# Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS) Analysis of the Lipid Molecular Species Composition of Yeast Subcellular Membranes Reveals Acyl Chain-based Sorting/Remodeling of Distinct Molecular Species En Route to the Plasma Membrane

Roger Schneiter,* Britta Brügger,‡ Roger Sandhoff,‡ Günther Zellnig,§ Andrea Leber,* Manfred Lampl,* Karin Athenstaedt,* Claudia Hrastnik,* Sandra Eder,* Günther Daum,* Fritz Paltauf,* Felix T. Wieland,‡ and Sepp D. Kohlwein*

*Spezialforschungsbereich Biomembrane Research Center, Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, A-8010 Graz, Austria; ‡Biochemie-Zentrum Heidelberg, Universität Heidelberg, D-69120 Heidelberg, Germany; and §Institut für Pflanzenphysiologie, Karl-Franzens Universität, A-8010 Graz, Austria

*Abstract.* Nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) was employed to determine qualitative differences in the lipid molecular species composition of a comprehensive set of organellar membranes, isolated from a single culture of *Saccharomyces cerevisiae* cells. Remarkable differences in the acyl chain composition of biosynthetically related phospholipid classes were observed. Acyl chain saturation was lowest in phosphatidylcholine (15.4%) and phosphatidylethanolamine (PE; 16.2%), followed by phosphatidylserine (PS; 29.4%), and highest in phosphatidylinositol (53.1%). The lipid molecular species profiles of the various membranes were generally similar, with a deviation from a calculated average profile of $\sim\pm$ 20%. Nevertheless, clear distinctions between the molecular species profiles of different membranes were observed, suggesting that lipid sorting mechanisms are operating at the level of individual molecular species to maintain the specific lipid composition of a given membrane. Most notably, the plasma membrane is enriched in saturated species of PS and PE. The nature of the sorting mechanism that determines the lipid composition of the plasma membrane was investigated further. The accumulation of monounsaturated species of PS at the expense of diunsaturated species in the plasma membrane of wild-type cells was reversed in *elo3Δ* mutant cells, which synthesize C24 fatty acid-substituted sphingolipids instead of the normal C26 fatty acid-substituted species. This observation suggests that acyl chain-based sorting and/or remodeling mechanisms are operating to maintain the specific lipid molecular species composition of the yeast plasma membrane.

Key words: *Saccharomyces cerevisiae* • subcellular fractions • lipids • cell membrane • mass fragmentography

THE eukaryotic cell is compartmentalized into organelles of distinct chemical composition and biological function. These subcellular organelles are not normally created de novo, but are inherited to the daughter cell after division in the mother (Nunnari and Walter, 1996; Warren and Wickner, 1996). Organelles are thus potential carriers of inheritable information that is not encoded by the genetic information of the genome.

The nature of such organelle specific information is poorly understood, but the fact that each of the organellar membranes has a distinct and often characteristic lipid composition raises the possibility that membranes contain structural information, encoded in their lipid composition. How this lipid composition of a given membrane is maintained, however, is only poorly understood. Possible mechanisms include lipid sorting during vesicular (and/or nonvesicular) trafficking and/or remodeling of lipids within their target membrane.

Lipid migration and/or remodeling must be regulated, as is indicated by the nonrandom lipid composition of organellar membranes. Some lipids even appear characteristic for specific membranes, such as cardiolipin for the inner mitochondrial membrane (Krebs et al., 1979), lyso-

bisphosphatidic acid (LBPA) for the inner membranes of late endosomes (Kobayashi et al., 1998), semi-LBPA for the tubular regions of the trans-Golgi network (Cluett et al., 1997), and sterols and sphingolipids for the plasma membrane (Lange et al., 1989). It is not known if this heterogeneous distribution of phospholipid classes is also reflected in a nonrandom distribution of individual lipid molecular species. A particular lipid molecular species is defined by its head group and its precise acyl chain substituents. Biological membranes are thus composed of dozens of chemically distinct lipid molecular species. However, the question of whether the major molecular species of, for example, phosphatidylserine (PS)[1] synthesized in the ER membrane has the same acyl chain composition as the PS that resides in the cytosolic leaflet of the plasma membrane, remains to be answered. Resolving this question is of paramount importance, as it would indicate whether the mechanisms that are operating to adjust the lipid composition of organellar membranes are discriminating between different lipid classes, i.e., are selective for the head group substituent only, or that they recognize individual lipid molecular species, i.e., are selective for both the precise acyl chain and head group substituents of a lipid.

The lack of answer to this important question is due in part to technical difficulties encountered in determining the phospholipid molecular species composition of subcellular membranes by traditional techniques such as column, thin-layer, and high-performance liquid-chromatography, or more recently, by fast-atom bombardment mass spectrometry. These approaches are time consuming and lack the sensitivity required for the analysis of highly purified subcellular membranes. We took advantage of a newly developed analytical method to characterize the lipid composition of yeast organellar membranes at the resolution of individual lipid molecular species by nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS; Brügger et al., 1997). Electrospray ionization (ESI) coupled with collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) potentially represents the most sensitive, discriminating, and direct method for the qualitative and quantitative analysis of subpicomole amounts of lipids from biological samples (Han and Gross, 1994; Kerwin et al., 1994; Kim et al., 1994).

The major glycerophospholipid classes in yeast are the same as those of higher eukaryotic cells and the biochemical pathways for their synthesis have been well established (for reviews see Paltauf et al., 1992; Moreau and Cassagne, 1994; Kent, 1995; Daum et al., 1998; van Meer, 1998). In yeast, phosphatidylcholine (PC, ~45%), phosphatidylethanolamine (PE, ~20%), and phosphatidylinositol (PI, ~15%) constitute the major three glycerophospholipid classes. PS (~5%), phosphatidic acid (PA, ~3%) and cardiolipin (~2%, but enriched to 15% in the inner mitochondrial membrane) are the three minor species (e.g., Daum et al., 1999). Whereas the PE and PI content be-

tween the different subcellular membranes does not vary greatly, PS is enriched along the secretory pathway and constitutes ~13% of the glycerophospholipids in late secretory vesicles and ~34% of the glycerophospholipids of the plasma membrane. This enrichment of PS appears to be compensated for by a concomitant reduction of PC in the respective membranes (Zinser et al., 1991; van Meer, 1998).

The major saturated fatty acids in glycerophospholipids of higher eukaryotic cells are palmitic (C16:0) and stearic acid (C18:0). Oleic (C18:1), linoleic (C18:2), and arachidonic acid (C20:4) are the major unsaturated species (Keenan and Morré, 1970). Since *Saccharomyces cerevisiae* does not synthesize polyunsaturated fatty acids, the acyl chain composition of yeast lipids is rather simple. Unsaturated palmitoleic (C16:1, ~50%) and oleic (~25%) fatty acids together account for 70–80% of all fatty acids, with the remaining being mainly palmitic (~15%) and stearic acid (C18:0, ~5%). As in higher eukaryotes, saturated fatty acyl chains also predominate the *sn-1* position of glycerophospholipids in yeast (Wagner and Paltauf, 1994). Similarly, phospholipase $A_2$-dependent pathways for the generation of individual lipid molecular species appear to be conserved (Lands and Crawford, 1976; Wagner and Paltauf, 1994).

In view of this rather simple fatty acid composition, yeast appears ideally suited to study the role of individual lipid molecular species in membrane function, how such lipid species are generated and remodeled, and how the molecular species composition of a given membrane is established and maintained. As a first step towards such an understanding of membrane function at the level of individual molecular species, a qualitative nano-ESI-MS/MS analysis of the molecular species composition of 11 different yeast subcellular membranes was performed.

## Materials and Methods

### Isolation of Subcellular Membranes

The wild-type *S. cerevisiae* strain used was X2180-1A (MATα *SUC2 mal gal2 CUP1*). 10 liter fermentation cultures of wild-type cells were grown at 24°C in liquid YPD medium (1% Bacto yeast extract, 2% Bacto peptone [Difco Laboratories Inc.], 2% glucose). Peroxisomes were isolated from the wild-type strain D273-10B (American Type Culture Collection 25657) cultivated under inducing conditions (0.3% Bacto yeast extract, 0.5% Bacto peptone, 0.5% $KH_2PO_4$, pH 6.0, 0.1% oleic acid [Merck], 0.2% Tween 80 [Merck]). The *elo3Δ* mutant strain EMA40 (MATa *ura3 trp1 leu2 elo3::URA3*), together with the corresponding wild-type strain EMY30 (MATα *ura3 trp1 leu2*), were obtained from G. Loison (Sauof: Recherche Centre de Labège, France; Silve et al., 1996) and cultivated in liquid YPD medium at 30°C before isolation of the plasma membrane.

For the isolation of mitochondria, microsomes, nuclei, peroxisomes, vacuoles, and lipid particles, late exponential phase cultures were harvested by centrifugation and converted to spheroplasts essentially as described by Daum et al. (1982). Mitochondrial subfractions enriched in markers of the outer membrane, the inner membrane, and the contact sites were isolated after swelling and shrinking of intact mitochondria as described in Pon et al. (1989). Microsomal membranes were obtained from the postmitochondrial supernatant by differential centrifugation at 40,000 and 100,000 $g$; the 100,000 $g$ supernatant being the soluble cytosolic fraction. Nuclei were enriched by sucrose density gradient centrifugation of the postmitochondrial supernatant as described (Hurt et al., 1988; Aris and Blobel, 1991). Vacuoles were isolated as described by Uchida et al. (1988), with a modification that yields the lipid particle fraction (Leber et al., 1994). Peroxisomes were isolated as previously described (Zinser et al., 1991).

---

Plasma membranes were isolated following the procedure described by Serrano (1988) which relies on the disruption of intact cells by glass beads.

Golgi membranes were isolated from early exponential phase wild-type cells as described by Lupashin et al. (1996). Deuterium oxide, sucrose, and ATP were purchased from Sigma Chemical Co.

### Protein Analysis

Protein was quantified by the method of Lowry et al. (1951) using BSA as standard. Before quantification, proteins were precipitated with 10% TCA and solubilized in 0.1% SDS, 0.1 M NaOH. Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to Hybond-C nitrocellulose filters (Nycomed Amersham Inc.). Relative enrichment and degree of contaminations of subcellular fractions were determined by immunoblotting. Antigens were detected by antibodies against the respective protein followed by peroxidase-conjugated secondary antibodies and enhanced chemiluminescent signal detection using SuperSignal™ (Pierce Chemical Co.). Signal intensity was quantified by densitometric analysis using the wand tool present in NIH Image 1.61 (http://rsb.info.nih.gov/nih-image/download.html). Rabbit polyclonal antisera against the following proteins were employed: Kar2p/BiP (1:5,000; M. Rose, Princeton University, Princeton, NJ); Kre2p, (1:1,000, affinity-purified serum; H. Bussey, McGill University, Montreal, Canada; Lussier et al., 1995); Gas1p (1:5,000; H. Riezman, Biocenter Basel, Basel, Switzerland; Conzelmann et al., 1988); carboxypeptidase Y (CPY; 1:500; R. Schekman, University of California, Berkeley, CA); Erg6p (1:100,000; Leber et al., 1994); porin and MDH (both 1:1,000; G. Schatz, Biocenter Basel, Basel, Switzerland; Aac1p (1:1,000; W. Neupert, University of Munich, Munich, Germany); Pox1p (1:1,000; W.-H. Kunau, University of Bochum, Bochum, Germany). The mouse mAb against the soluble cytosolic 3-phosphoglycerate kinase PGK (mAb 22C5-D8) was purchased from Molecular Probes and used at 2 μg/ml.

### Lipid Analysis and Mass Spectrometry

Lipid extracts of subcellular membrane fractions (Bligh and Dyer, 1959) were dried under a stream of nitrogen and redissolved in a small volume (20–200 μl) of methanol/chloroform (2:1) containing either 10 mM ammoniumacetate (added from a 100 mM stock solution in methanol) for positive ion analyses or no additive for negative ion measurements. Total phospholipid content of samples was measured by the method of Rouser et al. (1970). For the quantification of free ergosterol, lipid extracts of subcellular membrane fractions were dried in a vacuum concentrator and sulfated before mass spectrometry as described (Sandhoff et al., 1999).

Mass spectrometric analyses were performed with a triple quadrupole instrument model QUATTRO II (Micromass) equipped with a nano-electrospray source operating at a typical flow rate of 20–50 nl/min. The instrument was used either in the single-stage MS mode for the detection of total negative and positive ions or in tandem MS mode for product ion, precursor ion, or neutral loss scan analysis, as previously described (Brügger et al., 1997). When used in single stage MS mode, relative peak intensity of different phospholipid classes may depend on the lipid concentration of the sample analyzed. PA, for example, was found to strongly quench the ionization of other lipids in samples with a high lipid concentration. The relative intensity of each $(M - H)^-$ and $(M + H)^+$ ion was determined from phospholipid class specific scan analyses as described (Brügger et al., 1997). To identify the fatty acid composition of each molecular species, product ion scan analyses of the different molecular species and parent scan analyses for all major fatty acids were performed.

Before nano-ESI-MS/MS analysis, the lipid extracts were centrifuged in a benchtop centrifuge for 5 min, and then a 3–10 μl aliquot was transferred into the electrospray capillary (type D; Micromass). The spray was started by applying 400–700 V to the capillary. For each spectrum, 20–100 repetitive scans of 10-s duration were averaged. All tandem MS experiments were performed with argon as collision gas at a nominal pressure of 2 mTorr.

Collision energy settings employed were as follows: +par 184, 30 eV; +nl 141 and +nl 185, 28 eV; −par 241, 45 eV; −nl 87, 28 eV; −par 195, 50 eV; product ion scans, 30–50 eV; fatty acid specific scans, 40–50 eV.

### Electron Microscopy

For ultrastructural analysis of subcellular membranes by EM, freshly prepared membrane fractions were fixed in 1% glutaraldehyde in 0.06 M phosphate buffer, pH 7.2, containing 1 mM CaCl$_2$ and 0.6 M mannitol for 45 min at room temperature. Membranes were then washed three times

for 20 min each in phosphate buffer, postfixed in 1% OsO$_4$ for 45 min at room temperature, washed twice for 10 min each in phosphate buffer, dehydrated in a graded series of ethanol (50–100%, with en bloc staining in 1% uranylacetate in 70% ethanol overnight), and embedded in Spurr's resin. 70 nm ultra-thin sections were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

## Results

### Isolation of Organellar Membranes from S. cerevisiae

The aim of the present work was to determine qualitative differences between the lipid molecular species composition of distinct organellar membranes. To minimize alterations in the lipid composition due to culture variations, as many membranes as possible were isolated from a single batch of cells. The fractionation strategy designed to simultaneously isolate nine different organellar membranes is outlined in Fig. 1. A 10 liter fermentation culture of wild-type cells was grown to late exponential phase, harvested, and one fifth of the total cell mass was used for the isolation of the plasma membrane fraction, which required disintegration of intact cells by glass beads (Serrano, 1988). The remainder of the culture was converted to spheroplasts, split into three aliquots, each of which was then specifically processed for the isolation of particular fractions, i.e., nuclei (Hurt et al., 1988; Aris and Blobel, 1991), vacuoles and lipid particles (Uchida et al., 1988; Leber et al., 1994), and mitochondria, which were further subfractionated into inner and outer mitochondrial membranes and contact sites (intermediate density fraction; Pon et al., 1989). The postmitochondrial supernatant was subfractionated into heavy (40,000 $g$) and light (100,000 $g$) microsomes, and the soluble cytosolic fraction (Zinser and Daum, 1995). Since Golgi membranes could not be isolated in sufficient yield and quality from a late exponential phase culture, an early exponential phase culture of the same wild-type strain was processed in parallel for the isolation of this organelle (Lupashin et al., 1996). The isolation of peroxisomes from yeast requires prior induction of the organelle by oleic acid. Peroxisomes were thus isolated from an independent culture cultivated under inducing conditions (Veenhuis et al., 1987).
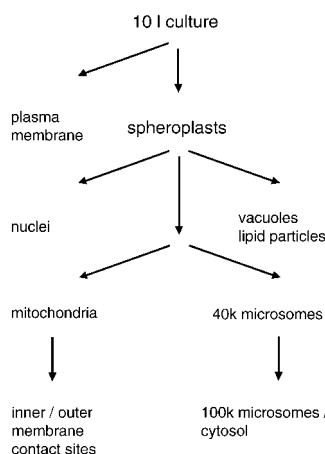


*Figure 1.* Outline of the fractionation scheme designed to simultaneously isolate nine subcellular membrane fractions from one single 10 l culture of *S. cerevisiae* wild-type cells.
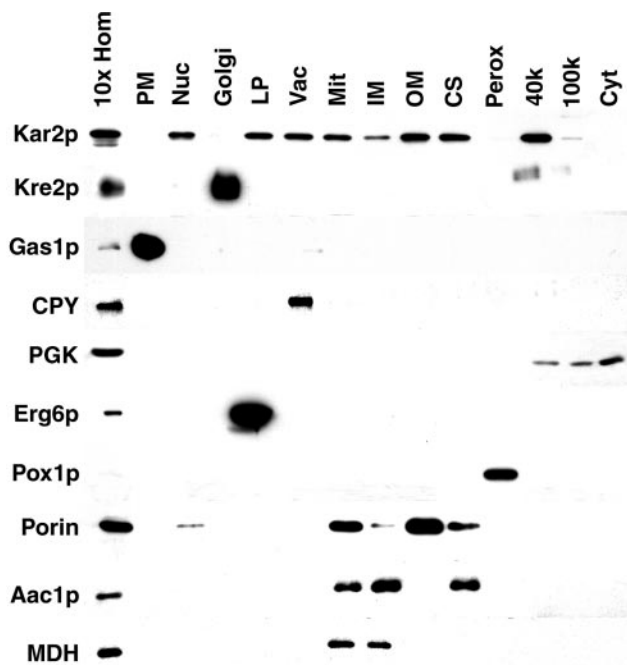
Kar2p
Kre2p
Gas1p
CPY
PGK
Erg6p
Pox1p
Porin
Aac1p
MDH

10x Hom  PM  Nuc  Golgi  LP  Vac  Mit  IM  OM  CS  Perox  40k  100k  Cyt

*Figure 2.* Immunoblot analysis of subcellular fractions. 10 μg protein of the cell homogenate (10× Hom) and 1 μg of each of the subcellular fractions were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with antibodies against the marker proteins indicated. Hom, cell homogenate; PM, plasma membrane; Nuc, nuclei; LP, lipid particles; Vac, vacuoles; Mit, mitochondria; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane; CS, contact sites; Perox, peroxisomes; 40k, 40,000 *g* microsomes; 100k, 100,000 *g* microsomes; Cyt, cytosol.

### Biochemical Characterization of Subcellular Fractions

Biochemical and morphological criteria were subsequently employed to assess the quality of the isolated membranes. Relative enrichment and degree of cross-contamination of the fractions was judged by immunoblot analysis with antibodies against the following marker proteins: Kar2p/BiP, a HSP70 family member and soluble lumenal ER protein, served as marker for the ER (Norming-ton et al., 1989); α1,2-mannosyltransferase (Kre2p; Lussier et al., 1995), was used as marker for the Golgi membrane; Gas1p, a glycosylphosphatidylinositol (GPI)-anchored membrane protein (Conzelmann et al., 1988), was the marker for the plasma membrane; CPY served as a lumenal marker for the vacuole; 3-phosphoglycerate kinase (PGK) was used as marker for soluble cytosolic protein; Δ24-sterol C-methyltransferase (Erg6p) served as marker protein for the lipid particle fraction (Leber et al., 1994); Acyl-CoA oxidase (Pox1p) was a marker for peroxisomes (Thieringer et al., 1991); porin was used as marker for the outer mitochondrial membrane (Daum et al., 1982); ADP/ATP carrier protein (Aac1p) marked the inner mitochondrial membrane; and malate dehydrogenase (MDH) was used as marker of the mitochondrial matrix. Immunoblot analyses of all the subcellular fractions probed with these antibodies are shown in Fig. 2 and a quantification of the results is given in Table I.

As shown in Fig. 2 and Table I, the plasma membrane was the subcellular fraction with the highest enrichment for its marker protein. The GPI-anchored Gas1p was enriched 185.5-fold. The isolated nuclei were 3.7-fold enriched for Kar2p. Enrichment factors of 10–15 for ER proteins in isolated yeast nuclear fractions have been reported for cells harvested at an early logarithmic phase (Hurt et al., 1988; Aris and Blobel, 1991). The lower enrichment seen in our preparations likely is due to the late growth phase at which these cells were harvested. Kar2p was detected in various fractions, which is in line with the contamination of most organelles by the poorly defined microsomes.

The Golgi membrane was 23.5-fold enriched for the medial-Golgi apparatus marker Kre2p, which was also detected in the heavy microsome fraction (2.4-fold). Based on enzyme activity, this particular Golgi membrane fraction has been reported to be 205-fold enriched for the cis-Golgi membrane localized GDPase (Lupashin et al., 1996). Since early and late Golgi compartments differ in density (Cunningham and Wickner, 1989), the Golgi fraction analyzed in this study is more representative of an early Golgi compartment when compared with the Kex2p enriched late Golgi compartment whose lipid composition has previously been analyzed (McGee et al., 1994; Leber et al., 1995).

*Table I. Relative Enrichment of Marker Proteins in Subcellular Fractions*

| Organelle | Marker protein | Enrichment | | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | PM | Nuc | Golgi | LP | Vac | Mit | IM | OM | CS | Perox | 40k | 100k | Cyt |
| | | | | | | | *-fold* | | | | | | | |
| ER | Kar2p | | 3.7 | | 3.8 | 2.9 | 2.4 | 1.0 | 5.3 | 5.5 | | 7.0 | | |
| Golgi | Kre2p | | | 23.5 | | | | | | | | 4.1 | 0.3 | |
| PM | Gas1p | 185.5 | | | | | | | | | | | | |
| Vac | CPY | | | | | 15.2 | | | | | 1.1 | | 0.7 | |
| Cyt | PGK | | | | | | | | | | | 2.2 | 2.4 | 5.2 |
| LP | Erg6p | | | | 156.0 | | | | | | | | | |
| Perox | Pox1p | | | | | | | | | | 30.0 | | | |
| OM | Porin | | 1.8 | | | 0.9 | 6.2 | 0.9 | 10.3 | 5.0 | | | | |
| IM | Aac1p | | | | | | 12.6 | 20.7 | | 17.5 | | | | |
| Matrix | MDH | | | | | | 4.9 | 3.0 | | | | | | |

Enrichment of the respective antigen in the homogenate is set to 1. No signal was detected for fields containing no value. PM, plasma membrane; Vac, vacuoles; Cyt, cytosol; LP, lipid particles; Perox, peroxisomes; OM, outer mitochondrial membrane; IM, inner mitochondrial membrane.
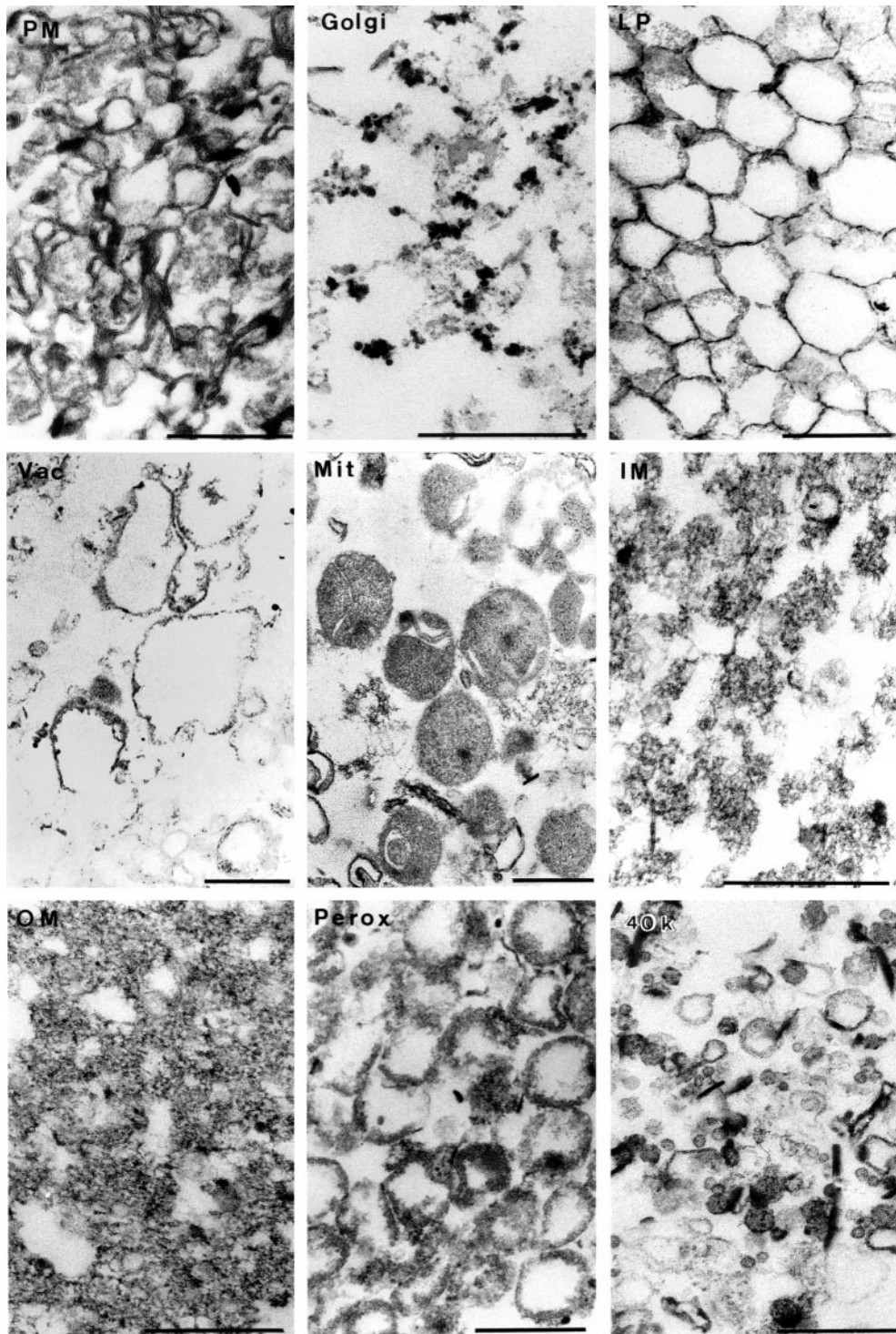
*Figure 3.* Morphological analysis of subcellular membrane fractions. Subcellular fractions were isolated as outlined in Fig. 1, fixed, and processed for EM as described in Materials and Methods. PM, plasma membrane; LP, lipid particles; Vac, vacuoles; Mit, mitochondria; IM, inner mitochondrial membrane; OM, outer mitochondrial membrane; Perox, peroxisomes; 40k, 40,000 *g* microsomes. Bars, 500 nm.

The lipid particle fraction was highly enriched for its marker protein Erg6p, but also contained Kar2p, consistent with the proposed relationship of this compartment with the ER membrane (Leber et al., 1998).

The vacuolar fraction was 15.2-fold enriched in the lumenal vacuolar CPY. Mitochondria were enriched for porin, a major protein of the outer mitochondrial membrane (Daum et al., 1982), and for the ADP/ATP carrier protein, Aac1p, of the inner mitochondrial membrane. Both markers were further enriched in their corresponding subfractions. The marker protein for the mitochondrial matrix, MDH, was enriched in the mitochondrial fraction, but was also present in the inner mitochondrial membrane fraction, as has been observed previously (Zinser and Daum, 1995).

The dense microsomal fraction (40,000 *g*) was 7.0-fold enriched for Kar2p. None of the marker proteins were selectively enriched in the light microsomal fraction (100,000 *g*). The soluble cytosolic fraction was 5.2-fold enriched for phosphoglycerate kinase and did not show any enrichment for other marker proteins tested.

*Table II. Thickness of Subcellular Membranes*

| Fraction | Width | (n) |
|---|---|---|
| | *nm* | |
| 40k microsomes | 7.5 ± 0.8 | (5) |
| Plasma membrane | 9.2 ± 0.4 | (8) |
| Golgi membrane | 7.3 ± 0.4 | (7) |
| Lipid particles | 4.5 ± 0.4 | (7) |
| Vacuole | 6.8 ± 0.6 | (6) |
| Outer mitochondrial membrane | 6.7 ± 0.2 | (8) |
| Inner mitochondrial membrane | 7.4 ± 0.5 | (8) |

*Table III. Phospholipid and Sterol Composition of Subcellular Membranes*

| Fraction | Protein/Phospholipid | Ergosterol/Phospholipid |
|---|---|---|
| | *μg/μg* | *mol/mol* |
| 40k microsomes | 3.3 ± 0.6 | 0.18 ± 0.04 |
| Plasma membrane | 2.2 ± 0.4 | 0.46 ± 0.03 |
| Vacuole | 0.9 ± 0.1 | 0.07 ± 0.001 |
| Lipid particles | 2.1 ± 0.1 | 0.24 ± 0.04 |
| Inner mitochondrial membrane | 5.5 ± 0.6 | 0.12 ± 0.01 |
| Outer mitochondrial membrane | 1.5 ± 0.1 | 0.02 ± 0.01 |
| Contact sites | 2.4 ± 0.3 | 0.05 ± 0.01 |
| Peroxisomes | 5.7 ± 0.1 | 0.03 ± 0.01 |

Data represent mean values and SD of two determinations.

Peroxisomes were enriched ~30-fold for Pox1p relative to the corresponding homogenate (not shown), with no major cross-contaminations detectable.

### Morphological Characterization of Membrane Fractions

To further assess the purity and homogeneity of the isolated fractions, membranes were fixed and examined by ultra-thin section EM. The results of this analysis are shown in Fig. 3. A distinct membrane/vesicle morphology for each of the isolated fractions was readily visible and the fractions appeared homogenous for the type of membrane isolated. The 40,000 *g* microsomal fraction contained the most morphologically inhomogeneous materials, such as big and small vesicles next to stacked sheets of membranes.

Lipid bilayer thickness has been discussed as a possible sorting determinant for integral membrane proteins (Bretscher and Munro, 1993). We thus measured membrane thickness of the different fractions on high magnification prints of electron micrographs. The values obtained from this analysis are listed in Table II. The average thickness of the membranes was 7.1 ± 0.4 nm. The plasma membrane was significantly thicker (9.2 ± 0.4 nm), and the lipid particle membrane was significantly thinner (4.5 ± 0.4 nm), as expected for a lipid monolayer that delineates the lipid particles (Leber et al., 1994).

### Nano-ESI-MS/MS Analysis of the Lipid Composition of Subcellular Membranes

Lipids from the various subcellular membrane fractions were extracted, their phosphate content was determined, and set in relation to the protein content of the respective fraction (Table III). The ergosterol content of the membranes was determined by quantitative nano-ESI-MS/MS analysis of lipid extracts containing a defined amount of [$^{13}$C]cholesterol as internal standard, added to the fractions before lipid extraction (Sandhoff et al., 1999). Ratios of phospholipid to ergosterol content of the subcellular fractions are also listed in Table III. These analyses complement previously published data (Zinser et al., 1991,

1993), and show that the yeast plasma membrane has a phospholipid to ergosterol ratio of 2.2, which is comparable to a value of 1.9–2.9 reported for higher eukaryotic cells (Lange et al., 1989; Allan and Kallen, 1994; van Meer, 1998). Limiting amount of material did not allow quantification of the phospholipid content of the Golgi, nuclei, and mitochondrial fractions, which are therefore not represented in Table III.

Lipid extracts of the subcellular membrane fractions were then analyzed by nano-ESI-MS/MS. A comparison of negative ion mode spectra of the different membranes is shown in Fig. 4. For peak assignment, each major ion was subjected to product ion scan analyses (data not shown). Interpretation of the spectra was facilitated by the relative simple fatty acid composition of *S. cerevisiae* phospholipids. As can be seen from the overview shown in Fig. 4, the plasma membrane differed most from all other fractions. Free ceramide (Cer-C, containing phytosphingosine with a α-hydroxylated C26 fatty acid) was readily detectable in this membrane fraction, whereas the mature sphingolipids (M(IP)$_2$C, IPC-C, and IPC-D) were also detected in the heavy microsome fraction (indicated in black in Fig. 4). The m/z values of these sphingolipids were identical to those reported previously (Hechtberger et al., 1994; for review see Lester and Dickson, 1993; Dickson, 1998; Schneiter, 1999).

To determine the relative abundance of each molecular species within its lipid class, scans specific for single phospholipid classes were recorded for every membrane fraction (data not shown; Brügger et al., 1997). Specifically, the molecular species profile of PS was calculated from positive mode ion scans specific for the neutral loss of m/z 185 (specific for serine phosphate; Tables IV and V). The molecular species profile of PE was calculated from positive mode ion scans specific for the neutral loss of m/z 141 (specific for ethanolamine phosphate; Table VI). The molecular species profile of PC was calculated from positive ion precursor scans for m/z 184 (specific for choline phosphate; Table VII). The molecular species profile of PI was

*Figure 4.* Overview of the lipid molecular species distribution in different subcellular membranes. Negative ion mass spectra of the m/z range 500–1,000 of unprocessed lipid extracts prepared from the subcellular membranes indicated. Individual lipid classes are color coded: PA, magenta; PE, blue; PS, cyan; PI, red; MMPE, lilac; sphingolipids, black. Positions of major molecular species are indicated. PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; MMPE, monomethylphosphatidylethanolamine; PI, phosphatidylinositol; Cer, ceramide C; IPC, inositolphosphorylceramide; M(IP)$_2$C, mannosyldiinositolphosphorylceramide. The total carbon chain length (x) and number of carbon–carbon double bonds (y) of individual lipid molecular species is specified (x:y).
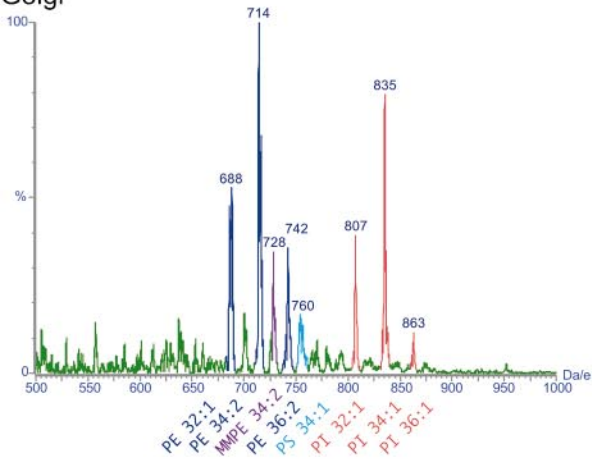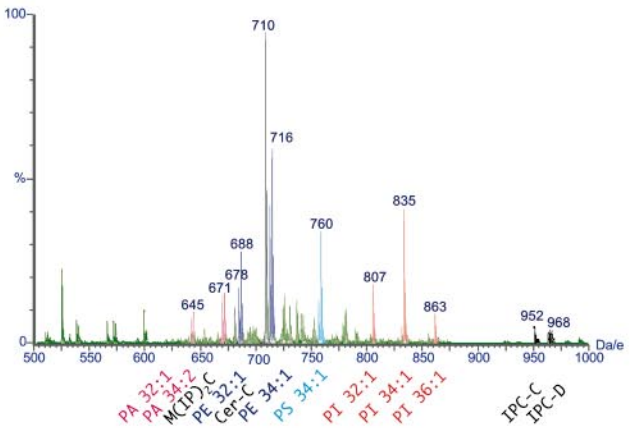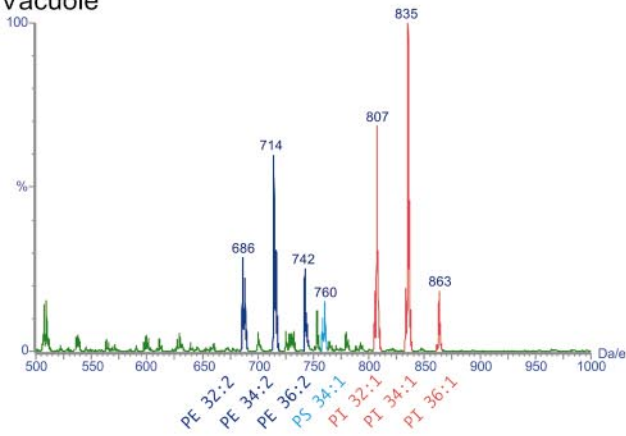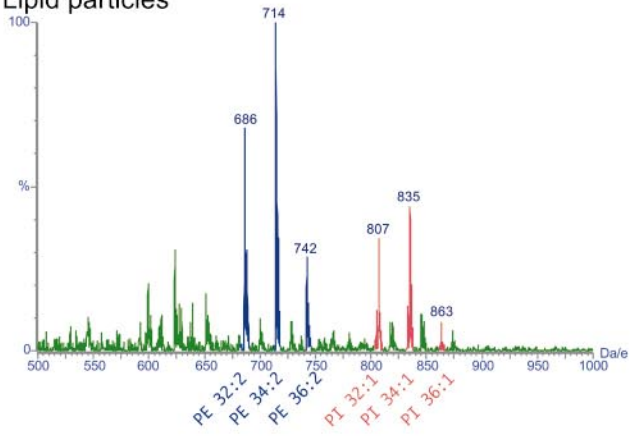
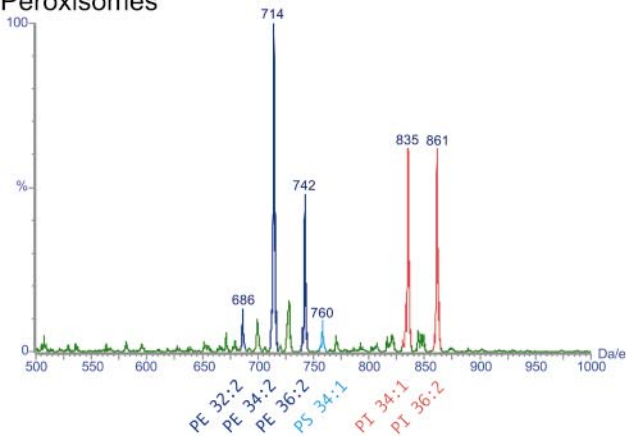**Nuclei**
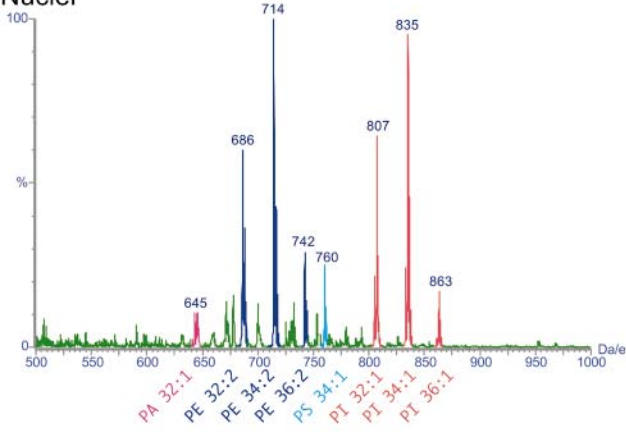
**40k Microsomes**
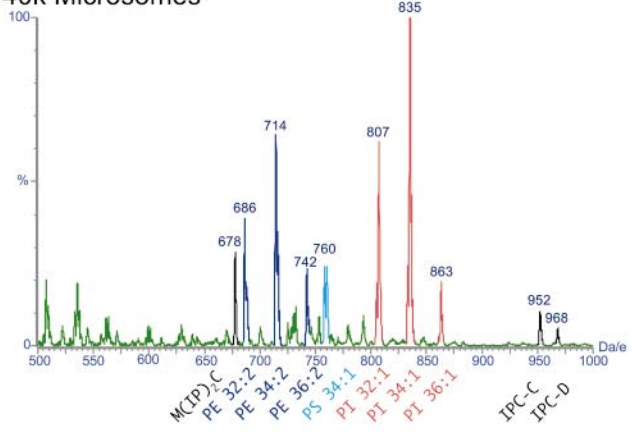
**Golgi**

**Plasma membrane**

**Vacuole**

**Lipid particles**
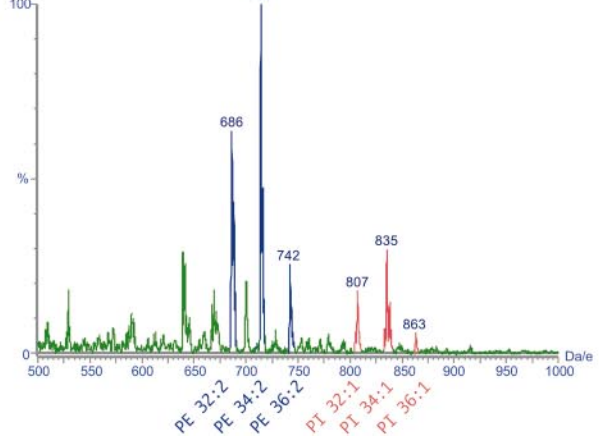
**Peroxisomes**

**Mitochondria**

Table IV. PS Species of Yeast Subcellular Membranes

| Fraction | x:y m/z [M + H]$^+$ | 28:0 680 | 32:1 734 | 32:2 732 | 34:1 762 | 34:2 760 | 36:2 788 | 36:3 786 | Saturated fatty acids |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Molecular species | | |
| Nuclei | | | 16.6 | 7.5 | 40.8 | 34.0 | | | 29.2 |
| 40k microsomes | | | 18.6 | 10.0 | 36.8 | 33.3 | | | 27.7 |
| Golgi membrane | | 10.6 | 13.2 | 8.5 | 37.7 | 28.3 | | | 36.1 |
| PM | | | 15.8 | 1.9 | 64.1 | 16.8 | 1.4 | | 40.0 |
| Vac | | | 17.2 | 11.2 | 34.6 | 33.3 | 1.3 | | 25.9 |
| LP | | | 10.1 | 25.0 | 21.9 | 40.0 | 3.0 | | 16.0 |
| Mit | | | 19.1 | 7.0 | 53.7 | 20.2 | | | 36.4 |
| OM | | | 16.0 | 11.5 | 36.8 | 34.8 | | | 26.4 |
| IM | | | 12.9 | 9.8 | 46.2 | 31.1 | | | 29.6 |
| CS | | | 17.0 | 10.0 | 36.5 | 34.2 | | | 26.8 |
| Average | | | 15.7 ± 2.2 | 10.2 ± 3.4 | 40.9 ± 8.3 | 30.6 ± 5.3 | | | 29.4 |
| Perox | | 4.2 | | | | 64.5 | 27.3 | 3.9 | 4.2 |

calculated from negative ion precursor scans for m/z 241 (specific for the dehydration product of inositol phosphate; Table VIII).

Since peroxisomes were isolated from cells cultivated in the presence of oleic acid (C18:1), the lipid molecular species profile of this membrane was shifted dramatically towards oleic acid–containing species (Fig. 4; Tables IV, VI–VIII). For the comparison of the molecular species profiles of the different membranes that follow, peroxisomes were thus not further taken into account. Moreover, molecular species containing double desaturated acyl chains were observed in peroxisomal phospholipids (Tables IV, VI–VIII). These are due to contaminations of the oleic acid preparation used to supplement the culture media by C18:2, as revealed by GC-MS analysis (data not shown).

A molecular species profile, calculated by averaging the values of ten different membranes, not including peroxisomes, is listed in Tables IV and VI–VIII. In the rightmost column of these Tables, the percentage of saturated acyl chains in the respective phospholipid class is given for each of the subcellular membranes.

## Molecular Species of PS

Averaged over all membrane fractions, PS 34:1 (i.e., composed of oleic acid [C18:1] and palmitic acid [C16:0]) constituted the main species of PS with 40.9%. This was followed by PS 34:2 (i.e., composed of oleic acid [C18:1] and palmitoleic acid [C16:1]) with 30.6%, PS 32:1 (15.7%), and PS 32:2 (10.2%). In the plasma membrane, PS 34:1 was the most prominent species (64.1%). Surprisingly, this rise in PS 34:1 at the plasma membrane was compensated by a greatly diminished PS 32:2 level (1.9%). A similar, albeit less pro-

Table V. Plasma Membrane PS Species of Wild-type and elo3Δ Mutant Cells

| Genotype | x:y m/z [M + H]$^+$ | 28:0 680 | 32:1 734 | 32:2 732 | 34:1 762 | 34:2 760 | 36:2 788 | Saturated fatty acids |
|---|---|---|---|---|---|---|---|---|
| | | | | Molecular species | | | | |
| Wild-type* | | 3.5 | 22.1 | 3.2 | 57.6 | 12.3 | 0.6 | 43.4 |
| elo3Δ | | 7.5 | 15.3 | 24.6 | 8.2 | 40.3 | 1.2 | 19.3 |

*Different wild-type from that analyzed in Tables IV, VI–VIII.

nounced, reduction was observed for PS 34:2 (16.8%), suggesting that PS species containing two unsaturated fatty acids are replaced by and/or remodeled to species containing one saturated fatty acid at the plasma membrane.

Interestingly, in the lipid particle monolayer membrane, a PS profile contrasting that of the plasma membrane was observed. Diunsaturated fatty acid–substituted PS species, i.e., PS 34:2 (40.0%) and PS 32:2 (25.0%), were enriched at the expense of saturated fatty acid–containing species. The Golgi membrane, on the other hand, was greatly enriched in the short chain lipid PS 28:0, i.e., containing C14:0/C14:0 (10.6%).

Yeast has a plasma membrane phospholipid asymmetry typical of eukaryotic cells, with PS mainly, if not exclusively, in the inner leaflet, and sterols and sphingolipids in the outer leaflet (Devaux, 1991; Schroit and Zwaal, 1991; Cerbon and Calderon, 1995). The yeast plasma membrane has been estimated to contain most of the PS (90%), PE (80–90%), and PI (85%) in the inner leaflet and the sphingolipids (30% of total phospholipids) on the outer leaflet (Patton and Lester, 1991; Hechtberger et al., 1994; Cerbon and Calderon, 1995; Balasubramanian and Gupta, 1996). The acyl chain composition of the sphingolipids in yeast is unusual in that they contain the saturated very long chain C26:0 fatty acid (Lester and Dickson, 1993; Hechtberger et al., 1994; Schneiter and Kohlwein, 1997). The observation that monounsaturated PS species were greatly enriched at the expense of diunsaturated species suggested that the C26:0 containing sphingolipids may affect the acyl chain composition of lipids in the inner leaflet of the plasma membrane. To test this hypothesis, the PS profile of plasma membrane isolated from a yeast mutant, elo3Δ (also known as *SUR4/APA1/VBM1/SRE1/YLR372w*; David et al., 1988; García-Arranz et al., 1994; Silve et al., 1996; Oh et al., 1997), that is defective in the final step of elongating the C24 to the C26 fatty acid, and hence, does not contain any C26 fatty acid, was analyzed. Compared with the corresponding wild-type strain, in the plasma membrane from the elo3Δ mutant, PS 34:2 is the most abundant species (40.3%), followed by PS 32:2 (24.6%), PS 32:1 (15.3%), and PS 34:1 (8.2%; see Table V; Fig. 5). This dramatic shift towards diunsaturated fatty acid–containing species in cells that synthesize C24-substituted sphingolipids thus suggests that the precise acyl chain length of very long chain fatty

*Table VI. PE Species of Yeast Subcellular Membranes*

| Fraction | x:y m/z [M + H]+ | 30:1 662 | 32:1 690 | 32:2 688 | 34:1 718 | 34:2 716 | 34:3 714 | 36:1 746 | 36:2 744 | 36:3 742 | Saturated fatty acids |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nuclei | | 0.8 | 14.8 | 25.8 | 13.8 | 38.5 | | 1.0 | 3.5 | | 15.2 |
| 40k microsomes | | 1.0 | 9.9 | 28.4 | 14.4 | 42.4 | | 1.2 | 1.0 | | 13.3 |
| Golgi membrane | | 2.1 | 18.0 | 19.9 | 20.7 | 35.8 | | | 2.3 | | 20.4 |
| PM | | 2.2 | 19.8 | 12.4 | 34.6 | 22.0 | | 2.5 | 5.9 | | 29.6 |
| Vac | | 1.3 | 16.8 | 32.8 | 12.2 | 32.8 | | 0.4 | 3.0 | | 15.4 |
| LP | | 2.1 | 10.1 | 40.9 | 6.9 | 34.8 | | | 5.1 | | 9.6 |
| Mit | | 1.2 | 11.3 | 20.7 | 14.3 | 43.9 | | | 7.2 | | 13.4 |
| OM | | 1.3 | 13.0 | 27.8 | 10.0 | 41.2 | | | 4.6 | | 12.2 |
| IM | | 1.0 | 18.2 | 20.5 | 14.8 | 33.6 | | 0.6 | 9.9 | | 17.0 |
| CS | | 1.4 | 16.1 | 23.6 | 13.7 | 38.7 | | 0.3 | 5.5 | | 15.8 |
| Average | | 1.4 ± 0.4 | 14.8 ± 3.0 | 25.3 ± 5.9 | 15.5 ± 4.8 | 36.4 ± 4.6 | | 1.0 ± 0.6 | 4.8 ± 1.9 | | 16.2 |
| Perox | | | | 6.1 | 2.0 | 47.9 | 3.6 | | 33.6 | 6.7 | 1.0 |

## Molecular Species of PE

Averaged over all membranes, the most abundant PE species was PE 34:2 (36.4%), followed by PE 32:2 (25.3%), PE 34:1 (15.5%), and PE 32:1 (14.8%). The tendency towards enrichment of saturated species (PE 32:1 and PE 34:1) at the expense of diunsaturated species (PE 32:2 and PE 34:2) at the plasma membrane, noted for PS, was also observed for PE (see Table VI). Lipid particles were again enriched in the diunsaturated PE species, PE 32:2, as was the case for PS.

## Molecular Species of PC

The molecular species profile of PC did not display significant variation between the different membranes (Table VII). Overall, PC 32:2 (40.0%) was the most abundant species, followed by the 34:2 (25.4%), 32:1 (14.9%), and 34:1 (9.3%) species.

## Molecular Species of PI

The molecular species profile of PI was generally more heterogeneous than that of the other glycerophospholipids. This was mainly due to the fact that disaturated species of PI, but not of PS, PE, or PC, were present in all of the membranes (see Table VIII). The most abundant PI species was PI 34:1 (34.0%), followed by PI 32:1 (21.8%), PI 32:0 (7.5%), PI 34:0 (7.3%), and PI 34:2 (7.0%). PI thus contained a significantly higher proportion of saturated acyl chains (53.1%) than PS (29.4%), PE (16.2%), and PC (15.4%). The unusual short-chain substituted PI 28:0 was enriched at the plasma membrane (8.1%, compared with an average of 3.4%). The mitochondrial contact sites, on the other hand, were enriched in a second unusual short-chain containing PI, namely PI 30:0 (9.3%; see Table VIII). This enrichment of PI 30:0 in contact sites appeared to be at the expense of PI 34:1, which was reduced to 16.2%, compared with an average of 34.0%. PI 34:1, on the other hand, was enriched in the Golgi membrane (45.1%), but reduced at the plasma membrane (29.4%). PI 34:2 and PI 32:0 were both enriched in the lipid particle membrane.

## Overview of Lipid Classes

In general, the four main glycerophospholipid classes, PS, PE, PC, and PI, each were comprised of two main species, which together made up 56–72% of the respective lipid class, and two (or four in the case of PI) minor species that

---

*Table VII. PC Species of Yeast Subcellular Membranes*

| Fraction | x:y m/z [M + H]+ | 28:1 676 | 30:1 704 | 30:2 702 | 32:1 732 | 32:2 730 | 34:1 760 | 34:2 758 | 34:3 756 | 36:1 788 | 36:2 786 | 36:3 784 | Saturated fatty acids |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nuclei | | 1.6 | 2.7 | 0.8 | 15.3 | 39.2 | 10.2 | 25.9 | 2.0 | | 2.4 | | 16.0 |
| 40k microsomes | | 1.5 | 2.6 | 0.7 | 14.7 | 36.6 | 11.0 | 28.6 | 2.2 | | 2.2 | | 16.0 |
| Golgi membrane | | 3.2 | 5.8 | 1.6 | 15.7 | 42.5 | 5.7 | 19.4 | 0.5 | | 1.1 | | 15.5 |
| PM | | | 3.4 | 0.8 | 16.6 | 39.3 | 10.6 | 20.4 | 2.5 | | 1.9 | | 17.1 |
| Vac | | 2.0 | 2.8 | 0.9 | 15.3 | 38.3 | 9.7 | 25.4 | 1.6 | | 1.6 | | 15.7 |
| LP | | 1.5 | 2.9 | 1.0 | 15.4 | 41.8 | 7.5 | 25.6 | 1.7 | | 2.5 | | 14.5 |
| Mit | | 1.7 | 2.7 | 0.9 | 12.6 | 40.3 | 10.0 | 27.9 | 2.6 | | 1.3 | | 14.6 |
| OM | | 1.4 | 2.6 | 0.7 | 14.4 | 38.7 | 10.0 | 27.7 | 1.4 | | 1.9 | | 15.0 |
| IM | | 2.0 | 2.4 | 0.8 | 14.0 | 40.1 | 9.1 | 26.5 | 2.0 | | 2.4 | | 14.8 |
| CS | | 2.2 | 2.6 | 0.9 | 14.5 | 39.4 | 9.1 | 26.5 | 1.5 | | 2.2 | | 15.0 |
| Average | | 1.7 ± 0.5 | 3.1 ± 0.6 | 0.9 ± 0.2 | 14.9 ± 0.8 | 40.0 ± 1.0 | 9.3 ± 1.2 | 25.4 ± 2.2 | 1.8 ± 0.5 | | 2.0 ± 0.4 | | 15.4 |
| Perox | | | | | 1.9 | 3.3 | 11.4 | 31.5 | 2.3 | | 41.1 | 7.2 | 6.7 |

Table VIII. PI Species of Yeast Subcellular Membranes

| Fraction | x:y m/z [M − H]⁻ | 26:0 725 | 28:0 753 | 30:0 781 | 30:1 779 | 32:0 809 | 32:1 807 | 32:2 805 | 34:0 837 | 34:1 835 | 34:2 833 | 36:0 865 | 36:1 863 | 36:2 861 | Saturated fatty acids |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Molecular species | | | | | | | |
| Nuclei | | 2.0 | 2.7 | | 3.0 | 5.0 | 19.6 | 6.4 | 9.3 | 33.9 | 7.8 | | 7.8 | 2.4 | 51.2 |
| 40k microsomes | | 2.2 | 3.0 | | 2.2 | 9.3 | 18.9 | 4.8 | 5.9 | 37.0 | 8.9 | | 6.7 | 1.1 | 52.8 |
| Golgi membrane | | 2.6 | 1.9 | | 0.9 | 6.3 | 25.7 | 2.3 | | 45.1 | 6.7 | | 5.9 | 2.6 | 49.6 |
| PM | | 4.6 | 8.1 | | 5.2 | 9.5 | 23.1 | 3.5 | 3.5 | 29.4 | 4.0 | | 8.4 | 0.9 | 58.8 |
| Vac | | 1.9 | 4.4 | 3.1 | 3.1 | 7.0 | 22.2 | 4.9 | 8.5 | 34.8 | 4.6 | | 5.4 | | 57.7 |
| LP | | | | | | 11.3 | 24.2 | 6.3 | 8.5 | 33.1 | 11.2 | | 5.4 | | 51.2 |
| Mit | | 1.1 | 2.4 | | | 7.8 | 24.6 | 7.6 | 6.6 | 37.3 | 5.0 | | 7.6 | | 52.7 |
| OM | | 2.1 | 2.6 | 1.9 | | 5.0 | 22.1 | 4.9 | 5.2 | 39.4 | 8.3 | | 7.2 | 1.3 | 51.2 |
| IM | | | 3.4 | | 3.7 | 7.5 | 19.3 | 4.1 | 9.5 | 33.9 | 7.8 | 3.4 | 7.5 | | 56.0 |
| CS | | | 2.2 | 9.3 | | 6.5 | 18.2 | 3.0 | 8.8 | 16.2 | 5.3 | 2.6 | 7.4 | 0.5 | 50.3 |
| Average | | 2.4 ± 0.7 | 3.4 ± 1.3 | 4.8 ± 3.0 | 3.0 ± 1.0 | 7.5 ± 1.6 | 21.8 ± 2.2 | 4.8 ± 1.2 | 7.3 ± 1.8 | 34.0 ± 4.7 | 7.0 ± 1.8 | | 6.9 ± 0.8 | 1.5 ± 0.7 | 53.1 |
| Perox | | | | | | | | | | 40.8 | 9.2 | | 12.9 | 37.1 | 26.9 |

contributed 24–30% to the total. The two main PS species, PS 34:2 and PS 34:1, represented 71.5%, and the two minor species, PS 32:1 and PS 32:2, represented 25.9% of all PS. Similarly, the two major PE species, PE 34:2 and PE 32:2, comprised 61.7%, whereas the two minor species, PE 34:1 and PE 32:1, comprised 30.3% of PE. The PC species were dominated by PC 32:2 and PC 34:2, which together comprised 65.4% and were complemented by the two minor species, PC 32:1 and PC 34:1, which contributed 24.2%. PI contained two major species, PI 34:1 and PI 32:1 (55.8%), and four minor species, PI 32:0, PI 34:0, PI 34:2, and PI 36:1, which together made up 28.7%. Remarkably, not all possible combinations of the four major fatty acids were found esterified to the different glycerophospholipid classes. Disaturated fatty acid-containing species (32:0 and 34:0) were only observed for PI (see Fig. 6).

## Discussion

Nano-ESI-MS/MS analysis, together with improved procedures for the isolation of individual subcellular membranes from yeast, *S. cerevisiae* (Zinser and Daum, 1995), enabled us to perform what is, to our knowledge, the first analysis of the lipid molecular species composition of a comprehensive set of organellar membranes from a eukaryotic cell. The ESI-MS/MS analyses employed in this study were performed with intact phospholipid molecules, thus permitting direct structural assignments that avoid indirect inferences of identities of glycerophospholipid species inherent in HPLC- and GC/MS-based methods. Scan modes specific for the different phospholipid classes allowed us to resolve the complex mixture of lipid molecular species into readily assignable molecular species profiles for each of the different membranes. This analysis revealed a number of remarkable differences in the lipid molecular species composition of yeast subcellular membranes.

### Enrichment of Saturated Species of PS and PE at the Plasma Membrane

The molecular species composition of the plasma membrane deviated most from that of all other membranes.

Even though this was unexpected, it is not surprising, given that the lipid class composition of the plasma membrane with its high enrichment in sterols and sphingolipids, is much different from that of all the other membranes. It also contained large amounts of free ceramide-C, not readily detected in any other membrane. Furthermore, the molecular species profile of PS and PE at the plasma membrane was shifted dramatically towards species containing one saturated acyl chain (34:1) at the expense of diunsaturated species (32:2 and 34:2). A comparable enrichment of saturated PS species also has been reported for the plasma membrane of mammalian cells (Keenan and Morré, 1970; for review see van Meer, 1998). This enrichment of saturated species of PS and PE is particularly noteworthy in light of the fact that PS and PE constitute, 33.6% and 20.3%, respectively, the majority of the glycerophospholipids in the plasma membrane of yeast (Zinser at al., 1991; Zinser and Daum, 1995). Moreover, in contrast to all other glycerophospholipids, PS preferentially incorporates radiolabeled palmitic acid into position *sn-1*, suggesting that remodeling of the acyl chain composition of this lipid follows a unique pathway (Wagner and Paltauf, 1994).

As inferred from the established lipid asymmetry at the plasma membrane of eukaryotic cells, these PS species appear to be concentrated in the inner leaflet of the membrane and thus may interact with the saturated C26 very long acyl chain of sphingolipids from the opposing membrane leaflet. An effect of the C26-substituted sphingolipids on the molecular species composition of the plasma membrane was evident from the dramatic alteration of the PS profile observed in *elo3Δ* mutant cells. The degree of acyl chain saturation of plasma membrane lipids is, due to the higher packing density of saturated acyl chains, regarded as a critical parameter for the generation of detergent insoluble membrane domains (Schroeder et al., 1994; Brown and London, 1998).

The plasma membrane is the terminal compartment of lipids whose transport and/or maturation depends on a functional secretory pathway, i.e., the sphingolipids (Puoti et al., 1991), PS, and ergosterol (Pichler, H., and G. Daum, unpublished observation). The question that arises is how
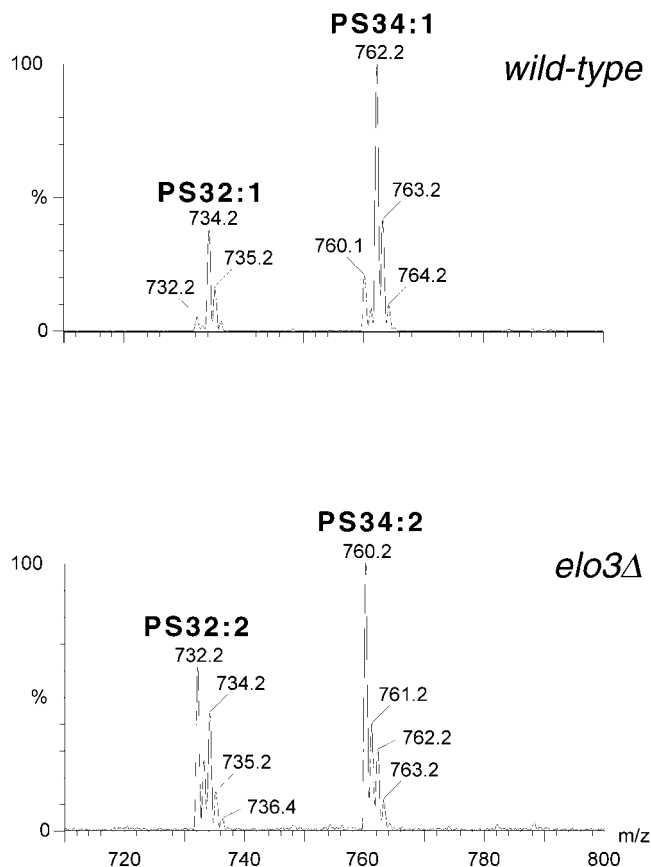
*Figure 5.* PS profile of the plasma membrane in *elo3Δ* mutant cells. Positive ion mass spectra specific for molecular species of PS (neutral loss of m/z 185) in the m/z range 700–800 of unprocessed lipid extracts prepared from plasma membranes of wild-type (EMY30) and *elo3Δ* (EMA40) mutant cells. Peak assignment to PS molecular species is indicated.



*Figure 6.* Summary showing the average molecular species profile with the corresponding fatty acid composition of each lipid class and their possible interconversion by the de novo pathway from CDP-DAG in the ER, or by contribution from the Kennedy pathway from local pools of DAG in the Golgi apparatus. The two major molecular species of each lipid class are boxed in gray. Linear increases and decreases in the relative abundance of 32:1 and 34:1 containing species along the biosynthetic route from PS via PE to PC are indicated by the triangles.

this peculiar lipid composition of the plasma membrane is established and maintained. Three alternative, but not necessarily exclusive, hypotheses may be considered: (i) lipid selection and/or remodeling in the Golgi apparatus, i.e., by synthesis of specific species of PE and PC via the Kennedy pathway, results in the formation of secretory vesicles that already have an established lipid asymmetry and a molecular species composition typical of the plasma membrane; (ii) the acyl chain composition of lipids delivered to the plasma membrane is subject to extensive remodeling within their target membrane, i.e., unsaturated fatty acids on PS and PE are replaced by saturated fatty acids, resulting in the in situ generation of the molecular species profile of the plasma membrane; and (iii) the molecular species composition of the plasma membrane is established by selective removal of species that do not fit the particular requirements of that membrane, i.e., by selective phospholipase-mediated degradation of diunsaturated fatty acid-substituted species, or by concentrating such species in endocytotic vesicles and delivery to the vacuole. The question whether the plasma membrane lipid composition is defined in the exocytic or the endocytic pathway may be answered by analyzing the molecular species profile of the appropriate vesicles (Allan and Kallen, 1994). Our previous observation that sphingolipids, ergosterol,
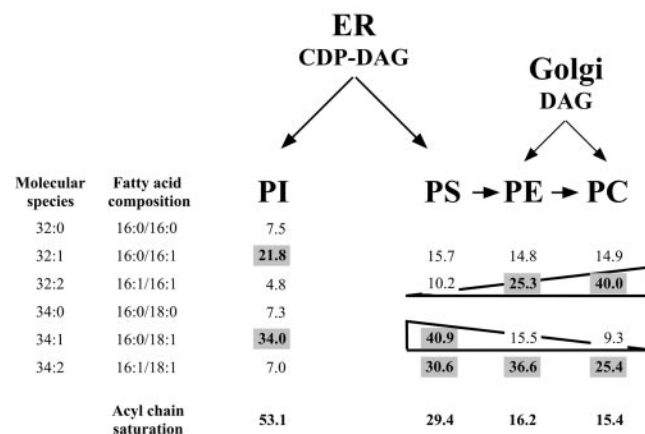
and PS are enriched in the late (Kex2p-enriched) Golgi compartment (~11% PS) and in late secretory vesicles (~13% PS) suggests a significant contribution of the Golgi/secretory pathway in establishing the lipid composition of the yeast plasma membrane (Zinser et al., 1991; Hechtberger et al., 1994; McGee et al., 1994; Leber et al., 1995).

### Enrichment of Diunsaturated Species of PS and PE in the Lipid Particle Membrane

In lipid particles, a phospholipid monolayer surrounds a hydrophobic core of triacylglycerols and steryl esters (Leber et al., 1994). Surprisingly, this membrane was particularly rich in diunsaturated fatty acid-substituted species of all major phospholipid classes, PS, PE, PC, and PI (see Tables IV, VI–VIII). The lipid particle membrane is the subcellular membrane with the highest content of PI and is furthermore rich in PC (36.4%) and PE (20.0%), but contains little PS (5.4%; Leber et al., 1994). The enrichment of lipid species with bulky head groups (PI and PC) and acyl chain substituents (diunsaturated species), suggests that during the biogenesis of this membrane type selected lipid species get incorporated into or are retained within the prospective lipid particle membrane.

### Methylation Intermediates in the Golgi Membrane

Intermediates in the de novo synthesis of PC via methylation of PE, i.e., monomethyl-PE (MMPE) and dimethyl-PE (DMPE), were detected in the Golgi membrane. No such methylation intermediates were detected in any of the other subcellular membranes (see Fig. 4). The de novo synthesis of PC occurs by sequential three-step methylation of PE first to MMPE, then to DMPE, and finally to PC. This reaction sequence is generally thought to occur in the ER and is catalyzed by two methyltranferases in yeast, Cho2p/Pem1p and Opi3p/Pem2p, with overlapping sub-

strate specificity (Kuchler et al., 1986; Kodaki and Yamashita, 1989). Cho2p catalyzes the first step, methylation of PE to MMPE, and Opi3p catalyzes the subsequent two steps from MMPE to PC (for review see Paltauf et al., 1992; Daum et al., 1998). The fact that methylation intermediates were observed in the Golgi fraction rather than in the ER led us to consider whether methylation may be, in part, a Golgi apparatus-localized process. Inspection of the two protein sequences revealed the presence of a COOH-terminal ER retention signal in Opi3p (KAKKNM), but no such signal could be found in Cho2p (TLDSLA; Jackson et al., 1990). The subcellular localization of Cho2p thus needs to be reinvestigated in more detail. Alternatively, since the Golgi membrane was isolated from exponentially growing cells, the presence of methylation intermediates in this fraction may be due to export of not yet fully matured phospholipids from the ER of rapidly dividing cells.

### Phospholipid Class-specific Differences in the Degree of Acyl Chain Saturation

Striking differences in the fatty acid distribution of phospholipids that are metabolically closely related (e.g., PS, PE and PC, and PS and PI), noted already in our earlier study of the acyl chain composition of total phospholipids (Wagner and Paltauf, 1994), were also observed in this study, suggesting that pathways exist for the generation of distinct phospholipid molecular species within the different phospholipid classes.

Consistent with the results obtained by Wagner and Paltauf (1994), we find that the percentage of saturated fatty acids is low in PC (15.4%) and PE (16.2%). PS (29.4%), the direct precursor to PE via the de novo pathway, has a significantly higher content of saturated fatty acids. Although both PS and PI are derived from cytidine diphosphate-diacylglycerol (CDP-DAG), the fatty acid distribution between these two phospholipid classes is remarkably different, with PI having the highest percentage of saturated fatty acids (53.1%; see Fig. 6).

These species differences may be explained by different substrate specificity (species preferences) of enzymes catalyzing individual steps in the synthesis of the major lipid classes, or by distinct subcellular localization of specific substrates for the synthesis of the different lipid classes. For example, the enzymes that catalyze the final step in the synthesis of PI and PS (PI synthase and PS synthase, respectively) may possess different preferences for distinct molecular species of their common substrate, CDP-DAG, hence yielding products with different acyl chain compositions. Alternatively, these two enzymes may be localized in different subdomains of the ER and therefore may have access only to local pools of CDP-DAG molecular species. Apart from such species differences established by de novo synthesis of the various lipid classes (Samborski et al., 1990), species differences may also be established later, by the salvage/Kennedy pathway for PE and PC synthesis from local pools of DAG species produced in the Golgi apparatus (Kearns et al., 1997). Furthermore, pathways may exist to generate specific molecular species by remodeling the acyl chain composition of existing lipids (MacDonald and Sprecher, 1991; Schmid et al., 1991); different

molecular species may have different rates of turnover (Ousley and Morell, 1992), or may be transported with different efficiency (Heikinheimo and Somerharju, 1998).

Minor lipid classes, such as plasmalogens and cardiolipin, and unusual molecular species of the major lipid classes were not analyzed in detail in this study. Molecular species of cardiolipin from yeast previously have been characterized and shown to be of mainly tetramonounsaturated type (Schlame et al., 1993). Low levels of plasmalogens, mainly of PE, were detected in most of the subcellular fractions analyzed and were also observed in lipid extracts from cells cultivated on synthetic minimal media, suggesting that *S. cerevisiae* has the enzymatic repertoire to synthesize these lipids de novo (data not shown).

The dynamic nature of the lipid molecular species composition of the different subcellular membranes has not been addressed in this study. Data reported here thus may merely serve as a point of reference for future more detailed and focused analyses of lipid molecular species profiles of, for example, the plasma membrane of various mutant cells or of vesicles of different origin. Moreover, comparison of molecular species profiles from a variety of growth conditions should provide a minimum set of lipid molecular species whose presence in a given membrane compartment is required for normal vegetative growth and another set composed of species that are synthesized only in response to specific environmental stimuli or during more specialized processes. The data presented here thus provide basic information integral to the interpretation of such future experiments.

### References

Allan, D., and K.-J. Kallen. 1994. Is plasma membrane lipid composition defined in the exocytic or the endocytic pathway? *Trends Cell Biol.* 4:350–353.

Aris, J.P., and G. Blobel. 1991. Isolation of yeast nuclei. *Methods Enzymol.* 194:735–748.

Balasubramanian, K., and C.M. Gupta. 1996. Transbilayer phosphatidylethanolamine movements in the yeast plasma membrane: evidence for a protein-mediated, energy-dependent mechanism. *Eur. J. Biochem.* 240:798–806.

Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

Bretscher, M.S., and S. Munro. 1993. Cholesterol and the Golgi apparatus. *Science.* 261:1280–1281.

Brown, D.A., and E. London. 1998. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* 14:111–136.

Brügger, B., G. Erben, R. Sandhoff, F.T. Wieland, and W.D. Lehmann. 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectroscopy. *Proc. Natl. Acad. Sci. USA.* 94:2339–2344.

Cerbon, J., and V. Calderon. 1995. Generation, modulation and maintenance of the plasma membrane asymmetric phospholipid composition in yeast cells during growth: their relation to surface potential and membrane protein activity. *Biochim. Biophys. Acta.* 1235:100–106.

Cluett, E.B., E. Kuismanen, and C.E. Machamer. 1997. Heterogeneous distribution of the unusual phospholipid semilysobisphosphatidic acid through the Golgi complex. *Mol. Biol. Cell.* 8:2233–2240.

Conzelmann, A., H. Riezman, C. Desponds, and C. Bron. 1988. A major 125-kd membrane glycoprotein of *Saccharomyces cerevisiae* is attached to the lipid bilayer through an inositol-containing phospholipid. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2233–2240.

Cunningham, K.W., and W.T. Wickner. 1989. Yeast KEX2 protease and mannosyltransferase I are localized to distinct compartments of the secretory pathway. *Yeast.* 5:25–33.

Daum, G., P.C. Böhni, and G. Schatz. 1982. Import of proteins into mitochondria. Cytochrome $b_2$ and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* 257:13028–13033.

Daum, G., N.D. Lees, M. Bard, and R. Dickson. 1998. Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae. Yeast.* 14:1471–1510.

Daum, G., G. Tuller, T. Nemec, C. Hrastnik, G. Balliano, L. Cattel, P. Milla, F. Rocco, A. Conzelmann, C. Vionnet, et al. 1999. Systematic analysis of yeast strains with possible defects in lipid metabolism. *Yeast.* 15:601–614.

David, D., S. Sundarababu, and J.E. Gerst. 1998. Involvement of long chain fatty acid elongation in the trafficking of secretory vesicles in yeast. *J. Cell Biol.* 143:1167–1182.

Devaux, P.F. 1991. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry.* 30:1163–1173.

Dickson, R.C. 1998. Sphingolipid functions in *Saccharomyces cerevisiae*: comparison to mammals. *Annu. Rev. Biochem.* 67:27–48.

García-Arranz, M., A.M. Maldonado, M.J. Mazón, and F. Portillo. 1994. Transcriptional control of yeast plasma membrane H$^+$-ATPase by glucose. *J. Biol. Chem.* 269:18076–18082.

Han, X., and R.W. Gross. 1994. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. *Proc. Natl. Acad. Sci. USA.* 91:10635–10639.

Hechtberger, P., E. Zinser, R. Saf, K. Hummel, F. Paltauf, and G. Daum. 1994. Characterization, quantification and subcellular localization of inositol-containing sphingolipids of the yeast, *Saccharomyces cerevisiae. Eur. J. Biochem.* 225:641–649.

Heikinheimo, L., and P. Somerharju. 1998. Preferential decarboxylation of hydrophilic phosphatidylserine species in cultured cells. *J. Biol. Chem.* 273:3327–3335.

Hurt, E.C., A. McDowall, and T. Schimmang. 1988. Nucleolar and nuclear envelope proteins of the yeast *Saccharomyces cerevisiae. Eur. J. Cell Biol.* 46:554–563.

Jackson, M.R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3153–3162.

Kearns, B.G., T.P. McGee, P. Mayinger, A. Gedvilaite, S.E. Phillips, S. Kagiwada, and V.A. Bankaitis. 1997. Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature.* 387:101–105.

Keenan, T.W., and D.J. Morré. 1970. Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. *Biochemistry.* 9:19–25.

Kent, C. 1995. Eukaryotic phospholipid biosynthesis. *Annu. Rev. Biochem.* 64:315–343.

Kerwin, J.L., A.R. Tuininga, and L.H. Ericsson. 1994. Identification of molecular species of glycerophospholipids and sphingomyelin using electrospray mass spectrometry. *J. Lipid Res.* 35:1102–1114.

Kim, H.-Y., T.-C. Wang, and Y.-C. Ma. 1994. Liquid chromatography/mass spectrometry of phospholipids using electrospray ionization. *Anal. Chem.* 66:3977–3982.

Kobayashi, T., E. Stang, K.S. Fang, P. de Moerloose, R.G. Parton, and J. Gruenberg. 1998. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature.* 392:193–197.

Kodaki, T., and S. Yamashita. 1989. Characterization of the methyltransferases in the yeast phosphatidylethanolamine methylation pathway by selective gene disruption. *Eur. J. Biochem.* 185:243–251.

Krebs, J.J., H. Hauser, and E. Carafoli. 1979. Asymmetric distribution of phospholipids in the inner membrane of beef heart mitochondria. *J. Biol. Chem.* 254:5308–5316.

Kuchler, K., G. Daum, and F. Paltauf. 1986. Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in *Saccharomyces cerevisiae. J. Bacteriol.* 165:901–910.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lands, W.E.M., and C.G. Crawford. 1976. Enzymes of membrane phospholipid metabolism. *In* The Enzymes of Biological Membranes. Vol. 2: Biosynthesis of Cell Components. Martonosi, A., editor. Plenum Publishing Corp., NY. 3–85.

Lange, Y., M.H. Swaisgood, B.V. Ramos, and T.L. Steck. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured fibroblasts. *J. Biol. Chem.* 264:3786–3793.

Leber, A., C. Hrastnik, and G. Daum. 1995. Phospholipid-synthesizing enzymes in Golgi membranes of the yeast, *Saccharomyces cerevisiae. FEBS Lett.* 377:271–274.

Leber, R., E. Zinser, G. Zellnig, F. Paltauf, and G. Daum. 1994. Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae. Yeast.* 10:1421–1428.

Leber, R., K. Landl, E. Zinser, H. Ahorn, A. Spok, S.D. Kohlwein, F. Turnowsky, and G. Daum. 1998. Dual localization of squalene epoxidase, Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. *Mol. Biol. Cell.* 9:375–386.

Lester, R.L., and R.C. Dickson. 1993. Sphingolipids with inositolphosphate-containing head groups. *Adv. Lipid Res.* 26:253–274.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Lupashin, V.V., S. Hamamoto, and R.W. Schekman. 1996. Biochemical requirements for the targeting and fusion of ER-derived transport vesicles with purified yeast Golgi membranes. *J. Cell Biol.* 132:277–289.

Lussier, M., A.M. Sdicu, T. Ketela, and H. Bussey. 1995. Localization and targeting of the *Saccharomyces cerevisiae* Kre2p/Mnt1p alpha 1,2-mannosyltransferase to a medial-Golgi compartment. *J. Cell Biol.* 131:913–927.

MacDonald, J.I.S., and H. Sprecher. 1991. Phospholipid fatty acid remodeling in mammalian cells. *Biochim. Biophys. Acta.* 1084:105–121.

McGee, T.P., H.B. Skinner, E.A. Whitters, S.A. Henry, and V.A. Bankaitis. 1994. A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. *J. Cell Biol.* 124:273–287.

Moreau, P., and C. Cassagne. 1994. Phospholipid trafficking and membrane biogenesis. *Biochim. Biophys. Acta.* 1197:257–290.

Normington, K., K. Kohno, Y. Kozutsumi, M.-J. Gething, and J. Sambrook. 1989. *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell.* 57:1223–1236.

Nunnari, J., and P. Walter. 1996. Regulation of organelle biogenesis. *Cell.* 84:389–394.

Oh, C.S., D.A. Toke, S. Mandala, and C.E. Martin. 1997. *ELO2* and *ELO3*, homologues of the *Saccharomyces cerevisiae ELO1* gene, function in fatty acid elongation and are required for sphingolipid formation. *J. Biol. Chem.* 272:17376–17384.

Ousley, A.H., and P. Morell. 1992. Individual molecular species of phosphatidylcholine and phosphatidylethanolamine in myelin turn over at different rates. *J. Biol. Chem.* 267:10362–10369.

Paltauf, F., S.D. Kohlwein, and S.A. Henry. 1992. Regulation and compartmentalization of lipid synthesis in yeast. *In* The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression. Jones, E.W., J.R. Pringle, and J.R. Broach, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 415–500.

Patton, J.L., and R.L. Lester. 1991. The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane. *J. Bacteriol.* 173:3101–3108.

Pon, L., T. Moll, D. Vestweber, B. Marshallsay, and G. Schatz. 1989. Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. *J. Cell Biol.* 109:2603–2616.

Puoti, A., C. Desponds, and A. Conzelmann. 1991. Biosynthesis of mannosyl-inositol-phosphoceramide in *Saccharomyces cerevisiae* is dependent on genes controlling the flow of secretory vesicles from the endoplasmic reticulum to the Golgi. *J. Cell Biol.* 113:515–525.

Rouser, G., S. Fkeischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 5:494–496.

Samborski, R.W., N.D. Ridgway, and D.E. Vance. 1990. Evidence that only newly made phosphatidylethanolamine is methylated to phosphatidylcholine and that phosphatidylethanolamine is not significantly deacylated–reacylated in rat hepatocytes. *J. Biol. Chem.* 265:18322–18329.

Sandhoff, R., B. Brügger, D. Jeckel, W.D. Lehmann, and F.T. Wieland. 1999. Determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *J. Lipid. Res.* 40:126–132.

Schlame, M., S. Brody, and K.Y. Hostetler. 1993. Mitochondrial cardiolipin in diverse eukaryotes. Comparison of biosynthetic reactions and molecular acyl species. *Eur. J. Biochem.* 212:727–735.

Schmid, P.C., S.B. Johnson, and H.H.O. Schmid. 1991. Remodeling of rat hepatocyte phospholipids by selective acyl turnover. *J. Biol. Chem.* 266:13690–13697.

Schneiter, R. 1999. Brave little yeast, please guide us to Thebes: sphingolipid function in *S. cerevisiae. Bioessays.* In press.

Schneiter, R., and S.D. Kohlwein. 1997. Organelle structure, function, and inheritance in yeast: a role for fatty acid synthesis? *Cell.* 88:431–434.

Schroeder, R., E. London, and D. Brown. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. USA.* 91:12130–12134.

Schroit, A.J., and R.F. Zwaal. 1991. Transbilayer movement of phospholipids in red cell and platelet membranes. *Biochim. Biophys. Acta.* 1071:313–329.

Serrano, R. 1988. H$^+$-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. *Methods Enzymol.* 157:533–544.

Silve, S., P. Leplatois, A. Josse, P.-H. Dupuy, C. Lanau, M. Kaghad, C. Dhers, C. Picard, A. Rahier, M. Taton, et al. 1996. The immunosuppressant SR31747 blocks cell proliferation by inhibiting a steroid isomerase in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 16:2719–2727.

Thieringer, R., H. Shio, Y. Han, G. Cohen, and P.B. Lazarow. 1991. Peroxisomes in *Saccharomyces cerevisiae*: immunofluorescence analysis and import

of catalase A into isolated peroxisomes. *Mol. Cell. Biol.* 11:510–522.

Uchida, E., Y. Ohsumi, and Y. Anraku. 1988. Purification of yeast vacuolar membrane $H^+$-ATPase and enzymological discrimination of three ATP-driven proton pumps in *Saccharomyces cerevisiae. Methods Enzymol.* 157:544–562.

van Meer, G. 1998. Lipids of the Golgi membrane. *Trends Cell Biol.* 8:29–33.

Veenhuis, M., M. Mateblowski, W.H. Kunau, and W. Harder. 1987. Proliferation of microbodies in *Saccharomyces cerevisiae. Yeast.* 3:77–84.

Wagner, S., and F. Paltauf. 1994. Generation of glycerophospholipid molecular species in the yeast *Saccharomyces cerevisiae.* Fatty acid pattern of phospholipid classes and selective acyl turnover at *sn-1* and *sn-2* positions. *Yeast.* 10:1429–1437.

Warren, G., and W. Wickner. 1996. Organelle inheritance. *Cell.* 84:395–400.

Zinser, E., and G. Daum. 1995. Isolation and biochemical characterization of organelles from the yeast, *Saccharomyces cerevisiae. Yeast.* 11:493–536.

Zinser, E., C.D.M. Sperka-Gottlieb, E.-V. Fasch, S.D. Kohlwein, F. Paltauf, and G. Daum. 1991. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote *Saccharomyces cerevisiae. J. Bacteriol.* 173:2026–2034.

Zinser, E., F. Paltauf, and G. Daum. 1993. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J. Bacteriol.* 175:2853–2858.