering lipid particles. After the last step of flotation, crude vacuoles were homogenized very gently in 5 mM MES–Tris, pH 6.8–0.6 M sorbitol by using a loose-fitting Dounce homogenizer. This sample (7 ml) was layered on top of a 30-ml cushion consisting of 3 volumes of 0.6 M sorbitol and 1 volume of 0.6 M sucrose in 5 mM MES–Tris, pH 6.8, and centrifuged for 1 h at 27,000 rpm in an SW-27 rotor (Beckmann) (43). The pellet formed during this centrifugation step contained enriched vacuoles. According to microscopic observations, lipid particles remaining in this fraction seemed to adhere to the vacuolar membrane. The purified lipid particle fraction was collected from the top of the gradient.

Characterization of subcellular fractions. (i) Marker enzymes. Marker enzymes and antisera used for immunotitration of isolated membrane fractions are listed in Table 1. The microsomal enzyme GDP-mannosyl transferase I (Mat) was assayed essentially as described by Babczinski et al. (3) with some modifications. The concentration of GDP-[^{3}H]mannose in the assay was 20 μM (50 Ci/mol). The assay mixture contained 10 mM Tris Cl (pH 7.3), 7 mM $MgCl_2$, 0.1% Triton X-100 (wt/vol), and 70 μg of dolichol monophosphate per ml in a total volume of 0.1 ml. At 0, 0.5, and 1 min, 40 μl of the assay mixture was withdrawn; the reaction was terminated by addition of 0.5 ml of chloroform-methanol, 2:1 (vol/vol), and the reaction product was extracted. The organic phase was washed once with 0.5 volume of $MgCl_2$ solution (0.034%) and once with 0.5 volume of methanol-water-chloroform (48:47:1, by volume). Radioactivity was determined in 10 ml of scintillation cocktail (Lipoluma; Baker) in a Packard Tricarb 1500 liquid scintillation counter. The reaction was linear with respect to time and protein in the range between 0 to 1 min and 0 to 20 μg of protein per ml.

The peroxisomal marker enzyme, catalase, was assayed photometrically at 240 nm in a buffer containing 50 mM KH_2PO_4 (pH 7.5), 0.1% Triton X-100, and 0.114% perhydrol (2). Succinate dehydrogenase (1), invertase (18), NADPH-cytochrome *c* reductase (34), and α-D-mannosidase (33) were assayed according to published procedures.

(ii) Immunological characterization of subcellular fractions. Antibodies against porin (outer mitochondrial membrane), ADP-ATP carrier (inner mitochondrial membrane), and the 40-kDa microsomal protein were raised in rabbits as described earlier (12). Antiserum against the yeast nuclear 38-kDa protein was generously provided by E. Hurt (EMBL, Heidelberg, Federal Republic of Germany), antiserum against plasma membrane ATPase was a kind gift of R. Serrano (EMBL, Heidelberg, Federal Republic of Germany), and antiserum against yeast ribosomal protein L16 was donated by J. Woolford (Carnegie-Mellon, Pittsburgh, Pa.). Antibody against phosphatidylserine synthase was generated as described previously (25). Immunotitrations were performed after separating proteins on 10 or 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (27) and transferring them to nitrocellulose sheets by following standard procedures (19). Rabbit antibodies bound to antigens were detected by using alkaline phosphatase or peroxidase-conjugated goat anti-rabbit secondary antibodies by following the manufacturer's instructions.

(iii) Activity analyses of phospholipid-synthesizing enzymes. CDP-diacylglycerol synthase (26), phosphatidylinositol synthase (26), phosphatidylserine synthase (38), and phosphatidylserine decarboxylase (27a) were analyzed as previously described. Phospholipid *N*-methyltransferases were assayed by the method of Yamashita et al. (44), except that 5 mM $MgCl_2$ was added to the assay mixture. Glycerol-3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase were assayed by the methods of Schlossmann and Bell (35) and Schutgens et al. (36). Acyl-CoA synthetase was assayed as described by Mishina et al. (32). Sterol Δ^{24}-methyltransferase activity was determined by following the incorporation of radioactivity from *S*-adenosyl-[^{3}H]methionine into the sterol fraction (31). Unsaponifiable lipids containing zymosterol as a substrate were prepared from *S. cerevisiae* dissolved in ethanol (0.75 mg/ml) and added to the assay to a final concentration of 12 μM.

(iv) Miscellaneous analytical procedures. Protein was quantitated by the method of Lowry et al. (29) by using bovine serum albumin as the standard. The assays were performed in the presence of 0.2% SDS. Proteins from density gradient fractions were trichloroacetic acid precipitated and solubilized in 0.2% SDS–0.5 M NaOH prior to determination.

Lipids were extracted from organelle membranes by the method of Folch et al. (16). Neutral lipids were separated by thin-layer chromatography by using silica gel 60 plates and a solvent containing light petroleum-diethylether-acetic acid (70:30:2, by volume). Individual phospholipids were separated by two-dimensional thin-layer chromatography by using chloroform–methanol–25% ammonia (65:35:5, by volume) for development in the first direction and chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, by volume) for the second direction. Total and individual phospholipids were quantitated as described by Broekhuyse (6). Ergosterol and ergosteryl ester concentrations were determined after separation by thin-layer chromatography, by direct densitometry at 275 nm by using a Shimadzu CS 930 thin-layer chromatography scanner with ergosterol as a standard. Accuracy of this method was within 5%. Individual sterols were analyzed after alkaline hydrolysis of the lipid extract by gas-liquid chromatography using an HP.1 capillary column (Hewlett-Packard). Injector and detector temperatures were both 310°C, oven temperature was 270°C, and the N_2 flow rate was 50 ml/min.

Fluidity of individual subcellular membranes was determined in vitro by measuring the fluorescence anisotropy with trimethylammonium diphenylhexatriene (TMA-DPH) as a probe. Analyses were carried out in a Shimadzu RF 540 spectrofluorometer at 30°C as described earlier (39).

RESULTS

Isolation and characterization of subcellular fractions of *S. cerevisiae*. By employing a linear density gradient centrifugation protocol, we isolated mitochondria which were largely devoid of microsomal contaminations. In contrast to a procedure published earlier (12) which used a pH 7.4 buffer system, we observed that at pH 6.0 the amount of microsomal membranes in mitochondrial preparations was significantly reduced. Nuclei, secretory vesicles, plasma membranes, vacuoles, peroxisomes, and lipid particles were isolated following new or improved procedures as described in Materials and Methods. Table 1 summarizes marker enzymes and polyclonal antibodies used for the characterization of subcellular fractions. In order to isolate sufficient amounts of individual membranes such as those of mitochondria, secretory vesicles, vacuoles, or peroxisomes, different growth conditions (carbon sources) or strains (e.g., *sec1* mutant) had to be employed (Table 1). The possibility that different growth conditions might influence membrane compositions and enzyme activities has to be considered. In our experience, however, lipid compositions of whole cells and isolated membrane fractions as well as specific activities of enzymes involved in lipid biosynthesis were rather similar in the strains and under the culture conditions employed in this study (data not shown). Figure 1 and Table 2 show the results of marker enzyme measurements and immunotitration experiments using polyclonal antibodies against organelle-specific proteins in individual subcellular fractions.

The microsomal fraction can be separated by differential or density gradient centrifugation into several subfractions, which are characterized by the various marker enzymes. In the heavy microsomal fraction (20,000 to 30,000 × *g* pellet), GDP-mannosyl transferase was enriched approximately 4.5-fold over the homogenate, whereas the 40-kDa protein and NADPH-cytochrome *c* reductase were enriched to a lesser extent (Table 2). The light microsomal fraction prepared by centrifugation of the 30,000 × *g* supernatant at 100,000 × *g* was characterized by the presence of the 40-kDa protein and NADPH-cytochrome *c* reductase, whereas GDP-mannosyl transferase was not enriched in this fraction. Interestingly, the activity of phosphatidylserine synthase did not parallel the distribution of any of the microsomal or other marker proteins tested (see below) (Fig. 1).

Fractions obtained by density gradient centrifugation of the 30,000 × *g* organelle pellet were characterized by their content of succinate dehydrogenase and their GDP-mannosyl transferase activities (Fig. 1). The distribution of mitochondrial membranes over the gradient was confirmed with immunoblots by using anti-ADP-ATP carrier antibody (inner mitochondrial membrane) and antiporin antibody (outer mitochondrial membrane) (data not shown). GDP-mannosyl transferase activity was detected over the whole gradient; the specific activity, however, was lowest in fractions containing mitochondria. The distribution of the 38-kDa nuclear protein and L16 ribosomal protein, as detected by use of

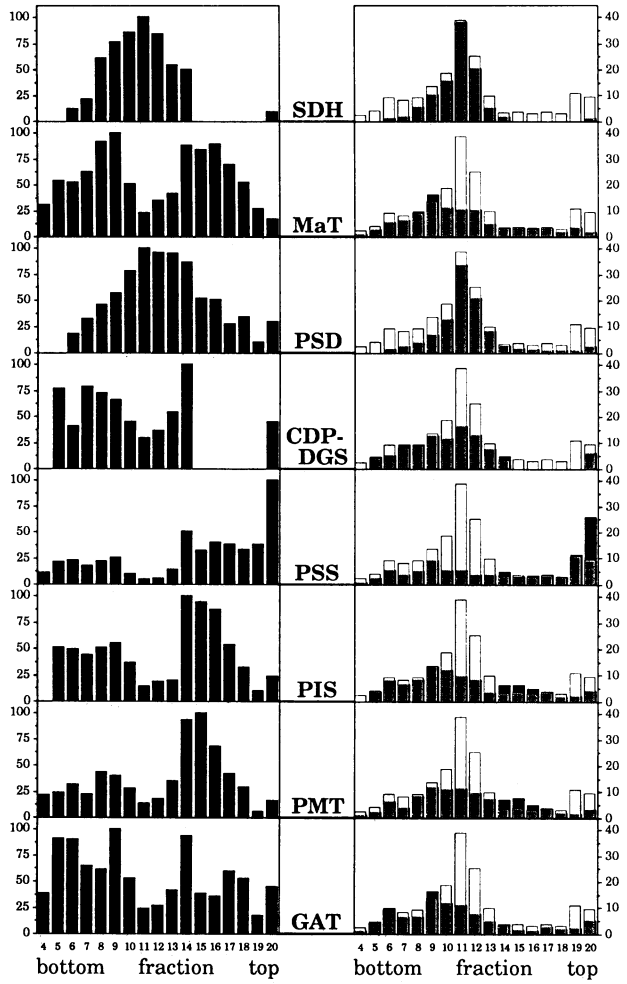

FIG. 1. Separation of mitochondria and heavy microsomes by sucrose density gradient centrifugation. A 30,000 × *g* organelle pellet of yeast cells was layered on top of a linear sucrose gradient (30 to 60%, wt/wt) in 5 mM MES–1 mM KCl, pH 6.0, and centrifuged as described in Materials and Methods. Fractions of 2 ml were collected and analyzed for marker enzymes and lipid-synthesizing enzymes. The left side shows relative specific activities (in percent) of enzymes over the gradient, with the fraction containing the highest specific activity set at 100%. The right panel shows the enzyme activity (in percent) in each fraction with the total amount of activity layered onto the gradient set at 100% (dark bars). White bars represent the relative protein concentration over the density gradient; the fraction with the highest protein concentration (fraction 11) contained approximately 1.90 mg of protein per ml. Conditions and references for enzyme assays are described in Materials and Methods and Table 1. Abbreviations: SDH, succinate dehydrogenase; MaT, GDP-mannosyl transferase; PSD, phosphatidylserine decarboxylase; CDP-DGS, CDP-diacylglycerol synthase; PSS, phosphatidylserine synthase; PIS, phosphatidylinositol synthase; PMT, phospholipid *N*-methyltransferases; GAT, glycerophosphate acyltransferase.

antisera, parallels the distribution of GDP-mannosyl transferase. This suggests that the 38-kDa nuclear protein is located in a part of the nuclear envelope which is connected to the endoplasmic reticulum. A 15-kDa protein, presumably a histone (23a), cross-reacting with antiserum against the 38-kDa protein, was only present in the higher-density bottom fractions of the gradient. Fractions containing mitochondrial membranes were very low in the L16 ribosomal protein and nuclear proteins (data not shown).

Plasma membrane preparations from wild-type strain X-2180 were enriched approximately 100-fold over the homogenate as estimated by the amount of immunoreactive plasma membrane ATPase (Table 2). Cross-contamination with heavy microsomes represented by GDP-mannosyl transferase activity was moderate. In secretory vesicles, prepared from the *S. cerevisiae* mutant *sec1* after a shift to the restrictive temperature for 2 h prior to membrane isolation, invertase was highly enriched. In addition, significant amounts of plasma membrane ATPase were present in these preparations. This observation is not surprising, because the plasma membrane ATPase is transferred to its final destination via secretory vesicles (5, 23). Significant contamination of secretory vesicles with heavy microsomes (GDP-mannosyl transferase activity) and vacuoles (α-D-mannosidase activity) was observed. The vacuolar preparation was highly enriched over the total homogenate and was only marginally contaminated with heavy microsomes. An additional purification step (see Materials and Methods), introduced to the original protocol for the isolation of vacuoles published by Uchida et al. (40), resulted in an improved resolution of vacuoles from lipid particles. However, the increased amount of steryl esters in vacuolar preparations, compared with the amount in other membranous fractions (see Table 4), and microscopic analysis suggest that considerable amounts of lipid particles were still adhering to vacuoles. Yeast nuclear preparations contain microsomal marker proteins to some extent, as one would expect on the basis of the observation that the nuclear membrane and endoplasmic reticulum form a continuous membrane system (for a review see reference 17). Peroxisomes prepared from induced cells by sucrose density gradient centrifugation were highly enriched in catalase and contained only minor mitochondrial (approximately 10%) and microsomal (less than 5%) contaminations.

Subcellular distribution of lipid-synthesizing enzymes. Most of the cellular capacity to synthesize phospholipids is contained in the 30,000 × *g* pellet. Mitochondria and heavy microsomal membranes were isolated from this fraction by density gradient centrifugation and analyzed with respect to activities of lipid-synthesizing enzymes. The results are shown in Fig. 1. Specific activities of glycerol-3-phosphate acyltransferase, phosphatidylserine synthase, phosphatidylinositol synthase, and the phospholipid *N*-methyltransferases were lowest in gradient fractions containing mainly mitochondrial membranes. A good correlation was found between the specific activities of phosphatidylinositol synthase and phospholipid *N*-methyltransferase and the specific activity of the microsomal marker GDP-mannosyl transferase, suggesting that these two phospholipid-synthesizing enzymes are located in typical microsomes. Total and specific activities of phosphatidylserine synthase and the amount of immunoreactive phosphatidylserine synthase (data not shown) were highest in a membrane fraction floating on top of the gradient at 30% (wt/wt) sucrose. Therefore, the existence of a specialized subfraction of microsomes containing phosphatidylserine synthase but little or none of the other enzymes must be considered. The presence of some phosphatidylserine synthase activity in other fractions, such as the nucleus, vacuoles, secretory vesicles, or mitochondria (Table 3) might be the consequence of a more or less tight attachment of this phosphatidylserine synthase-containing fraction to the respective membranes.

TABLE 2. Cross-contamination between subcellular fractions

Marker[a]	Relative enrichment (fold)[b]							
	Plasma membrane	Secretory vesicles	Vacuoles	Nucleus	Peroxisomes	Heavy microsomes	Light microsomes	Mitochondria
Plasma membrane ATPase	100	≈7	<0.1	—	—	—	—	—
NADPH-cytochrome *c* reductase	0.4	1.1	<0.01	—	—	2.8	5.8	0.4
GDP-mannosyl transferase	2.1	4.2	1.3	0.6	0.7	4.5	1.0	1.5
40-kDa protein	<0.1	0.5	<0.1	≈1	<0.1	≈1	≈4	<0.01
Succinate dehydrogenase	<0.1	0.5	<0.02	0.3	<0.1	2.0	0.02	4.0
α-D-Mannosidase	0.3	3.5	42.5	—	—	—	—	—
Invertase	—	16.7	—	—	—	—	—	—
Catalase	—	—	—	—	≈15	—	—	0.2
38-kDa nuclear protein	—	—	—	≈12	—	—	—	—

[a] For growth conditions and membrane preparation, see Materials and Methods and Table 1.
[b] The relative specific activities of marker enzymes or the relative amount of the marker proteins in the homogenate are set at 1. —, Not determined.

The distribution of activity of phosphatidylserine decarboxylase, an enzyme reported earlier to be located in the inner mitochondrial membrane (26), showed good correlation with that of succinate dehydrogenase (Fig. 1). In contrast to phosphatidylserine decarboxylase, the activity distributions of CDP-diacylglycerol synthase and glycerol-3-phosphate acyltransferase over the density gradient were rather nonhomogeneous. Fractions containing large amounts of mitochondria contribute significantly to the total CDP-diacylglycerol synthase and glycerol-3-phosphate acyltransferase activities (Fig. 1). The highest specific activity of glycerol-3-phosphate acyltransferase was observed in a specialized subcellular fraction referred to as lipid particles (Table 3) (8). This result suggests that the distribution of this enzyme over the whole density gradient might well be the result of an unspecific association of lipid particles with membranous structures.

Peroxisomes and the plasma membrane seem to be largely devoid of any of the lipid-synthesizing enzymes tested. Only a slight enrichment of phospholipid *N*-methyltransferase activity was detected in the peroxisomal fraction. Yeast peroxisomes were not enriched in acyl-CoA synthetase and dihydroxyacetone phosphate acyltransferase activities, which are characteristic for mammalian peroxisomes (21).

Lipid particles contain very high activities of glycerol-3-phosphate acyltransferase and sterol Δ^{24}-methyltransferase activities, which is in good agreement with data reported earlier (8, 9, 30). However, the very low protein concentration in this fraction did not allow an accurate estimation of specific enzyme activities. Phosphatidylserine synthase and phosphatidylinositol synthase activities were not detectable in lipid particles.

Yeast vacuoles show a considerable level of glycerol-3-phosphate acyltransferase activity, which is most likely due to cross-contamination with lipid particles. Microscopy observations and the slightly increased ergosteryl ester-to-ergosterol ratio in vacuoles (Table 4) support this notion.

Activities of lipid-synthesizing enzymes in secretory vesicles can be primarily attributed to cross-contamination with microsomes (compare with Table 2). Large amounts of secretory vesicles, which accumulate in *S. cerevisiae sec1* under nonpermissive conditions, are perhaps preferred target membranes for the attachment of microsomal subfrac-

TABLE 3. Distribution of phospholipid-synthesizing enzymes among subcellular fractions in *S. cerevisiae*[a]

Subcellular fraction	Relative specific activity[b]				
	GAT	PIS	PSS	PLMT	SMT
Plasma membrane	<0.1	0.2	<0.1	<0.1	0.3
Secretory vesicles	2.2	4.5[c]	4.3[c]	0.8	0.9
Vacuoles	4.2	1.0	1.1	<0.1	0.8
Nucleus	1.2	0.2	4.0	0.1	0.7
Peroxisomes	0.7	0.3	0.5	1.5	—
Microsomes[d]					
Gradient fraction 9	7.7	1.2	3.7	5.5	
Gradient fraction 15	2.7	2.2	5.8	9.4	2.2[e]
Gradient fraction 20	3.5	0.5	14.6	2.2	
Light microsomes	0.9	0.8	1.0	0.5	0.9
Mitochondria	2.0	0.4	0.7	1.0	1.0
Lipid particles	≈41	ND	ND	3.2	≈84

[a] The relative specific enzyme activities of the homogenate are set at 1. Specific activities of the enzymes tested vary to some extent, but not dramatically, in the initial lysates of the various strains used.
[b] GAT, Glycerol-3-phosphate acyltransferase; PIS, phosphatidylinositol synthase; PSS, phosphatidylserine synthase; PLMT, phosphatidylethanolamine *N*-methyltransferase and phospholipid *N*-methyltransferase; SMT, sterol Δ^{24}-methyltransferase; ND, not detectable; —, not determined.
[c] High relative specific activities might be an artifact caused by accumulation of secretory vesicles in *S. cerevisiae sec1* mutants at the restrictive temperature.
[d] See Fig. 1.
[e] Relative specific activity in heavy microsomal fraction (Tables 1 and 2).

TABLE 4. Phospholipid, ergosterol, and ergosteryl ester content in subcellular fractions of *S. cerevisiae*

Subcellular fraction	Ratio of[a]:			
	Phospholipid: protein (mg/mg)	Ergosterol: protein (mg/mg)	Ergosterol: phospholipid (mol/mol)	Ergosteryl ester: ergosterol (mol/mol)
Plasma membrane	0.23	0.40	3.31	<0.01
Secretory vesicles	0.71	0.30	0.75	<0.01
Vacuoles	0.51	0.05	0.18	0.29
Nucleus	0.08	0.03	0.64	0.32
Peroxisomes	0.38	0.08	0.39	<0.01
Light microsomes	0.06	0.008	0.26	0.04
Mitochondria	0.09	0.01	0.20	0.05
Inner mitochondrial membrane	0.15	0.03	0.32	<0.01
Outer mitochondrial membrane	0.91	<0.01	0.01	0.06
Lipid particles	—	—	—	13.30

[a] —, Uncertain estimations due to very low concentrations of proteins and phospholipids.

TABLE 5. Phospholipid composition of subcellular fractions of *S. cerevisiae*

Subcellular fraction	% of total phospholipid[a]						
	PtdCho	PtdEtn	PtdIns	PtdSer	CL	PA	Others
Plasma membrane	16.8	20.3	17.7	33.6	0.2	3.9	6.9
Secretory vesicles	35.0	22.3	19.1	12.9	0.7	1.2	8.8
Vacuoles	46.5	19.4	18.3	4.4	1.6	2.1	7.7
Nucleus	44.6	26.9	15.1	5.9	<1.0	2.2	4.3
Peroxisomes	48.2	22.9	15.8	4.5	7.0	1.6	ND
Light microsomes	51.3	33.4	7.5	6.6	0.4	0.2	0.5
Mitochondria	40.2	26.5	14.6	3.0	13.3	2.4	ND
Inner mitochondrial membrane	38.4	24.0	16.2	3.8	16.1	1.5	ND
Outer mitochondrial membrane	45.6	32.6	10.2	1.2	5.9	4.4	ND

[a] PtdCho, Phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid; others, other phospholipids; ND, not detectable.

TABLE 6. Fluorescence anisotropy of TMA-DPH in isolated membrane fractions of *S. cerevisiae*

Subcellular fraction	Fluorescence anisotropy[a]
Plasma membrane	0.191
Secretory vesicles	0.173
Vacuoles	0.139
Nucleus	0.167
Peroxisomes	0.172
Light microsomes[b]	0.152
Inner mitochondrial membrane[b]	0.157
Outer mitochondrial membrane[b]	0.134

[a] For the definition of fluorescence anisotropy and for technical details, see reference 39.
[b] Data from Sperka-Gottlieb et al. (39).

tions. However, significant differences in the specific activities of phosphatidylserine synthase, phosphatidylinositol synthase, and phospholipid *N*-methyltransferases were detected in secretory vesicle preparations and in the microsomal fraction. This observation might serve as a further indication of the heterogeneous character of the microsomal fraction.

Lipid composition of subcellular fractions. In order to characterize yeast subcellular fractions, their relative content of protein, phospholipids, ergosterol, and ergosteryl esters was analyzed (Table 4). Among the isolated organelles tested, peroxisomes, vacuoles, and secretory vesicles, in an increasing order, are characterized by a high phospholipid-to-protein ratio. In the plasma membrane and in secretory vesicles, a high ergosterol-to-protein ratio was observed which in contrast was very low in mitochondrial membranes, in light microsomes, and in the nuclear membrane. A marked increase in the ergosterol-to-phospholipid ratio was observed in membranes along the secretory pathway. The ratio is highest in the plasma membrane, followed by the secretory vesicles, microsomes, and nucleus. The outer mitochondrial membrane exhibits the highest phospholipid-to-protein ratio but the lowest ergosterol-to-phospholipid ratio. Lipid particles consist mainly of neutral lipids and contain very small amounts of phospholipids and proteins. They are characterized by a high triacylglycerol content (data not shown) and a high ergosteryl ester-to-ergosterol ratio of 13.3:1, which is in sharp contrast to membranous subcellular fractions. These results are in good agreement with data reported earlier by Clausen et al. (9). It is conceivable that lipid particles are the major location of ergosteryl esters within the yeast cell and that the presence of ergosteryl esters in membrane preparations of vacuoles and nuclei is the result of cross-contamination with lipid particles.

The pattern of individual phospholipids extracted from yeast subcellular membranes is summarized in Table 5. In all membrane fractions tested, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine are the predominant glycerophospholipid classes. Cardiolipin is present in large amounts (16% of total phospholipids) in the mitochondrial inner membrane but also in considerable amounts in the outer mitochondrial membrane (6% of total phospholipids) and in the peroxisomal membrane (7% of total phospholipids). According to specific activities of marker proteins, the relatively high cardiolipin content in peroxisomes cannot be attributed to cross-contamination with mitochondria. Similarly, the amount of cardiolipin detected in the outer mitochondrial membrane is higher than could be attributed to contamination with the inner mitochondrial membrane (39). A striking characteristic of the plasma membrane is the very high content of phosphatidylserine (30% of total phospholipid). The increase in phosphatidylserine in organelle membranes along the secretory pathway (microsomes, secretory vesicles, plasma membrane) parallels the relative content of ergosterol in these membranes (Table 4).

Fluidity of subcellular membranes. Lipid mobilities in individual membranes were analyzed by measuring the fluorescence anisotropy with TMA-DPH as a membrane probe. As summarized in Table 6, mitochondrial membranes, vacuoles, and microsomes are rather fluid (low anisotropy), compared with the more rigid plasma membrane, secretory vesicles, and nuclear and peroxisomal membranes.

DISCUSSION

Knowledge about the subcellular distribution of phospholipid-synthesizing enzymes is essential for an understanding of the assembly of phospholipids into functional biological membranes. Previous studies on the subcellular localization of phospholipid-synthesizing enzymes were based on the separation of individual membrane fractions by differential centrifugation techniques and the use of well-established marker enzymes (10, 26). From these studies it appeared that several of the enzymes involved in lipid synthesis were distributed differently in mammalian cells and in the yeast *S. cerevisiae*. These enzymes, namely phosphatidylserine synthase, phosphatidylinositol synthase, and glycerol-3-phosphate acyltransferase, were reported to be of microsomal origin in mammalian systems (for a review, see reference 4) but cofractionated with both the microsomal fraction and the outer mitochondrial membrane in *S. cerevisiae* (10, 26).

Several observations led us to reinvestigate the subcellular distribution of lipid-synthesizing enzymes in yeast. The differential localization of phosphatidylserine synthase to mitochondria and microsomes (10, 26) and the occurrence of this enzyme in crude preparations of peroxisomes (15a) could not be reconciled with the fact that phosphatidylserine synthase is encoded by a single-copy structural gene, *CHO1* (25), and is apparently not modified posttranslationally (37a, 38). A similar argument holds for phosphatidylinositol synthase, which was previously found to cofractionate with phosphatidylserine synthase (10, 26).

Following a new protocol for the isolation of yeast perox-

isomes (22) which is based on a pH 6.0 buffered gradient centrifugation system, we were able to show that highly purified peroxisomes are essentially free of any of the phospholipid synthetic activities analyzed. By using the same gradient system for the separation of yeast mitochondria and heavy microsomes, the distribution of marker proteins and phospholipid-synthesizing enzymes turned out to be different from results reported earlier (26). At pH 7.4, a substantial part of microsomal membranes cofractionated with mitochondria. At pH 6.0, most of this microsomal fraction was shifted to higher density in the gradient and could thus be separated from mitochondria. After density gradient centrifugation, GDP-mannosyl transferase was mainly associated with the heavy microsomal fraction and can therefore serve as a reliable marker for this compartment.

A major issue of this work was to determine the subcellular localization of phosphatidylserine synthase. Combination of differential and density gradient centrifugation revealed that the specific activity of phosphatidylserine synthase was highest in a microsomal subfraction, which could be collected from the top of a sucrose density gradient after centrifugation of the 30,000 × *g* organelle pellet (Fig. 1). Because none of the microsomal marker proteins cofractionated with phosphatidylserine synthase, it can be assumed that this enzyme is located in a distinct membranous fraction or particle population whose identity is unknown. It is not a phospholipid-synthesizing fraction (41), since other enzymes of phospholipid biosynthesis, such as phosphatidylinositol synthase or phospholipid *N*-methyltransferases, are not contained in this subfraction but rather cofractionate with the true endoplasmic reticulum marker, GDP-mannosyltransferase. In addition, substantial activity of phosphatidylinositol synthase was detected in secretory vesicles. This enrichment could not be completely ascribed to cross-contamination with microsomes. In contrast to previous results, neither phosphatidylserine synthase nor phosphatidylinositol synthase activity was enriched with mitochondria. The minimal phosphatidylinositol synthase activity associated with mitochondrial membranes could be accounted for by microsomal contamination when GDP-mannosyltransferase was used as a microsomal marker. No such corrections could be made for the activity of phosphatidylserine synthase, because GDP-mannosyltransferase (or other microsomal markers) did not cofractionate with this enzyme. It is therefore an open question whether association of phosphatidylserine synthase activity with mitochondria is an artifact of the fractionation procedure in a pH 7.4 buffered system or whether it reflects the physiological situation. The fact that phosphatidylserine synthase was absent from several other membranes, such as the plasma membrane, vacuoles, or peroxisomes, would speak against a nonspecific membrane association.

The only enzymes which appear to be exclusively microsomal, i.e., which cofractionate with GDP-mannosyltransferase, are the phospholipid *N*-methyltransferases. For CDP-diacylglycerol synthase, the situation is different. From the density gradient pattern (Fig. 1) one can conclude that this enzyme, in accordance with previous results (26), is located both in microsomes and in mitochondria. In this case, the existence of isoenzymes cannot be excluded. Occurrence of CDP-diacylglycerol synthase in mitochondria and in microsomes would be reasonable because the product, CDP-diacylglycerol, is required in mitochondria for the synthesis of cardiolipin and in microsomes and phosphatidylserine synthase particles for the synthesis of phosphatidylinositol and phosphatidylserine. Association of CDP-diacylglycerol synthase with membranes that utilize its product would avoid translocation of large quantities of this phospholipid through the cytosol. CDP-diacylglycerol in high concentrations might have deleterious effects on proteins because of its negative charge and strong detergent effects.

Highest specific activity of glycerol-3-phosphate acyltransferase was found in the lipid particle fraction (Table 3) (see reference 9), which contains approximately 30% of the cellular activity of this enzyme. Because these particles store triacylglycerols in addition to steryl esters, it is plausible to ascribe glycerol-3-phosphate acyltransferase a role in triacylglycerol synthesis (8) in this specific compartment. However, phosphatidic acid is also indispensable as a general precursor to phospholipids, be it by de novo synthesis via CDP-diacylglycerol or by the salvage pathway via diacylglycerol. To serve this purpose, close vicinity of glycerol-3-phosphate acyltransferase to membranous compartments involved in phospholipid synthesis would be of advantage. Indeed, glycerol-3-phosphate acyltransferase activity was found in all fractions except peroxisomes and light microsomes, which could be due to the association of lipid particles with various organelles. Sterol Δ^{24}-methyltransferase was also found to be associated with lipid particles (Table 3) (see reference 30); the presence of this enzyme in the microsomal fraction confirms previous results by others (30) and might support the view of lipid particle association with these membranes.

Phosphatidylglycerolphosphate synthase (26) and phosphatidylserine decarboxylase could be unequivocally allocated to mitochondria (Fig. 1). The former enzyme is involved in the biosynthesis of cardiolipin, which has the highest cellular concentration in mitochondria (Table 5). Vacuoles and the plasma membrane are apparently devoid of the phospholipid-synthesizing enzymes analyzed in this study. In animal cells, peroxisomes significantly contribute to glycerophospholipid synthesis as they contain the enzymes catalyzing two initial steps of ether lipid biosynthesis (20). In yeast, however, neither dihydroxyacetone phosphate acyltransferase nor any other phospholipid-synthesizing activity could be detected in peroxisomes. The absence of ether lipid-synthesizing enzymes was not surprising, as *S. cerevisiae* does not contain ether lipids.

The lipid composition of biological membranes is thought to affect the functioning of membrane-associated processes. Differences in the lipid pattern of various subcellular organelles of yeast cells might provide a clue as to the role of lipids in these membranous compartments. The most characteristic features not previously reported are the following. The phosphatidylserine concentration is exceptionally high in the plasma membrane, where it comprises a third of total glycerophospholipids. Also, secretory vesicles are enriched in phosphatidylserine, followed by light microsomes. This increase in phosphatidylserine content along the secretory pathway is suggestive of a role of this phospholipid in protein secretion. The remarkable enrichment of the plasma membrane in ergosterol parallels the preferential location of cholesterol in the plasma membrane of animal cells (28). Secretory vesicles are second highest in ergosterol concentration, suggesting that these vesicles contribute to the flow of ergosterol from the endoplasmic reticulum to the plasma membrane. This assumption does not imply, however, that protein secretion and lipid transport must use the same vesicles. Available experimental evidence (13) would rather exclude such a cooperative process, at least with respect to

the transport of phosphatidylinositol and phosphatidylcholine. The phospholipid pattern of peroxisomes is characterized by a relatively high cardiolipin content. It can be speculated that cardiolipin plays a similar role in the import of proteins into peroxisomes as it supposedly does in mitochondria (15).

Fluorescence anisotropy, determined with the amphiphilic probe TMA-DPH, is a measure for lipid mobility or flexibility within a membrane. Membranes with the highest anisotropy are the plasma membrane, secretory vesicles, and peroxisomes; for the first two, the high ergosterol content would explain high membrane rigidity. TMA-DPH in peroxisomes gave anisotropy values identical to those found in secretory vesicles, despite the significantly (50%) lower ergosterol-to-phospholipid ratio in peroxisomes. Thus, while ergosterol certainly increases membrane rigidity, the role of proteins as a determinant of membrane rigidity is not as clear and most likely depends on the kind of proteins present in the respective membrane.

Knowledge of the subcellular location of phospholipid-synthesizing enzymes is prerequisite to defining routes of intracellular lipid traffic, to elaborating the underlying mechanisms, and finally to understanding how lipids are assembled into membranes. Maintenance of the correct phospholipid composition during membrane biogenesis or regeneration requires efficient regulatory mechanisms. There is increasing evidence that phospholipid synthesis in yeast is controlled in part at the level of transcription of genes encoding phospholipid-synthesizing enzymes (7). On the other hand, transformants overexpressing genes encoding phospholipid-synthesizing enzymes (e.g., phosphatidylserine synthase) still have normal membrane phospholipid patterns, despite increased enzyme activities in cell-free preparations (38). Phospholipid synthesis must therefore also be regulated at the stage of enzyme activities in living cells, and it is plausible to assume a coordinated interplay between phospholipid synthesis and transport. It will be a task for the future to identify the factors which control these events and thus which play a major role in the regulation of membrane assembly.

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