A Yeast Strain Lacking Lipid Particles Bears a Defect in Ergosterol Formation*

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Lipid particles of the yeast *Saccharomyces cerevisiae* are storage compartments for triacylglycerols (TAG) and steryl esters (STE). Four gene products, namely the TAG synthases Dga1p and Lro1p, and the STE synthases Are1p and Are2p contribute to storage lipid synthesis. A yeast strain lacking the four respective genes is devoid of lipid particles thus providing a valuable tool to study the physiological role of storage lipids and lipid particles. Using a *dgal1lro1are1are2* quadruple mutant transformed with plasmids bearing inducible *DGA1*, *LRO1*, or *ARE2* we demonstrate that TAG synthesis contributes more efficiently to lipid particle proliferation than synthesis of STE. Moreover, we show that proteins typically located to lipid particles in wild type such as Erg1p, Erg6p, Erg7p, and Ayr1p are refined to microsomal fractions of the *dgal1lro1are1are2* quadruple mutant. This result confirms the close relationship between lipid particles and endoplasmic reticulum. Most interestingly, the amount of the squalene epoxidase Erg1p, which is dually located in lipid particles and endoplasmic reticulum of wild type, is decreased in the quadruple mutant, whereas amounts of other lipid particle proteins tested were not reduced. This decrease is not caused by down-regulation of *ERG1* transcription but by the low stability of Erg1p in the quadruple mutant. Because a similar effect was also observed in *are1are2* mutants this finding can be mainly attributed to the lack of STE. The quadruple mutant, however, was more sensitive to terbinafine, an inhibitor of Erg1p, than the *are1are2* strain suggesting that the presence of TAG and/or intact lipid particles has an additional protective effect. In a strain lacking the two STE synthases, Are1p and Are2p, incorporation of ergosterol into the plasma membrane was reduced, although the total cellular amount of free ergosterol was higher in the mutant than in wild type. Thus, an esterification/deacylation mechanism appears to contribute to the supply of ergosterol to the plasma membrane.

Triacylglycerols (TAG) and steryl esters (STE) are the most important storage lipids in eukaryotic cells. TAG provides an energy source on one hand and a source of fatty acids for membrane phospholipid formation on the other hand. Mobilization of STE sets sterols free, which are also required for membrane proliferation, especially of the plasma membrane. In the yeast *Saccharomyces cerevisiae* as in other eukaryotic cells TAG and STE form the core of so called lipid particles (1), which are surrounded by a phospholipid monolayer with a small amount and a limited number of proteins embedded. Thus, formation of lipid particles is tightly linked to the synthesis of TAG and STE.

In the yeast, *ARE1* and *ARE2* encode two enzymes with overlapping acyl-CoA:sterol acyltransferase activities (2, 3). Zwietick et al. (4) demonstrated that both proteins are located to the same subcellular compartment, namely the endoplasmic reticulum, but exhibit different specificities for sterol substrates in vivo. Both enzymes share 16–17% amino acid identity with human acyl-CoA:cholesterol acyltransferase (3). Related enzymes with acyl-CoA:sterol acyltransferase activity are also present in other higher eukaryotes (5, 6). Are2p is the major yeast STE synthase catalyzing at least 65–75% of total cellular acyl-CoA:sterol acyltransferase activity (3). An *are1are2* double mutant completely lacks STE, indicating that Are1p and Are2p are the only enzymes forming STE in yeast.

Bioconversion of TAG in *S. cerevisiae* is mainly catalyzed by the two acyltransferases Lro1p and Dga1p (7). *LRO1* (LCAT-related open reading frame) is the only gene in yeast showing a significant sequence similarity to the human lecithin:cholesterol acyltransferase. Lro1p mediates esterification of DAG using the sn-2 acyl group of phospholipids as acyl donor and thus exhibits phospholipid:diacylglycerol acyltransferase activity (8). Lro1p catalyzes ∼35% of TAG synthesis in yeast (9–11) and is present in microsomal fractions. *DGA1* encodes an acyl-CoA:diacylglycerol acyltransferase, which shows 44% overall amino acid sequence homology to an acyl-CoA:diacylglycerol acyltransferase from the oleaginous fungus *Mortierella ramanniana* (12, 13). Dga1p, however, is unrelated to enzymes of the DGAT1 family, which are homologous to acyl-CoA:cholesterol acyltransferases identified in plants (14–16) and mammals (17), but belongs to the DGAT2 family. Localization studies unveiled that Dga1p is a lipid particle protein but is also present in microsomal fractions at a certain amount (10). Besides the major Lro1p and Dga1p routes of TAG synthesis, alternative pathways of TAG biosynthesis exist in yeast. Are1p and Are2p were shown to also exhibit minor TAG synthase activities (11, 18). Moreover, acyl-CoA and Lro1p independent TAG synthesis was detected in yeast lipid particles and micromeres in vitro (10). This pathway, however, appears to be negligible for TAG formation in vivo.

Although a yeast strain lacking *DGA1*, *LRO1*, *ARE1*, and *ARE2* is devoid of TAG, STE, and as a consequence, of lipid particles, growth of this quadruple mutant is not affected under standard conditions. This observation indicates that storage lipid synthesis is not essential in yeast (11, 18). It has been demonstrated, however, that lipid particles are not only a lipid
storage compartment, but also harbor certain proteins involved in lipid metabolism (10, 19, 23). Most of these proteins are also present in the endoplasmic reticulum, which is in line with the proposed relationship of these two compartments. Although models for the biogenesis of lipid particles have been suggested, this process is still unclear at the molecular level (1).

The study presented here provides novel insight into cellular properties of a yeast strain that lacks lipid particles. Special emphasis was given to the fate of polypeptides that are typically located in lipid particles of the wild type. We demonstrate retention of these proteins in microsomes of the dga1lro1are1are2 quadruple mutant. Moreover, we show that the cellular level of the squalene epoxidase enzyme Erg1p is decreased in this strain. As a result, the rate of ergosterol biosynthesis is reduced, the mutant becomes hypersensitive to the Erg1p inhibitor terbinafine, and incorporation of sterols into the plasma membrane is markedly decreased. Most properties can be restored when the dga1lro1are1are2 quadruple mutant is transformed with plasmids that bear inducible genes encoding enzymes of neutral lipid formation. These results are discussed in connection with the biogenesis of lipid particles in yeast.

MATERIALS AND METHODS

Strains and Culture Conditions—Strains used throughout this study are listed in Table I. Cells were grown aerobically to the late logarithmic growth phase \( (A_{600} \sim 11.0) \) at 30 °C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid), and 2% glucose (Merck). Media were inoculated with precultures grown aerobically for 48 h in YPD medium at 30 °C.

Strains harboring pGP316-derived plasmids (see Table I) were grown on uracil-free minimal medium (–URA) containing 0.67% yeast nitrogen base and 2% glucose supplemented with all amino acids and nucleotides except uracil. GAL1p promoter-controlled genes were induced by using –URA medium containing 2% galactose instead of 2% glucose.

Strain Construction—For the transformation of cells with wild type DGA1, LRO1, or ARE2 the respective open reading frames were inserted into plasmid pGP316 that is based on the centromeric shuttle vector pRS316 harboring the marker gene URA3 (24). pGP316 also contains a GAL1p-promoter sequence from pBS SK + (25), which was cloned into pBS116 cleaved with PvuII. The open reading frames were amplified by PCR with genomic DNA derived from a wild type yeast strain as template. Primers used for amplification are listed in Table II. The PCR fragments were generated with ExTaq DNA polymerase (TaKaRa, Japan) by using ~30 ng of genomic wild type DNA as a template in a standard PCR mixture containing PCR buffer (20 m\( M \)) of each deoxynucleoside triphosphate, and 0.1 μM of the primers in a total volume of 50 μl. After a denaturation step of 2 min at 94 °C the enzyme was added and fragments were amplified for 10 cycles of 15 s at 94 °C, 30 s at 55 °C, and 120 s at 72 °C and for 30 cycles of 15 s at 94 °C, 30 s at 68 °C, and 180 s at 72 °C, followed by a final elongation step of 10 min at 72 °C. The plasmid pGP316 was cleaved with HincII overnight at 37 °C. Overnight cultures \( (A_{600} \sim 0.7) \) of the dga1lro1are1are2 quadruple mutant strain H1246 were transformed by the high efficiency lithium acetate transformation protocol (26). Transformants were grown on –ura plates for 2–3 days at 30 °C. Only these clones that yielded large colonies were considered as positive transformants and further analyzed. To verify that these transformants harbor plasmids bearing the respective open reading frames under the GAL1p promoter, a forward primer binding within the GAL1p promoter region of the plasmid, and a reverse primer binding within the open reading frame (Table III) were designed, and analytical PCR with whole yeast cell extracts (27) using AmpliTag DNA polymerase was performed.

**Stress and Drug Sensitivity Tests**—Drugs listed below were added to YPD medium containing 2% agar immediately prior to pouring into Petri dishes. Drugs tested and their concentrations used were as follows: 0.1–8 μg of cycloheximide (stock in ethanol) per ml of YPD, 0.005–2.5 mg of terbinafine (stock in ethanol) per ml of YPD, 0.5–10 μg of cerulenin (stock in ethanol) per ml of YPD, 0.5–8% ethanol. All plates were incubated at 30 °C for 3–4 days. Temperature sensitivity was tested on YPD at 37 °C.

**Isolation and Characterization of Subcellular Fractions**—The yeast lipid particle fraction was obtained at high purity from cells grown to the late logarithmic phase as described by Leber et al. (28). The isolation of other subcellular fractions used in this study has been described by Zinser et al. (29).

**Protein Analysis**—Protein was quantified by the method of Lowry et al. (30) using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid at a final concentration of 10%. The protein pellet was solubilized in 0.1% SDS, 0.1% NaOH. Prior to protein analysis, samples of the lipid particle fraction were delipidated. Nonpolar lipids were extracted with 2 volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were precipitated as described above.

**SDS-polyacrylamide gel electrophoresis** was carried out by the method of Laemmli (31). Samples were dissociated at 37 °C to avoid hydrolysis of polypeptides, which may occur at higher temperatures. Western blot analysis was performed as described by Haid and Suisaa (32). Proteins were detected by enzyme-linked immunosorbent assay using rabbit antiserum as the first antibody and peroxidase-conjugated goat anti-rabbit IgG as the second antibody. Antibodies used in this study were directed against lipid particle proteins Erg1p, Erg6p, Erg7p, and Ayr1p.

**Lipid Analysis**—Lipids from yeast cells grown to the late logarithmic phase \( (A_{600} \sim 11.0) \) and from subcellular fractions were extracted as described by Foch et al. (33). For the quantification of neutral lipids extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (Limonat IV, CAMAG, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by using the solvent system light petroleum/diethyl ether/acetic acid (25:25:1; v/v) for the first third of the distance. Then, plates were dried briefly and further developed to the top of the plate by using the solvent system light petroleum/diethyl ether (49:1; v/v). Quantification of ergosterol and ergysterol esters was carried out by densitometric scanning at 275 nm using a Shimadzu dual-wavelength chromatoscaner CS-930 with ergosterol as a standard.

For the analysis of individual sterols 100 μg of protein of plasma membrane fractions were added to a mixture containing 0.6 ml of methanol (Merck), 0.4 ml of 0.5% (w/v) pyrogallol (Fluka) dissolved in methanol, 0.4 ml of 60% (w/v) aqueous KOH, and 5 μl of a cholesterol solution (2 mg/ml) as an internal standard (34). These samples were heated in a water bath for 2 h at 90 °C. Then, lipids were extracted three times with 1 ml of n-heptane (Merck), each. The combined extracts were dried under a stream of nitrogen and dissolved in 10 μl of pyridine. After adding 10 μl of \( N,O \)-bis(trimethylsilyl)-trifluoroacet-
amide (Sigma) the samples were diluted with 30 μl of ethyl acetate. GLC-MS analysis of silylated sterol adducts was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA), equipped with a HP 5972 mass selective detector, and HP 5-MS column (cross-linked 5% phenylmethyl silicone; dimensions: 30 m × 0.25 mm × 0.25 μm film thickness). The following temperature program was run: 1 min at 100 °C, 10 °C/min to 250 °C, and 3 °C/min to 300 °C. Sterols were identified based on their mass fragmentation pattern and by comparison to commercially available standards.

Free sterols were also analyzed by GLC-MS. Prior to the procedure mentioned above, 500 μg of protein of cell homogenates were extracted by the method of Folch et al. (33), and free sterols were separated from STE by thin layer chromatography (see above). Bands of free sterols were scraped off and extracted with chloroform/methanol (2:1; v/v) for 3 h. Then, the organic phase was collected and the remaining silica gel was re-extracted twice with chloroform/methanol (2:1; v/v) for 20 min, each. The organic phases were combined and taken to dryness. All procedures were carried out under light protection.

Measurement of TAG Synthase Activity—Yeast cells were grown on 500 ml of uracil-free minimal medium containing 2% glucose to the stationary growth phase. For induction of GAL1 promoter-controlled genes cells were harvested, washed twice, and inoculated into 500 ml of uracil-free minimal medium containing 2% galactose. Homogenates were prepared as described previously.

Northern Blotting—for the isolation of RNA from different yeast strains, 10 ml of 2% YPD medium were inoculated with the respective preculture (1:500) and grown for 18.5 h at 30 °C. Preparation of RNA was performed as described by Elion and Warner (35). For gel electrophoresis (36), 30 μg of RNA were loaded onto a 1.2% agarose gel. Then, the RNA was blotted by capillary transfer with 20 × SSC (3 M NaCl, 0.3 M sodium citrate) on a Bio-dyne B 0.45-μm membrane (Pall Gelman Laboratory, Ann Arbor, MI) for 23 h. For fixation of the RNA probes, the membrane was heated for 30 min at 120 °C. A random primed DNA fragment of ERG1 labeled with digoxigenin-dUDP, prepared by using the digoxigenin high prime DNA labeling and detection starter kit II (Roche Diagnostics), was used for hybridization at 42 °C overnight. Further steps were performed as described by the manufacturer.

Fluorescence Microscopy—Yeast cells were grown on uracil-free minimal medium containing 2% glucose to the stationary growth phase. For induction of GAL1 promoter-controlled genes, 2 ml of the preculture were harvested, cells were washed twice, and inoculated into uracil-free minimal medium containing 2% galactose. After various time periods, 100-μl aliquots were withdrawn, cells were centrifuged and stained with Nile Red (Sigma). Microscopic analysis was performed on a Zeiss Axiostar 35 microscope using a 100-fold oil immersion objective, a UV lamp, and a detection range between 450 and 490 nm. Images were taken with a CCD camera.

Computational Analysis—Molecular data about proteins were obtained from the Incyte Proteome BioKnowledge Library, the Saccharomyces Genome Data Base, and SwissProt.

RESULTS

Formation of Lipid Particles Can be Restored in a dga1lro1are1are2 Quadruple Mutant Preferentially by Expression of TAG-synthesizing Enzymes—Through the studies of Sandager et al. (11) it became evident that functionality of enzymes synthesizing TAG and STE is essential for the formation of lipid particles. This result although not surprising was obtained through experiments using a dga1lro1are1are2 quadruple mutant. In corresponding triple mutants bearing combinations of deletions of neutral lipid synthesizing enzymes lipid particles were still formed.

In the present study, we determined more precisely the efficiency of Dga1p, Lro1p, Are1p, and Are2p for lipid particle proliferation. For this purpose we constructed strains harboring inducible neutral lipid synthesizing enzymes in a dga1lro1are1are2 background (see Table I). Microscopic inspection using the fluorescent dye Nile Red revealed that after 15 min incubation under inducing conditions strains expressing Dga1p or Lro1p already harbored lipid particles (Fig. 1, B and C). During this time period of induction, the enzymatic activity of Dga1p increased from zero to 0.082 μg of diacylglycerol converted × mg−1 protein × h−1, and that of Lro1p from 0 to 0.023 μg of diacylglycerol converted × mg−1 protein × h−1. In contrast, the transforming expressing the major STE synthase Are2p (Fig. 1D) still lacked visible lipid particles after this time period similar to the control strain (Fig. 1A). It took 60 min of induction in the Are2p expressing transformant for lipid particles to become visible (Fig. 1F). Thus, we assumed that TAG formation led to a more efficient proliferation of lipid particles than STE biosynthesis.

In the dga1lro1are1are2 Quadruple Mutant Lipid Particle Proteins Are Retained in the Endoplasmic Reticulum—It has been hypothesized that lipid particles are derived from the endoplasmic reticulum and formed in a budding process that results in formation of a neutral lipid core associated with certain proteins (1). It was also assumed that proteins lacking transmembrane spanning domains were preferentially associated with the lipid particle surface compared with proteins containing transmembrane spanning domains (22). The dual localization of certain proteins in lipid particles and endoplasmic reticulum supported the view that these two compartments are closely related. The dga1lro1are1are2 quadruple mutant turned out to be a valuable tool to test this hypothesis in more detail. Western blot analysis using monospecific antibodies against the typical lipid particle proteins Ayr1p, Erg1p, Erg6p, and Erg7p was employed to localize these polypeptides in the lipid particle lacking quadruple mutant. For this purpose, high-purified yeast subcellular fractions of lipid particles, microsomes, vacuoles, plasma membrane, mitochondria, and cytosol from the wild type strain G175 and the respective fractions from the quadruple mutant H1246 were prepared and analyzed. Because the quadruple mutant lacked an obvious lipid particle floating layer in a Ficoll gradient, a zone corresponding to the wild type lipid particle fraction was analyzed. Fig. 2 clearly demonstrates that all proteins tested were, as expected, highly enriched in lipid particles of the wild type (lane 1), but completely absent from the corresponding fraction of the quadruple mutant (lane 3). In wild type, the four proteins tested behaved differently insofar as Erg7p was more or less...
FIG. 1. Formation of lipid particles. Fluorescence microscopic analysis of the quadruple mutant strains YDS10 (A, empty plasmid), YDS11 (B, expression of DGA1), YDS12 (C, expression of LRO1), and YDS13 (D, expression of ARE2). Western blot analysis of 30,000 × g microsomes from the quadruple mutant strains YDS10 (lane 1, empty plasmid), YDS11 (lane 2, expression of DGA1), YDS12 (lane 3, expression of LRO1), and YDS13 (lane 4, expression of ARE2) was performed using primary antibodies against Erg6p and Ayr1p as described under “Materials and Methods.”

FIG. 2. The lipid particle proteins Erg7p, Erg6p, and Ayr1p are enriched in microsomal fractions of the dga1lro1are1are2 quadruple mutant. Western blot analysis of lipid particles (1) and 30,000 × g microsomes (2) from wild type strain G175, and lipid particle fraction (3), 30,000 × g microsomes (4), and 40,000 × g microsomes (5) from the quadruple mutant H1246 was performed as described under “Materials and Methods.” Primary antibodies from rabbit were directed against Erg1p, Erg6p, Erg7p, and Ayr1p.

exclusively localized to lipid particles, Erg6p and Ayr1p were present at major amounts in lipid particles and only at a minor amount in microsomes, whereas Erg1p was almost evenly distributed between the two compartments (lanes 1 and 2). In the quadruple mutant, Erg7p, Erg6p, and Ayr1p were found to be enriched in microsomal fractions (lanes 4 and 5) compared with wild type (lane 2). The amount of Erg1p in microsomes of the quadruple mutant, however, was found to be surprisingly low. This decrease was not because of mislocalization of Erg1p to other compartments as demonstrated by Western blot analysis with other subcellular fractions (data not shown).

Induction of plasmid born Dga1p, Lro1p, or Are2p, respectively, in a quadruple mutant partially restored lipid particle formation and redirection of Ayr1p and Erg6p from microsomes to the newly formed lipid particles. Fig. 3 shows that the amounts of Erg6p and Ayr1p in microsomes decreased upon induction of DGA1, LRO1, and ARE2 expression in the quadruple mutant background. Concomitantly, Erg6p and Ayr1p appeared in lipid particle fractions of the respective transformants (data not shown). It has to be noted that induction of Are2p caused redirection of Erg6p to lipid particles less efficiently than expression of Dga1p and Lro1p (see Fig. 3). This result is in line with our view that Dga1p and Lro1p restore lipid particle proliferation more efficiently than Are2p.

The Amount of Erg1p Is Significantly Decreased in the dga1lro1are1are2 Quadruple Mutant—Results described above suggested that the cellular level of Erg1p was decreased in the dga1lro1are1are2 quadruple mutant. To test whether or not this effect was specific for Erg1p we compared the amounts of the four lipid particle proteins Erg1p, Erg6p, Erg7p, and Ayr1p in homogenates from wild type to the quadruple mutant strain. As can be seen from Fig. 4A, Erg1p is indeed the only polypeptide present at significantly lower concentration in the mutant than in wild type.

To distinguish between lack of TAG, STE, or complete absence of lipid particles as the reason for the low level of Erg1p, we analyzed cell homogenates from the respective multiple deletion mutants. Fig. 4B shows that only dga1lro1 and dga1lro1are1 mutants unveiled amounts of Erg1p similar to wild type. In contrast, the level of Erg1p was strongly reduced in strains are1are2, dga1lro1are2, dga1are1are2, and lro1are1are2. These data indicate that the amount of Erg1p is mainly regulated by the presence of active STE forming enzymes, especially Are2p. Decrease of the transcription rate of ERG1 and/or destabilization of Erg1p in the mutant strains were considered as possible mechanism(s) behind this regulation. Northern blot analysis, however, revealed no decrease in the transcriptional level of ERG1 in mutants compared with wild type (Fig. 5A). In contrast, the stability of Erg1p strongly decreased in strains lacking Are1p and Are2p after blocking cellular protein biosynthesis by addition of cycloheximide to growing yeast cultures (Fig. 5B). Whereas the level of Erg1p remained stable in the wild type (lane 1), the majority of this enzyme was degraded within 5 h of incubation in dga1lro1are1are2 (line 2) and are1are2 (line 2). Interestingly, this defect is more pronounced in the quadruple mutant than in the are1are2 strain.

The Supply of Ergosterol to the Plasma Membrane of the Quadruple Mutant Is Decreased—Whereas ergosterol of the yeast is stored in the form of fatty acyl esters in lipid particles, free ergosterol is incorporated into membranes, especially the plasma membrane. To test whether synthesis of STE and storage in lipid particles affect the supply of sterols to the plasma membrane through an esterification/hydrolysis mechanism, the plasma membranes of dga1lro1are1are2 and are1are2 mutants were analyzed (Fig. 6A). The amounts of ergosterol in the plasma membrane of the quadruple mutant H1246 was only 60%, and in an are1are2 double mutant 80% of the wild type level. We conclude that (i) formation/deacylation of STE contributes to the supply of sterols to the plasma membrane, but, moreover, (ii) the presence of TAG or lipid particles seems to have an additional effect on this process. GC/MS analysis demonstrated that in wild type, double mutant and quadruple mutant ergosterol was the only detectable sterol in the plasma membrane (data not shown). Thus, the reduced amount of ergosterol in the plasma membrane of the mutants was not compensated by incorporation of other sterols. The decrease of ergosterol in the plasma membrane, however, does not reflect
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**Fig. 4.** The cellular level of Erg1p is decreased in the absence of Are1p and Are2p. A, Western blot analysis of Erg1p, Erg6p, Erg7p, and Ayr1p was performed with homogenates from wild type G175 (1) and the dga1lro1are1are2 quadruple mutant (2). B, Western blot analysis of Erg1p with homogenates from the mutant strains are1are2 (1), dga1lro1 (2), dga1lro1are1 (3), dga1lro1are2 (4), dga1lro1are2 (5), and lro1are1are2 (6).

**Fig. 5.** Erg1p is unstable in the dga1lro1are1are2 quadruple mutant and in the are1are2 double mutant. Northern blot analysis (A) of ERG1 was carried out as described under “Materials and Methods” using samples from wild type G175 (1), the quadruple mutant dga1lro1are1are2 (2), and the double mutant are1are2 (3). Western blot analysis (B) of Erg1p was performed with homogenates from these strains grown for time periods indicated after addition of 5 μg/ml cycloheximide to cells grown to the late logarithmic phase.

The level of free ergosterol in total cell extracts. As can be seen from Fig. 6B, free ergosterol was even enriched in the quadruple mutant and in are1are2 compared with wild type. Thus, it was the disability of the mutant strains to form STE that caused the observed defect in the supply of sterols to the plasma membrane.

The dga1lro1are1are2 Quadruple Mutant Is Highly Sensitive to Terbinafine—Although it was shown that synthesis of storage lipids is not essential in yeast (11) it was tempting to speculate that the lack of TAG and/or STE might cause phenotypic alterations. One possibility for such alterations could be increased stress and/or drug sensitivity of the quadruple mutant, probably because of the fact that excess of fatty acids and/or sterols formed but not stored in lipid particles may become harmful for membranes. Alternatively, the lowered level of ergosterol in plasma membranes from strains lacking ARE1 and ARE2 might cause changes in the uptake of drugs into cells. To test these hypotheses, we performed growth tests in the presence of cerulenine (inhibitor of fatty acid biosynthesis), terbinafine (inhibitor of sterol biosynthesis), sorbitol (osmotic stress), cycloheximide (inhibitor of protein biosynthesis), hydrogen peroxide (oxidative stress), ethanol (non-fermentable carbon source), and at 37 °C (temperature stress). These experiments demonstrated that the dga1lro1are1are2 quadruple mutant was hypersensitive against terbinafine (Fig. 7), an inhibitor of the squalene epoxidase Erg1p, whereas all other reagents or conditions did not affect the growth behavior of the mutant compared with wild type (data not shown). Growth of the mutant was completely inhibited at a concentration of 5 μg of terbinafine/ml of medium (see Fig. 7), whereas growth of wild type was only slightly reduced at a concentration of 30 μg of terbinafine/ml (data not shown). This finding is in line with our observation that the amount of squalene epoxidase Erg1p was dramatically down-regulated in the quadruple mutant. The question, however, remained whether terbinafine sensitivity was because of the total block of STE synthesis, the decreased level of Erg1p in the quadruple mutant, or the lack of TAG and/or lipid particles in the quadruple mutant. Slightly increased sensitivity of the are1are2 double mutant against terbinafine has been reported before by Zweytick et al. (4), although only at a drug concentration of 30 μg/ml. Thus, it appears that hypersensitivity of dga1lro1are1are2 against terbinafine results from a combination of the low amount of Erg1p present in this mutant and the loss of TAG and/or lipid particles. To clarify this point, we performed terbinafine sensitivity assays with various multiple deletion strains. Whereas the double mutants are1are2 and dga1lro1, and the triple mutants dga1lro1are2 and lro1are1are2 grew similar to wild type in the presence of 5 μg of terbinafine/ml, the triple mutants dga1lro1are1 and dga1lro1are2 unveiled the same phenotype as the quadruple mutant (Fig. 7). This finding was confirmed by growth tests using yeast strains with inducible lipid particle proliferation. Expression of DGA1 or LRO1, respectively, which led to rapid formation of lipid particles (see Fig. 1), resulted in the loss of terbinafine sensitivity (see Fig. 7). In contrast, expression of ARE2 did not overcome this sensitivity within the time range tested, which is in line with our observation that lipid particle formation was only weakly induced by plasmid driven expression of Are2p (see Fig. 1). We conclude from these experiments that a combination of two defects is responsible for hypersensitivity against terbinafine in the yeast S. cerevisiae,
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G175 (wild type)  
H1246 (dga1lro1are2)  
H1112 (are1)  
H1226 (are2)  
H1237 (dga1lro1are2)  
H1126 (dga1lro1)  
H1262 (lro1are2)  
H1239 (lro1are2)  
YDS10 (empty plasmid)  
YDS11 (expression of DGA1)  
YDS12 (expression of LRO1)  
YDS13 (expression of ARE2)

**Fig. 7.** The dga1lro1are2 quadruple mutant is hypersensitive to terbinafine. Cell suspensions of strains listed in the figure were spotted at dilutions (1, 1/10, and 1/100) on YPD containing 5 µg/ml terbinafine. Incubation was carried out at 30 °C.

Lipid particles are storage compartments for TAG and STE in all eukaryotic cells (1) including the yeast *S. cerevisiae* (28). Studies from our laboratory, however, showed that lipid particles also contain several enzymes embedded in the surface phospholipid monolayer, which are involved in lipid metabolism (10, 19, 20, 22, 23). Only one of these enzymes, namely Dga1p (10), contributes to neutral lipid formation in lipid particles. In contrast, Lro1p, Are1p, and Are2p are microsomal enzymes (10, 19, 20, 22, 23). Only one of these enzymes, namely Dga1p (10), contributes to neutral lipid formation in lipid particles. In contrast, Lro1p, Are1p, and Are2p are microsomal enzymes. The subcellular distribution of these enzymes raises the question as to the interplay of these two compartments during lipid particle biogenesis. The availability of the dga1lro1are2 quadruple mutant (11) allowed us to obtain more insight into the metabolic network of neutral lipid formation, the biogenesis of lipid particles, and their physiological relevance in yeast.

Because three enzymes of sterol biosynthesis, namely Erg1p, Erg6p, and Erg7p, are located to lipid particles, it was tempting to speculate that lack of lipid particles might affect sterol homeostasis. In this study, we demonstrate that these three enzymes were retained to the endoplasmic reticulum, and in addition the level of Erg1p was dramatically decreased in the dga1lro1are1are2 quadruple mutant (see Figs. 2 and 4). Because the cellular level of Erg1p was also decreased in a mutant lacking Are2p, the major STE synthase in yeast, we assumed that block of STE synthesis was the primary reason for this effect. Mechanisms relevant for the decrease of the Erg1p level in the mutants may be (i) a decreased rate of transcription; (ii) inhibition or negative regulation of translation; or (iii) destabilization of the polypeptide. Enrichment of free sterols (see Fig. 6B) in are1are2 and the quadruple mutant may account for the former two effects and explain the finding that *ERG1* expression is positively regulated by reduced amounts of intracellular ergosterol (37). Whereas translation of *ERG1* may still be negatively affected in the mutant strains, our results clearly demonstrated that the transcription rate of *ERG1* was not changed. Most importantly, however, we showed that lack of STE resulted in destabilization of Erg1p (see Fig. 5). The cell biological and molecular reason for this observation is not yet clear. The idea that association of Erg1p with the surface of lipid particles may have a stabilizing effect on the polypeptide may only be partially correct, because the level of Erg1p is also low in are1are2 strains that still form lipid particles although with TAG as the only neutral lipid component. The additional protection of Erg1p by lipid particles as a structural element or by TAG as a lipid particle component may be deduced from the lability of the polypeptide in the dga1lro1are1are2 quadruple mutant (see Fig. 5).

The low level of Erg1p in strains lacking ARE2 resulted in hypersensitivity of the quadruple mutant against terbinafine, a specific inhibitor of Erg1p (see Fig. 7). However, growth of dga1lro1are2, lro1are2, and are2 are mutant strains, which also contain enhanced amounts of free ergosterol and reduced levels of Erg1p (see Fig. 4), was only marginally affected by the inhibitor. Contrary, the triple mutant dga1lro1are1 did not grow on terbinafine while showing the wild type level of Erg1p. Thus, it is very likely that the amount of Erg1p present in strains is not the only reason for the observed drug sensitivity. Because induction of lipid particle proliferation by expression of DGA1 or LRO1 resulted in insensitivity to terbinafine, the absence of TAG and/or lipid particles appears to have an additional effect. Thus, a combination of TAG and STE deficiency, which has so far not been described, might cause terbinafine hypersensitivity of the respective mutants.

Leber et al. (20) demonstrated that Erg1p is dually located to the endoplasmic reticulum and lipid particles. In contrast to other lipid particle proteins, enzymatic activity of Erg1p in *vitro* was only detectable with isolated endoplasmic reticulum fractions. It was argued that a reductase present only in the endoplasmic reticulum may be the missing cofactor in lipid particles, and interaction of the endoplasmic reticulum with lipid particles may lead to activation of Erg1p in the latter compartment. Erg1p is known to exhibit only low specific activity and is therefore a rate-limiting enzyme in ergosterol biosynthesis (38). Thus, the deposition of enzymatically inactive Erg1p in lipid particles may provide an additional possibility of regulation of total cellular enzyme activity at the organelle level. Investigations with higher eukaryotes unveiled an involvement of cytosolic protein factors in squalene epoxidase regulation (39). Although there is no proof for a similar regulatory mechanism in yeast, involvement of lipid particles and/or lipid particle proteins in squalene epoxidase regulation appears to be very likely.

Incorporation of ergosterol into the plasma membrane of the quadruple mutant was decreased to ~60% of the wild type level (see Fig. 6A) and not compensated by increased levels of sterol precursors. Surprisingly, this dramatic change of the plasma membrane lipid composition did not alter osmotic stability and general sensitivity to drugs in the quadruple mutant. The discrepancy between ergosterol levels in total dga1lro1are1are2 cells and in the plasma membrane of this mutant (see Fig. 6A) gave new rise to the speculation that lipid particles may participate in the transport of ergosterol to the plasma membrane. This finding is in line with previous studies (40) postulating that lipid particles play an important role in the distribution of ergosterol in the yeast cell. Thus, lipid particles may not only be involved in ergosterol metabolism and serve as storage for STE, but may also act as intermediate transport vehicles for sterols and thus play a regulatory role in sterol/sterol ester homeostasis in yeast. Interestingly, the amount of ergosterol in the plasma membrane of an are1are2 double mutant was not as dramatically reduced as in the quadruple mutant. This result indicates that lipid particles may have an additional effect on the supply of ergosterol to the plasma membrane besides their ability to store STE.

How are lipid particles formed in yeast? All lipid particle proteins identified so far are part of lipid-biosynthetic pathways, which are also present in the endoplasmic reticulum.
Typical lipid particle proteins lack transmembrane domains (22). This observation led to the speculation that these polypeptides may have left the bilayer environment of the endoplasmic reticulum together with a nascent core of neutral lipids during the process of lipid particle biogenesis. Thus, the endoplasmic reticulum is assumed to be the origin of lipid particles. This view was confirmed in this study by showing that Erg7p, Erg9p, Ayr1p, and also Erg1p are retained in microsomal fractions of a yeast strain lacking lipid particles (see Fig. 2). During lipid particle biogenesis small neutral lipid droplets formed between the two leaflets of the endoplasmic reticulum may be enwrapped by the cytosolic leaflet of the endoplasmic reticulum membrane. Proteins lacking transmembrane domains may be preferentially incorporated into these nascent droplets that bud off when reaching a certain size. Experiments with plants demonstrating that nascent oil bodies were found between the two leaflets of the endoplasmic reticulum (10), whereas Lro1p was absent from the former line with the observation that Dga1p in contrast to Lro1p is mainly involved in TAG storage. Thus, the process of lipid particle biogenesis. Thus, the endoplasmic reticulum (10), whereas Lro1p was absent from the former line with the observation that Dga1p in contrast to Lro1p is mainly involved in TAG storage. Thus, the process of lipid particle biogenesis. 

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