Good Fat, Essential Cellular Requirements for Triacylglycerol Synthesis to Maintain Membrane Homeostasis in Yeast*

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Storage triacylglycerols (TAG) and membrane phospholipids share common precursors, i.e. phosphatidic acid and diacylglycerol, in the endoplasmic reticulum. In addition to providing a biophysically rather inert storage pool for fatty acids, TAG synthesis plays an important role to buffer excess fatty acids (FA). The inability to incorporate exogenous oleic acid into TAG in a yeast mutant lacking the acyltransferases Lro1p, Dga1p, Are1p, and Are2p contributing to TAG synthesis results in dysregulation of lipid synthesis, massive proliferation of intracellular membranes, and ultimately cell death. Carboxypeptidase Y trafficking from the endoplasmic reticulum to the vacuole is severely impaired, but the unfolded protein response is only moderately up-regulated, and dispensable for membrane proliferation, upon exposure to oleic acid. FA-induced toxicity is specific to oleic acid and much less pronounced with palmitoleic acid and is not detectable with the saturated fatty acids, palmitic and stearic acid. Palmitic acid supplementation partially suppresses oleic acid-induced lipotoxicity and restores carboxypeptidase Y trafficking to the vacuole. These data show the following: (i) FA uptake is not regulated by the cellular lipid requirements; (ii) TAG synthesis functions as a crucial intracellular buffer for detoxifying excess unsaturated fatty acids; (iii) membrane lipid synthesis and proliferation are responsive to and controlled by a balanced fatty acid composition.

In the aqueous cellular environment, fatty acyl chains esterified in glycerophospholipids constitute the hydrophobic barrier of biological membranes. Thus, fatty acid (FA) composition is a crucial determinant of cellular membrane function. Establishment of the specific FA profiles in lipid species of various organelle membranes (1) relies on an intricate balance between endogenous FA synthesis, recycling of FA from lipid turnover and intracellular flux, e.g. during secretion or endocytosis and cellular growth, which must be accounted for by mechanisms that establish and maintain lipid homeostasis in these dynamic membrane systems (4). We have recently shown that TAG degradation provides metabolites that are critical for efficient cell cycle progression at the G1/S transition (3). Net supply with FA in growing cells, however, is procured by endogenous synthesis (5); nevertheless, yeast also has a high capacity for FA uptake (6), which may become essential in the absence of endogenous synthesis, e.g. in fatty-acid synthase mutants or in the presence of the fatty-acid synthase inhibitor cerulenin. The broad substrate specificity of up to six acyl-CoA synthetases may explain the ability of yeast to take up various “non-natural” FA (6), which may be present in their environment. FA derived from exogenous sources, endogenous de novo synthesis, or from lipid turnover react, as coenzyme derivatives, with glycerol 3-phosphate to form PA, which is the central precursor both for membrane phospholipids and for storage TAG. Esterification of the phosphate residue gives rise to cellular phospholipids, whereas PA dephosphorylation and one additional acylation step yield TAG, which are stored in lipid droplets. The mechanisms that direct and regulate the flux of activated FA either into membrane lipid or storage lipid (i.e. TAG) synthesis are unknown. However, upon FA overload, cells produce excess TAG, which results in proliferation and accumulation of lipid droplets.

In mammals, adipocytes are the preferred sites of excess TAG storage, whereas cells of nonadipose tissues have a rather limited capacity for neutral fat deposition; if this storage capacity is exceeded, FA and lipid overload may lead to abnormal cell function and ultimately cell death, also referred to as lipotoxicity (7). Thus, TAG synthesis appears to play an important role in buffering excess FA to prevent their incorporation into metabolically critical molecules, such as diacylglycerols (8) and sphingolipids (7, 9, 10), which may serve essential signaling and structural functions. Ultimately, after prolonged exposure to lipotoxic FA, cells may undergo apoptosis or necrosis (8–11).

In this study we have established and further explored a yeast model system for FA-induced lipotoxicity to address the molec-
Fatty Acid-induced Lipotoxicity in Yeast

ular mechanisms that control FA fluxes and to understand the physiological consequences of a lipotoxic insult. This yeast strain lacks all four acyltransferases involved in TAG synthesis, namely the diacylglycerol acyltransferase ortholog Dga1p (12), lecithin-cholesterol acyltransferase-related open reading frame Lro1p (lecithin-cholesterol acyltransferase ortholog (13)), and both acyl-CoA:cholesterol O-acyltransferase-related enzymes, Are1p and Are2p (acyl-CoA:cholesterol O-acyltransferase orthologs (14, 15)). Quite astonishingly, this mutant is viable despite the complete absence of TAG and sterol esters and, accordingly, also lacking any of lipid droplets, which are the storage compartment for both types of neutral lipids (16, 17). However, the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant YJP1078 becomes highly sensitive to the presence of unsaturated FA, such as oleic acid and palmitoleic acid. These fatty acids, which are preferentially incorporated into TAG in wild-type cells, promote cell death in a time- and concentration-dependent manner, which becomes apparent only after 3–6 h of incubation with the lipotoxic FA. Short term incubation, however, leads to massive alterations of cellular phospholipid profiles and the accumulation of intracellular membranes; the delivery of carboxypeptidase Y to the vacuole is blocked; however, the unfolded protein response (UPR) is only moderately up-regulated during membrane proliferation upon unsaturated FA challenge. Notably, UPR is not required for the proliferation of membranes, as a pentuple mutant additionally deleted for the UPR sensor kinase, Ire1p, displays a similar time course of membrane proliferation upon exposure to oleic acid. These data demonstrate the following: (i) FA uptake is not limited by the cellular requirements; (ii) in the absence of TAG synthesis excess FA are channeled into membrane phospholipid synthesis; (iii) UPR is not involved in excess membrane proliferation in mutants defective in TAG synthesis; and (iv) FA-induced cell death is not an immediate response to oleic acid exposure, but rather the consequence of induced defects of cellular membrane trafficking.

MATERIALS AND METHODS

Media and Culture Conditions—Standard YPD media contained 1% yeast extract (Difco), 2% glucose (Merck), 2% Bactopeptone (Difco); YPD minimal media with 2% glucose, 2% galactose (Sigma), or 2% raffinose (Acros Organics) as the carbon sources contained 0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate, supplemented with the respective amino acids and bases (adjusted to pH 6). Yeast transformants carrying expression plasmids were grown in uracil-free minimal medium. Sporulation medium contained 0.25% yeast extract, 1% potassium acetate, and 0.1% glucose. Ampicillin-resistant Escherichia coli transformants were selected in LBA media (1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 100 μg/ml ampicillin (Amresco)).

Geneticin resistance was determined on YPD plates containing 200 mg/liter geneticin (G418, Calbiochem). Expression of GFP fusions under control of a CUP1 promoter was induced by the addition of 0.5 mM CuSO4 to the medium. Expression of GFP fusions under the control of the MET25 promoter was induced in the absence of methionine in the media. Expression of GFP fusions or glutathione S-transferase fusions under the control of the GAL1/10 promoter was induced in media containing 2% galactose instead of glucose.

For fatty acid treatment, palmitic acid, palmitoleic acid, stearic acid, and oleic acid (Sigma) were dissolved in pre-warmed Brij58 (Sigma) and added to the culture media or agar plates at the indicated concentrations (final concentration of Brij58 was 1%). For liquid cultures, cells were grown overnight, shifted to fresh medium, grown to log phase, and exposed to the respective fatty acids for the indicated periods of time. Control media contained 1% Brij58, which, however, did not affect growth. For growth tests, cells were cultivated overnight in liquid media, adjusted to A600 = 0.5, and serially diluted in 1:10 steps, and 2 μl of the respective dilutions were spotted onto media plates containing 2% agar.

Yeast Strains and Plasmids—Yeast strains used in this study were wild-type BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), are1Δ mutant (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yor048wΔ::KanMX4), are2Δ mutant (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ynr019wΔ::KanMX), are1Δ are2Δ double mutant, YJP1076 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ycr048wΔ::KanMX4 ynr019wΔ::KanMX), dga1Δ lro1Δ double mutant, YJP1075 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yor245cΔ::KanMX4 ynr008wΔ::KanMX4), an are1Δ are2Δ dga1Δ lro1Δ quadruple deletion strain, YJP1078 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ycr048wΔ::KanMX4 ynr019wΔ::KanMX4 yor245cΔ::KanMX4 ynr008wΔ::KanMX4), and an are1Δ are2Δ dga1Δ lro1Δ are2Δ pentuple mutant (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ycr048wΔ::KanMX4 yor019wΔ::KanMX4 yor245cΔ::KanMX4 ynr008wΔ::KanMX4 yhr079cΔ::URA3). Deletion strains were obtained from Euroscarf. Double mutants and the quadruple mutant were constructed by standard genetic crosses and tetrad dissection; double mutations (i.e. dga1Δ lro1Δ and are1Δ are2Δ) were typically identified in nonparental di-type tetrads (based on geneticin resistance) and verified by colony PCR, using gene deletion-specific primers. These double mutants were used for further crosses to select triple and quadruple mutants, which were identified by colony PCR.

The construction of the ire1Δ are1Δ are2Δ dga1Δ lro1Δ pentuple mutant was performed as follows. The IRE1 open reading frame was replaced by the URA3 gene by homologous recombination in the are1Δ are2Δ dga1Δ lro1Δ background using the primers 5′-CATTTAAAAAAAACAGCATATCTGAAGAAATT-TAATTTTAGACTTTGAAAAAGCTTTTCAATTTCAATTTCAT-CATC-3′ and 5′-TAACATATTGCAATATAATTCCACACACAGGAAGACGGAGGCGATACAGGAGGTAATAC-CTGATATA-3′. URA3 was amplified by PCR, using the plasmid pCGCU as the template. PCR products were transformed into the are1Δ are2Δ dga1Δ lro1Δ background, and positive transformants were selected on SC plates lacking uracil. Pentuple mutants were verified by colony PCR, using gene deletion-specific primers.

E. coli TOP10F′ (proAB, lacIq, lacZDMD15, Tn10(Tet')}, mcrAΔ(mrr-hsdRMS-mcrB), ϕ80lacZM15, ΔlacX74, deoR, recA1, araD139Δ ara, leu), 7697galL, galK, ρ-lps(strptomycin'), endA1, nupG) was used for plasmid amplification and
purification. Transformation of yeast and E. coli cells was done following standard procedures (18).

**Cloning Procedures**—For localization studies, reading frame ELO3 was amplified by PCR and cloned into the pUG35 vector (19), harboring a MET25 promoter and the GFP reading frame 3′ to the multiple cloning site. Yeast wild-type BY4742 genomic DNA was used as the template, and primers 5′-ACGTAGTCTAGATGGAACACTACATCTCTAGTTATAGCA-3′ and 5′-ACGTAGTCGACAGCTTCCCTGGAAGAGACCTTGG-3′ were used for ELO3 amplification. The fragments were cleaved with XbaI and SalI and inserted into the multiple cloning site of pUG35, to yield a C-terminal GFP fusion. Plasmid pCGAU (GFP-skl) expressing the peroxisomal marker under control of the CLIP1 promoter and harboring a LIR3 selection marker was kindly provided by Dr. Uros Petrovic, Ljubljana, Slovenia.

**ARE1, ARE2, LRO1, and DGA1** were cloned into pCGCU and pCGGU plasmids, containing monomeric GFP, a LIR3 selection marker, and the CLIP1 or GAL10 promoter, respectively. Primers used were as follows: 5′-ACGTAGATTCATGACGAGACTCTAGATTTGTTGAGGACGCCTATTACATC-3′ and 5′-ACGTAGCATGCTAAAGGTAGTCAACAAGCTATATAAGGA-3′ for ARE1 amplification; 5′-ACGTAGATTCATGGAACAGGAAAGAGTTCTACTTGGAGGCA-3′ and 5′-ACGTAGCATGCTAGAACTCATTACATTCAGGACACCATCTGACTGACTG-3′ for ARE2 amplification; 5′-ACGTAGATTCATGGAACAGGAAAGAGTTCTACTTGGAGGCA-3′ and 5′-ACGTAGCATGCTAGAACTCATTACATTCAGGACACCATCTGACTGACTG-3′ for LRO1 amplification; and 5′-ACGTAGATTCATGACGAGACTCTAGATTTGTTGAGGACGCCTATTACATC-3′ and 5′-ACGTAGCATGCTAAAGGTAGTCAACAAGCTATATAAGGAGA-3′ for DGA1 amplification. Fragments were cleaved with EcoRI and SphiI and ligated into the multiple cloning site of pCGCU or pCGGU.

**LRO1 and DGA1** were also cloned into pYES263 (Euroscarf), harboring a GAL1/10 promoter, glutathione S-transferase coding sequence, and the LIR3 marker. Primers used were as follows: 5′-ACGTAGATTCATGACGAGACTCTAGATTTGTTGAGGACGCCTATTACATC-3′ and 5′-ACGTAGCATGCTAAAGGTAGTCAACAAGCTATATAAGGAGA-3′ for ARE1 amplification, and 5′-ACGTAGATTCATGACGAGACTCTAGATTTGTTGAGGACGCCTATTACATC-3′ and 5′-ACGTAGCATGCTAAAGGTAGTCAACAAGCTATATAAGGAGA-3′ for ARE2 amplification, and 5′-ACGTAGATTCATGACGAGACTCTAGATTTGTTGAGGACGCCTATTACATC-3′ and 5′-ACGTAGCATGCTAAAGGTAGTCAACAAGCTATATAAGGAGA-3′ for DGA1 amplification. Fragments were cleaved with BamHI and NotI and inserted into the multiple cloning site of pYES263.

**β-Galactosidase Assay**—Induction of the UPR was measured using a reporter construct with a CYC1 minimal promoter containing 4× UPR1 fused to the lacZ gene (pCJ104 plasmid, kindly provided by Stephen Jesch, Ithaca, NY). Cells were grown overnight in YND medium lacking uracil, transferred to fresh medium to an 0.0001 to 0.1% for 4–8 h. Aliquots were withdrawn and the cell number determined using CASY1® technology (Schärfe System GmbH). 300 cells were plated onto YPD plates and incubated for 2 days at 30 °C. Strains expressing Dga1-GFP and Lro1-GFP constructs were grown overnight in the presence of 0.5 mM CuSO4 in minimal medium lacking uracil, shifted to fresh medium for 4 h, and viable cell counts analyzed as described above.

**Carboxypeptidase Y Processing**—Cells were grown overnight, shifted to fresh medium, and incubated for 4 h. Fatty acids were added at a concentration of 0.1%; cells were harvested at indicated time points, and proteins were precipitated with 5% trichloroacetic acid (final concentration) for 10 min on ice. Pellets were spun down at 12,000 rpm for 5 min, resuspended in 1× SDS loading buffer, and incubated for 5 min at 80 °C. Proteins were separated on 8% SDS-polyacrylamide gels and electroblotted to nitrocellulose membranes (Bio-Rad); after blocking with 5% dry milk powder in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 8.0), blots were probed with anti-CPY antibody (Rockland Inc.).

**Lipid Analysis**—For total lipid analyses, yeast cells were homogenized with glass beads in a Merckenschlager homogenizer (B. Braun Biotech International) under CO2 cooling, and lipids were extracted with chloroform/methanol, 2:1 (v/v) (21, 22). Neutral lipid separation and analysis was performed by TLC on silica gel plates (Merck), essentially as described (23–25), using light petroleum/diethyl ether/acetic acid (32:8:0.4, per volume) as the solvent. Lipids were visualized on TLC plates by carbonization after dipping plates into 3.2% H2SO4 and 0.5% MnCl2, followed by heating at 120 °C for 30 min. Lipids were quantified by densitometric scanning at 450 nm (Camag TLC scanner 3), using triolein as the standard (24, 25). Whole cell lipid extracts were converted to fatty acid methyl esters using 14% boron trifluoride/methanol (26). Gas chromatography/mass spectrometry analysis was performed on a Trace-DC Ultra-DSQ-MS system (ThermoElectron, Waltham, MA).

**Electron Microscopy**—Cells were prepared for transmission electron microscopy essentially as described (27). Yeast cells were fixed with 1.5% KMnO4 in distilled water for 15 min at room temperature, dehydrated in a graded series of ethanol (50–100%), embedded in Epon resin, and specimens polymerized for 48 h at 60 °C. Ultrathin sections were stained with 2% lead citrate for 5 min and 2% uranyl acetate for 15 min. Ultrathin sections were observed with a Philips CM 10 transmission electron microscope.

**Fluorescence Microscopy**—Vital staining was typically performed with 1 ml of cell suspension. Fluorescence dyes, Nile Red, MitoTracker® Red CM-H2XROS (Invitrogen), and the yeast vacuole membrane marker, FM4–64 (Invitrogen), were added from stock solutions in DMSO at a final concentration of 1 μg/ml. Fluorescence microscopy was performed after 10–20 min of incubation with the vital dye, without subsequent washing of cells. Nile Red fluorescence was excited at 543 nm and emission detected simultaneously between 550 and 570 nm for lipid droplets, and between 600 and 650 nm for lipid droplets plus intracellular membranes, as described previously (28). MitoTracker® Red CM-H2XROS was excited at 543 nm and emission detected between 580 and 650 nm. The yeast vacuole membrane marker FM4–64 was excited at 488 nm and emission detected between 580 and 660 nm. SytoxGreen®TM (Invitrogen)
was used as a viability stain, and fluorescence was excited at 488 nm and emission detected between 500 and 550 nm; the same settings were used for GFP detection. Microscopy was performed on Leica TCS4d and Leica SP2 confocal microscopes, the latter equipped with acousto optical beam splitter and spectral detection, using 100× oil immersion (NA 1.4) and 40× oil immersions objectives (NA 1.25). Transmission images were acquired using differential interference contrast optics. Images were adjusted for contrast and color and assembled using Adobe Photoshop™ CS (Adobe Inc.).

RESULTS

Mutant Lacking Neutral Lipids Displays Delayed Growth and Morphological Defects

The are1Δ are2Δ dga1Δ lro1Δ quadruple mutant lacks all four enzymes responsible for the synthesis of TAG and steryl esters (SE) (16, 29). Accordingly, as shown by staining with the lipophilic dye Nile Red (Fig. 1A), this mutant is devoid of lipid droplets (LD). Notably, in this mutant, the Nile Red fluorescence emission spectrum appears different from that in the wild type (28); fluorescence of the endomembrane system between 600 and 650 nm is low in wild-type cells, and in marked contrast, the quadruple mutant displays intense membrane fluorescence in this emission range, consistent with a changed hydrophobicity of the environment of dye (30). The fluorescence characteristics of Nile Red make it particularly suited to differentiate the more hydrophobic lipid droplets (emission detection between 550 and 570 nm) and the less hydrophobic ER membrane (emission detection between 600 and 650 nm) in the mutants (Fig. 1A) (28); however, it also requires defined spectral detection to avoid mis-interpretation of membrane-associated fluorescence signals as “lipid droplets.” To assess in greater detail the impact of lacking TAG and SE synthesis on cellular organelle and membrane morphology, we analyzed mitochondria, vacuoles, endoplasmic reticulum, and peroxisomes, by fluorescence microscopy, using vital dyes and organelle-specific GFP fusion constructs. The ER marker Elo3-GFP (31, 32) was expressed in wild type and the YJP1078 quadruple mutant. No gross alterations of ER morphology were observed between the wild-type and the mutant at the level of light microscopy (supplemental Fig. 1). Mitochondria stained with MitoTracker™ also displayed no significant morphological alterations in the mutant, consistent with normal respiratory function that was also assessed by testing growth on nonfermentable carbon sources (data not shown). Interestingly, and despite recent observations that per-
oxisomes and lipid droplets closely interact (33), peroxisome formation in the quadruple mutant upon exhaustion of glucose appeared very similar to wild type as well. Peroxisome number and overall shape, as determined by microscopic inspection of the peroxisomal marker GFP-skl (34, 35), was indistinguishable from wild type (supplemental Fig. 1A). As a major morphological phenotype, we observed a highly fragmented vacuole in the mutant (supplemental Fig. 1A, panel i; similar fragmentation phenotypes suggestive of impaired membrane fusion are frequently observed in mutants defective in lipid metabolism, such as fatty acid elongation mutants (31) or phospholipase C mutants (36).

Next, we tested the cellular TAG and SE requirements for growth. Growth of the YJP1078 mutant strain was severely impaired in comparison with the wild type during the first 6–8 h of cultivation (Fig. 1B), similarly to the lipase-deficient tgl3Δ tgl4Δ mutants, which are unable to degrade TAG (2, 3). This observation is consistent with specific requirements for TAG, presumably to provide lipid precursors for membrane proliferation, during the initial phase of growth (2, 3, 37, 38). In stationary phase, mutant cells reached about 80–90% of the wild-type cell density. The dga1Δ lro1Δ double mutant displayed a less severe growth defect, as it still has residual capacity to synthesize TAG; in contrast, the are1Δ are2Δ double mutant grew like wild type (not shown), demonstrating that not the formation of SE but rather TAG is limiting for growth of the mutants.

The contribution of the individual acyltransferases to cellular growth and lipid droplet formation was assessed by expressing ARE1, ARE2, DGA1, and LRO1 individually in the quadruple mutant (39). As shown in supplemental Fig. 1, B and C, expression of the DGA1 gene encoding the acyl-CoA-dependent DAG acyltransferase restored growth and induced LD formation to a level comparable with wild type. Expression of the LRO1 gene (phospholipid-dependent DAG acyltransferase), on the other hand, was less effective, and the acyl-CoA:cholesterol O-acyltransferase-related sterol acyltransferase Are2p restored growth and LD formation only to a minor extent. ARE1 expression was not sufficient to drive LD formation, although it was previously shown to display minor TAG synthesizing capacity (16, 29). The capacity to synthesize TAG and LD, based on microscopic analysis, parallels the growth characteristics of the quadruple mutant transformed with the individual acyltransferases (supplemental Fig. 1, B and C). These data underscore the cellular requirements for TAG for rapid initiation of growth and demonstrate the specific requirement for TAG rather than sterol ester synthesis, for lipid droplet formation (39).

Lack of TAG Synthesis Renders Mutant Cells Highly Sensitive to Unsaturated Fatty Acids—The metabolic regulators that control the flux of FA into membrane or storage lipids are currently unknown. Because TAG are a preferred storage form for unsaturated FA, we next tested the response of mutants lacking enzymes involved in TAG and SE synthesis to treatment with various FA. In a first approach, saturated (palmitic acid, C16:0, and stearic acid, C18:0) and unsaturated FA (palmitoleic acid, C16:1, and oleic acid, C18:1) were applied over a wide range of concentrations (0.0001–1%) to wild-type and single, double, and quadruple mutants, lacking diacylglycerol and sterol acyltransferases; these FA represent the most abundant species in yeast (40). As shown in Fig. 2A, C16:0 and C18:0 saturated FA do not affect growth of either wild-type or of any of the mutant strains. Addition of unsaturated FA, on the other hand, strongly inhibited growth of the dga1Δ lro1Δ double mutant and the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant. Growth of the quadruple mutant was completely abolished at a concentration of 0.01% oleic acid, whereas the dga1Δ lro1Δ double mutant was more tolerant and required 0.1% oleic acid for complete growth inhibition. Similarly, supplementation with palmitoleic acid also severely inhibited growth of the double and quadruple mutants, albeit at a 2-fold higher concentration than oleic acid. This unsaturated FA-induced toxicity was indeed due to the lack of TAG synthesis because ectopic expression of DAG acyltransferases in the mutants fully (Dga1p) or partially (Lro1p) reversed the growth deficiencies on oleic and palmitoleic acid-containing media (Fig. 2A); expression of the sterol acyltransferases, Are1p or Are2p, in the quadruple mutant did not restore growth in the presence of unsaturated FA (data not shown). These data demonstrate that TAG synthesis, catalyzed by DAG acyltransferases Dga1p and Lro1p, is indispensable for excess unsaturated FA detoxification. Notably, palmitic acid supplementation partially restored growth of the quadruple mutant in the presence of oleic acid (see below).

Specificity and time dependence of FA toxicity was also assessed by survival analyses, which showed a clear time and concentration dependence, and oleate concentrations as low as 0.0005% (=15 μM, which corresponds to the critical micellar concentration) already significantly impaired viability (supplemental Fig. 2, A and B); incubation with 0.005% palmitoleate or oleate for 3 h rendered more than 70 or 90%, respectively, of mutant cells nonviable. By 12 h of incubation, less than 10% of mutant cells survived palmitoleate treatment, and virtually none of the cells survived oleate treatment (supplemental Fig. 2, C and D). However, in time course experiments we noticed that short term incubation up to 30 min with potentially toxic concentrations did not result in a marked change in survival rates, demonstrating that yeast cells apparently have a significant but obviously limited capacity to buffer excess FA, and that FA toxicity is not an acute response to the inability to synthesize TAG. In addition to plating assays, we also assessed viability in liquid culture by incubating cells with 0.001% of the respective FA, as indicated, for up to 12 h prior to SytoxGreenTM staining and fluorescence microscopy (Fig. 2B). Whereas wild type was fully resistant to oleic acid treatment, a significant fraction of cells of the quadruple mutant became SytoxGreenTM-positive after palmitoleic or oleic acid supplementation, indicating loss of viability (41). Unsaturated fatty acid toxicity was significantly suppressed by ectopic expression of DGA1 and LRO1 genes and also by additional supplementation with palmitic acid.

Taken together, these data show the following: (i) uptake of FA is not regulated by intracellular fatty acid requirements in yeast; (ii) saturated FA supplementation is not toxic, even in the absence of TAG synthesis; (iii) TAG synthesis is the critical determinant of unsaturated FA detoxification.

Phospholipid Profiles Are Altered in the Quadruple Mutant—We next analyzed TAG and phospholipid content and composition of wild-type and mutant cells, with and without FA treatment (Tables 1 and 2). Cells were grown to early log phase, and
FA was added at a concentration of 0.001% for 30 min and up to 12 h. In the quadruple mutant, as expected, TAG were below the detection limit throughout the duration of the experiment, whereas TAG levels increased by 20–25% in the wild type supplemented with palmitate or oleate and doubled as cells reached stationary phase. The total phospholipid to ergosterol ratio remained largely unchanged in wild-type cells under untreated as well as FA supplemented conditions. In marked contrast, the quadruple mutant displayed 20% elevated phospholipid to ergosterol ratio already in the absence of FA, which increased even further in the presence of palmitate by 1.5-fold and, more pronounced, oleic acid (3-fold) after 12 h of fatty acid incubation. It should be noted, however, that the quadruple mutant also lacks steryl ester synthesis, which increases free ergosterol levels by 20% (29). Notably, the relative changes in FA profiles in phospholipids both in wild type and the quadruple mutant clearly indicated substantial modification of incorporated palmitic acid by elongation and desaturation, maintaining a more balanced ratio of C16 to C18 FA, and degree of desaturation in the membrane lipids. In wild type, oleic acid is preferentially incorporated into TAG, whereas the oleic acid content in its phospholipids is only moderately elevated (from 25 to 32%, after 30 min of incubation). In striking contrast, in the quadruple mutant treated with oleic acid, the ratio of saturated to unsaturated FA in the phospholipids dropped by 25–30%, and the ratio of C16 to C18 FA decreased by 2–4-fold, after 30 min and 12 h of incubation, respectively, compared with wild type. Mutants harbored about 70% oleate and only about 8% palmitoleate in their phospholipids, whereas wild type contained equal amounts of these unsaturated FA in its phospholipids, after incubation with 0.001% oleic acid for 12 h (Table 2). Thus, TAG synthesis provides an essential regulating valve to dispose
TABLE 1
Triacylglycerol and phospholipid content and composition of wild type and the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant in the presence or absence of FA supplementation

| TG/ERG ratio | Wild type | are1Δ are2Δ dga1Δ lro1Δ |
|--------------|-----------|-------------------------|
|          |            |                          |
| 0.5 h + Palmitate + Oleate | 1.20 | 1.20 |
| 12 h          | 1.20 | 1.20 |
| PL/ERG ratio  | Wild type | are1Δ are2Δ dga1Δ lro1Δ |
|              |          |                          |
| 0.5 h + Palmitate + Oleate | 1.20 | 1.20 |
| 12 h          | 1.20 | 1.20 |

of excess unsaturated FA. Because palmitic acid supplementation is not toxic to the mutants defective in TAG synthesis and does not significantly change the ratios of saturated versus unsaturated and 16 versus 18 carbon atoms containing acyl chains, we suggest that oleic acid-mediated toxicity is because of massively altered acyl chain distribution in membrane phospholipids. In support of this notion is the observation that co-supplementation with oleic acid and palmitic acid restored FA distribution in phospholipids to levels more closely resembling wild-type distribution. In particular, ratios of saturated to unsaturated FA are wild-type-like under these conditions (Table 2).

ER Membranes Proliferate upon Unsaturated Fatty Acid Supplementation—The observed severe changes in phospholipid acyl chain distribution suggested a major impact on membrane morphology and function. Indeed, in YJP1078 quadruple mutant cells treated with unsaturated FA, highly light-diffracting structures appeared in transmission microscopy (Fig. 3A). Fluorescence microscopy, using the lipophilic dye Nile Red and recording in the 600–650 nm “membrane detection channel” (see also Fig. 1A), showed highly aberrant structures that formed in the presence of unsaturated FA but not in the presence of palmitate. These structures extended from the endoplasmic reticulum and co-localized with the fluorescent ER marker protein Elo3-GFP (Fig. 3B). Thus, in the absence of TAG synthesis, excess unsaturated FA appear to be channeled into phospholipids and induce major membrane proliferations of the ER. This view is supported by transmission electron microscopy, which unveils a remarkable proliferation of highly ordered, parallel sheets of membranes extending from the ER in mutants treated with unsaturated fatty acid (Fig. 3C). These membrane proliferations are much more pronounced in the presence of oleate compared with palmitoleate; palmitate had no apparent effect on ER morphology; however, mitochondrial morphology in the mutant appeared somewhat dilated on palmitate treatment, which is currently under investigation. Wild-type cells showed massive lipid droplets in the vicinity of ER membranes, as expected, upon treatment with saturated or unsaturated FA (Fig. 3C).

Induction of Membrane Proliferations Is a Very Rapid Process and Generates ER Stress—The potential correlation between ER membrane proliferations and FA-induced loss of viability of the quadruple mutant prompted us to compare the time courses for both events. SytoxGreen™ staining of the quadruple mutant and control mutant cells transformed with the DGA1-expressing plasmid were performed in parallel upon supplementation with 0.001% oleic acid. Rearrangement of the ER membrane, as indicated by Nile Red staining, became evident within 15–30 min after addition of oleic acid (Fig. 4A), consistent with a rapid incorporation of the FA into membrane lipids. In contrast, significant loss of viability of the mutant was detectable only after about 2–3 h of treatment, and viability decreased steadily thereafter. Thus, the appearance of membrane proliferations did not correlate with loss of viability, which suggests that the mutant cells are able to tolerate substantial membrane rearrangements and ER proliferations that need to exceed a certain threshold level for the induction of cell death. The massive ER proliferation also suggested an impact on the UPR, which is a major signaling pathway involved in ER quality control (42, 43). UPR in wild-type and the quadruple mutant was determined using a UPR-lacZ reporter construct, which expresses β-galactosidase under control of the UPR promoter element (44). Not unexpectedly, the quadruple mutant showed about 2-fold higher basal levels of UPR-controlled reporter gene expression, compared with wild type (Fig. 4B). UPR was not affected in wild-type cells treated with oleic acid, whereas the quadruple mutant showed up to 9-fold increased UPR-dependent β-galactosidase expression after 12 h of oleate supplementation. As a positive control, UPR induction in wild-type cells by tunicamycin occurred 60–150-fold compared with untreated cells (data not shown and see Refs. 43, 44) and demonstrates that UPR induction in mutant cells challenged with oleic acid represents a rather attenuated and delayed response.

We next investigated whether UPR was required for the induction of membrane proliferations by deleting the Ire1 gene in the quadruple mutant. Ire1p is the major membrane kinase in the nuclear membrane involved in transmitting the unfolded protein signal to the level of a transcriptional response to activate UPR target genes (42). UPR is tightly linked to the lipid metabolic pathways (45), and changes in FA concentration of 0.001%. Lipids were extracted at the indicated time points. The label percentage was normalized to ergosterol. Lipid concentration of 0.001%.

FIGURE 2. are1Δ are2Δ dga1Δ lro1Δ quadruple mutant is highly sensitive to exogenously supplied oleic and palmitoleic acid. A, growth tests of yeast strains on agar plates containing different FA. Wild-type, are1Δ are2Δ dga1Δ lro1Δ, are1Δ are2Δ dga1Δ lro1Δ, DGA1[are1Δ are2Δ dga1Δ lro1Δ] (quadruple mutant transformed with a plasmid expressing DGA1 wild-type gene), and LRO1[are1Δ are2Δ dga1Δ lro1Δ] (quadruple mutant transformed with a plasmid expressing the LRO1 gene) were grown to stationary phase and diluted to A600nm = 0.5, and serial dilutions of 1:10 were spotted onto agar plates containing different FA dissolved in 1% Brij58. Palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1) control is in the presence of 1% Brij58 only, w/o, without FA supplementation. B, determination of cell vitality of yeast strains on FA addition using SytoxGreen™ staining. Wild-type (top panel), are1Δ are2Δ dga1Δ lro1Δ [1st middle panel], and DGA1[are1Δ are2Δ dga1Δ lro1Δ] and LRO1[are1Δ are2Δ dga1Δ lro1Δ] (2nd middle panel) were grown overnight, shifted to fresh YPD medium, and grown for 4 h. Cells were incubated in the presence of indicated FA (0.001%) for 12 h prior to SytoxGreen™ staining. FA toxicity is most pronounced for oleate > palmitoleate and not detectable on supplementation with saturated FA. Dga1p and, to a lesser extent, Lro1p expression in the quadruple mutant rescues unsaturated FA toxicity. Addition of palmitic acid (bottom panel) restored viability to the quadruple mutant, in the presence of oleic acid. Scale bar, 10 μm; DIC, differential interference contrast.
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### TABLE 2
Triacylglycerol and phospholipid content and composition of wild type and the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant in the absence or presence of FA supplementation

Cells were grown to early log phase, and palmitate or oleate was added at a sub-lethal concentration of 0.001%. Lipids were extracted at the indicated time points, and FA was analyzed by gas chromatography/mass spectrometry after conversion to FA methyl esters. Mean values are of two independent measurements; S.E. was typically below ± 5%. Sat means saturated FA and unsat means unsaturated FA.

| Strain          | Time | Fatty acid | Triacylglycerols | Phospholipids | Sat/unsat | 16:0 | 16:1 | 18:0 | 18:1 |
|-----------------|------|------------|------------------|---------------|-----------|------|------|------|------|
|                 |      |            | %                | %             |           |      |      |      |      |
| Wild type       | 0.5  | Palmitate  | 30.6             | 35.5          | 10.3      | 22.4 | 21.2 | 39.8 | 10.0 | 25.2 | 0.48 | 1.73 |
|                 | 0.5  | Oleate     | 36.2             | 31.0          | 11.4      | 21.0 | 23.3 | 40.2 | 8.1  | 26.0 | 0.47 | 1.86 |
|                 | 0.5  | Palmitate  | 28.3             | 25.5          | 5.3       | 40.3 | 20.4 | 37.2 | 7.6  | 32.6 | 0.40 | 1.43 |
|                 | 12   | Oleate     | 28.2             | 35.8          | 5.6       | 26.3 | 15.6 | 43.5 | 11.2 | 28.0 | 0.47 | 1.52 |
|                 | 12   | Palmitate  | 37.5             | 31.6          | 8.6       | 20.3 | 21.6 | 41.3 | 7.2  | 28.4 | 0.41 | 1.78 |
|                 | 12   | Oleate     | 25.4             | 19.5          | 5.0       | 48.0 | 16.7 | 36.0 | 6.9  | 37.6 | 0.32 | 1.18 |
| are1Δ are2Δ dga1Δ lro1Δ | 0.5  | Palmitate  | 17.4             | 43.5          | 8.1       | 28.2 | 17.4 | 43.5 | 8.1  | 28.2 | 0.36 | 1.68 |
|                 | 0.5  | Oleate     | 19.3             | 47.4          | 7.7       | 25.2 | 19.3 | 47.4 | 7.7  | 25.2 | 0.37 | 2.03 |
|                 | 0.5  | Palmitate  | 13.5             | 23.0          | 7.9       | 51.8 | 13.5 | 23.0 | 7.9  | 51.8 | 0.29 | 0.61 |
|                 | 12   | Oleate     | 16.5             | 40.3          | 9.1       | 32.7 | 16.5 | 40.3 | 9.1  | 32.7 | 0.35 | 1.36 |
|                 | 12   | Palmitate  | 22.6             | 43.5          | 12.2      | 21.4 | 22.6 | 43.5 | 12.2 | 21.4 | 0.54 | 1.97 |
|                 | 12   | Oleate     | 12.0             | 9.7           | 6.4       | 69.5 | 12.0 | 9.7  | 6.4  | 69.5 | 0.23 | 0.29 |
| are1Δ are2Δ dga1Δ lro1Δ | 12   | Palmitate + oleate | 18.1           | 27.5          | 8.2       | 46.3 | 18.1 | 27.5 | 8.2  | 46.3 | 0.35 | 0.84 |

Position in the ER may lead to ER stress and subsequent UPR induction and cell death (46). Interestingly, this pentuple mutant lacking Ire1p in addition to the four acyltransferases required for TAG synthesis is viable in the absence of unsaturated FA, or if supplemented with palmitic acid, and did not display any increased sensitivity to oleic acid, compared with the quadruple mutant lacking the acyltransferases only (Fig. 4C). On oleic acid supplementation, membrane proliferations appeared after 30–60 min also in the pentuple mutant lacking Ire1p (Fig. 4, D and E). Notably, the morphology of these membrane proliferations resembles tubuloreticular structures (Fig. 4E) rather than membrane sheets, as observed in mutants with functional Ire1p (Fig. 3C). These tubuloreticular membranes are highly reminiscent of ERAC structures, an ER-associated compartment that is induced on overexpression of several membrane proteins (73). UPR is dispensable for ERAC induction, consistent with the observation that these membrane proliferations appear quite prominent in oleic acid-challenged pentuple mutants lacking Ire1p (Fig. 4E).

CPY Trafficking Is Defective in the Quadruple Mutant upon Addition of Oleic Acid—CPY is a post-translationally modified glycoprotein, which allows monitoring of protein trafficking—CPY processing after addition of saturated as well as unsaturated FA. As a control, a sec13-1 mutant was analyzed, which is defective in a component of the COPII complex involved in ER to Golgi secretory vesicle formation. After a shift to the restrictive temperature of 37 °C, Sec13p is rapidly inactivated, and a prominent 67-kDa ER form of CPY accumulates already 20 min after the shift (Fig. 5). In the absence of exogenous FA or on supplementation with palmitic acid, CPY processing in the YIP1078 quadruple mutant was like wild type, and it led to the complete conversion to the vacuolar 61-kDa form of the protein. In contrast, growth in the presence of oleic acid resulted in a block in CPY processing from the 67-kDa ER to the 69-kDa Golgi form within 20 min, comparable with the block in the sec13-1 mutant after shift to the restrictive temperature, demonstrating that the ER exit is severely impaired in the mutant under these conditions. Most notably, CPY processing was reverted to wild-type levels if both oleic acid and palmitic acid were supplemented to the quadruple mutant. Also, the formation of membrane proliferations was reverted if both FA were present, demonstrating the critical requirement of a balanced FA composition for maintaining membrane homeostasis, in the absence of TAG synthesis.

**DISCUSSION**

What limits the amount of membrane in a cell? Establishing and maintaining a balanced FA composition during cellular growth are of utmost importance for biological membrane structure and function (48). The molecular mechanisms controlling composition of the molecular species of individual subcellular membranes (1), however, are largely unknown. In recent years, a number of acyltransferases have been identified, which catalyze the incorporation of activated FA that are derived either from de novo synthesis or lipid turnover (49, 50). In addition, acyl transfer may also be governed by direct exchange from a phospholipid to diacylglycerol (13), adding to the complexity of cellular lipid remodeling and membrane lipid homeostasis. Membrane-forming glycerophospholipids and TAG, which primarily serve as biophysically rather inert FA storage compounds, are synthesized from common precursors, phosphatidic acid and diacylglycerol, in the endoplasmic reticulum and are diverted either way dependent on the physiological requirements. Recent evidence suggests a cell cycle-regulated switch that controls the flux of FA into and out of TAG (3), underscoring an important buffering function for TAG synthesis to provide membrane lipid precursors, presumably FA, in a timely coordinated manner.

Yeast cells typically do not feed on FA; thus, the net supply for membrane and storage lipid synthesis in growing cells mostly relies on endogenous FA synthesis (5), which in wild-type cells is well controlled by several levels of regulation (5, 51). In particular, the level of unsaturated FA is exquisitely controlled by a
regulatory circuit that involves the membrane-bound transcription factors Mga2p and Spt23p that are processed dependent on the membrane environment, and regulate expression of the only yeast desaturase OLE1 (52, 53). In the absence of endogenous synthesis, i.e. in mutants defective in FA synthase or in Ole1p desaturase-deficient cells, cellular growth depends on the presence of exogenous fatty acids, which are readily taken up by the cell and incorporated into all cellular lipids (5). Indeed, yeast has evolved efficient mechanisms to take up FA from the environment, a process that appears to be directly coupled to the activation step, which provides the substrates for acyltransferases or β-oxidation enzymes (6). In a recent study, Lockshon et al. (41) identified numerous yeast mutants that are highly sensitive to unsaturated FA treatment. Initially designed to identify novel factors involved in peroxisome biogenesis, it became evident from that study that unsaturated FA are actually growth-inhibitory to some mutants. It was concluded that lack of peroxisomal β-oxidation of excess unsaturated FA might have a negative impact on membrane fluidity, in particular of the plasma membrane (41). FA degradation, which in yeast takes place exclusively in peroxisomes, may thus be required for establishing a balanced FA composition of subcellular membranes. This view is also supported by a recent study, which uncovered a close association of peroxisomes with lipid...
The inability of mutant cells lacking TAG synthesis to sustain unsaturated FA supplementation implies a number of conclusions. First, uptake of (unsaturated) FA in yeast is not regulated by the cellular requirements and is largely dependent on the availability of FA in the culture medium (61). The rapid appearance of oleic acid in phospholipids of the mutant demonstrates the absence of an efficient feedback mechanism to regulate lipid acylation in the presence of excess unsaturated FA. Indeed, this is a very artificial situation because of the different mechanism of unsaturated FA-induced toxicity, however, must exist in the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant lacking TAG synthesis and lipid droplets altogether; oleic acid was toxic in the presence of glucose as the carbon source, which efficiently suppresses peroxisome induction (54). Furthermore, repression of peroxisome formation upon exhaustion of glucose was not affected in the mutant, which suggests that peroxisome biogenesis is independent of TAG synthesis and lipid droplet formation.

Because TAG and glycerophospholipids share similar precursors in the endoplasmic reticulum, namely PA and DAG, it appears likely that an excess of FA interferes with balanced lipid formation. Indeed, DAG and PA levels are increased in the quadruple mutant lacking TAG synthesis,4 which leads to a defective transcriptional regulation of phospholipid synthesis (55). The distribution of the DAG backbone, and thus of cellular FA toward phospholipids or TAG, is mainly controlled by the activity of Mg2+-dependent phosphatidate phosphatase Pah1p (56–58), which is the yeast ortholog of human Lipin (59), and mutants lacking active Pah1p enzyme are characterized by a nuclear membrane expansion phenotype and reduced levels of TAG (57, 58, 60). However, pah1Δ-deficient mutants are not sensitive to (unsaturated) FA at concentrations employed in this study because of sufficient residual cellular phosphatidate phosphatase activity and a limited capacity to synthesize TAG.

Oleic acid becomes toxic to pah1Δ mutants at 10 times higher concentrations (0.1%),4 further corroborating the requirement for TAG synthesis as a means to detoxify excess unsaturated FA.

4 J. Petschnigg and S. D. Kohlwein, unpublished observations.

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FIGURE 5. ER-to-Golgi trafficking is blocked in the quadruple mutant on addition of oleic acid. Cells were grown overnight, shifted to fresh medium, and incubated for 4 h. FA were added at concentrations of 0.001%, and cells were harvested at indicated time points. Proteins were extracted and separated by 8% SDS-PAGE. Carboxypeptidase Y processing was monitored using a CPY-specific antibody (Rockland Inc.). proCPY, 67-kDa ER form; mCPY, mature 61-kDa vacuolar form; w/o, without FA supplementation. Anti-Tcm (ribosomal protein) was used as a loading control.

FIGURE 4. Proliferation of membranes in the are1Δ are2Δ dga1Δ lro1Δ quadruple mutant is a fast response to oleic acid addition and independent of the unfolded protein response and does not directly correlate with a loss of viability. A, quadruple mutant cells and DGA1 [are1Δ are2Δ dga1Δ lro1Δ] cells were grown overnight, transferred to fresh medium for 4 h, and treated with 0.001% oleic acid for the indicated time points. Staining with Nile Red and detection in the 600–650 nm fluorescence emission range demonstrate that appearance of membrane proliferations precedes loss of viability (indicated by SytoxGreen staining) by about 30–60 min. Scale bar, 5 μm. DIC, differential interference contrast. B, wild-type and mutant cells harboring a URE3-lacZ construct were cultivated overnight and grown for 4 h in fresh SD media prior to 0.001% oleic acid addition. At indicated time points, aliquots were withdrawn and subjected to β-galactosidase assay. Activity (fold increase; black bars in the presence of oleate) is given relative to untreated wild-type cells (white bars) at time point 0. Assays were performed in triplicate. C, growth tests of yeast strains on agar plates containing different FA. Wild-type, are1Δ are2Δ dga1Δ lro1Δ quadruple mutants, and lro1Δ cells were grown to stationary phase and diluted to 0.5, and serial dilutions of 1:10 were spotted onto agar plates containing oleic acid dissolved in 1% Brij58 at indicated concentrations. Additional deletion of the IRE1Δ gene in the quadruple mutant background does not affect fatty acid sensitivity of the strain. D, membrane proliferations also occur in the are1Δ are2Δ dga1Δ lro1Δ Δpentuple mutant treated with oleic acid. The pentuple mutant was grown for 4 h prior to treatment with 0.001% oleic acid. Nile Red staining and fluorescence detection in the 600–650 nm range shows massive membrane proliferations in the mutant on oleic acid supplementation, within 30–60 min. Scale bar, 5 μm. E, electron micrographs of the pentuple mutant treated with oleic acid. Cells were grown in minimal medium for 4 h prior to oleic acid addition (0.001%) for 3 h. Induction of membrane proliferations (arrowheads) in the are1Δ are2Δ dga1Δ lro1Δ pentuple mutant on oleic acid is very rapid and independent of Ire1p. Note the altered morphology of these membrane proliferations, compared with the quadruple mutant (Fig. 3C), which show striking similarity to ERAC structures (73). V, vacuole; EN, endoplasmic reticulum; N, nucleus; w/o, without FA supplementation. Scale bar, 1 μm.
yeast, Ole1p (52, 53, 62); accordingly, overexpression of OLE1 under control of the strong GAL1/10 promoter neither stimulated desaturation nor did it enhance cell death of the quadruple mutant (data not shown), consistent with the notion of multiple levels of control of desaturase activity in yeast (53). Second, saturated FA supplementation to mutants unable to synthesize TAG had no apparent effect on viability, in yeast. Although such FA are also taken up by the cell and incorporated into lipids (5, 6), their uptake rate may be limited by their solubility (critical micellar concentration for palmitic acid is about 10-fold lower, compared with oleic acid). Also, and most importantly, saturated FA are subject to elongation and/or desaturation (5, 51), resulting in a FA distribution more closely resembling the “natural” spectrum typically present in yeast membrane lipids. In the fission yeast, Schizosaccharomyces pombe, TAG synthesis is essential for long term survival and to sustain saturated FA treatment (8). Caspase-dependent (apoptotic) and independent (necrotic) lipotoxic pathways exist, triggered by enhanced synthesis of DAG, a potent signaling molecule, and may involve mitochondria, production of ROS, and cell-death regulators such as Pca1p (11). Chinese hamster ovary cells drastically expand their ER on palmitate supplementation, which activates the unfolded protein response (42, 63) and eventually induces apoptosis (64, 65). In fibroblasts, the cytotoxic effect of palmitic acid is presumably because of its role as a precursor for ceramides (7, 9, 10) rather than through a mechanism that involves TAG synthesis (66), and it can be suppressed by overexpression of stearoyl-CoA desaturase, SCD1. Indeed, palmitic acid also becomes toxic in yeast mutants that are compromised in desaturase activity (46). The cumulative effects of altered chain length distribution (C16:C18 ratio) and the degree of unsaturation are likely to have profound effects on membrane structure and function, in particular of the endoplasmic reticulum. A general effect on membrane fluidity as a cause of death (41) by an excess of oleic acid containing lipids of the quadruple mutant (up to 70% after 12 h of incubation) appears unlikely, however, because maintaining cells at lower temperatures of 16 or 25 °C, which is expected to require higher levels of unsaturated FA, did not improve oleate tolerance. In line with a crucial balance between saturated and unsaturated FA in membrane lipids is the observation that oleic acid-induced toxicity can be partially suppressed by the addition of palmitic acid. A simple competition mechanism for uptake and activation is unlikely; this observation rather suggests that the activity of acyltransferases discriminates and limits incorporation of activated FA into lipids, and that this activity may be dependent on the lipid environment.

Third, in the absence of TAG synthesis, excess unsaturated FA are channeled into phospholipid synthesis, which induces membrane proliferations and a block of secretion. This is a very rapid process, taking place within a few minutes after challenging mutant cells with FA. Loss of viability of the mutants and downstream execution of either apoptotic or necrotic cell death pathways lack significantly behind and argue against an instant toxic insult by unsaturated FA. ER stress response and lipid metabolism are tightly linked; for example, one of the most highly regulated genes involved in lipid synthesis, INO1, is a target of the UPR-triggered transcription factor, Hac1p (42, 67–69). Opi1p-mediated repression of UASINO-regulated phospholipid biosynthetic genes is suspended upon Hac1p expression, which stimulates phospholipid synthesis (45, 68).

It came as a surprise to discover that the unfolded protein response was only moderately up-regulated upon oleic acid-induced membrane proliferation in the absence of TAG synthesis, and that membranes continued to proliferate in the absence of the central UPR signaling kinase, Ire1p. This observation of UPR-independent membrane growth is not unprecedented, as shown for the induction of karmellae (70) or similar membrane proliferations (71, 72). However, the morphology of the membrane proliferations changed in the absence of Ire1p, giving rise to tubuloreticular structures, rather than parallel sheets of membrane, strikingly resembling ERACs (73), which are implicated in ER quality control. ERAC formation was also shown to be independent of UPR (73). It remains to be determined whether these structures represent a subcompartment of the ER that is characterized by a particular lipid composition or rather functions to sequester specific ER proteins.

What makes unsaturated fatty acids toxic in the absence of TAG synthesis? Lack of membrane proliferations in wild-type cells exposed to oleic acid suggests a specific feedback mechanism on phospholipid acylation that is dependent on the flux of fatty acid into TAG. A block in secretion is expected to be detrimental, however, and whether specific lipid molecular species enriched in unsaturated FA are responsible for this defect remains to be uncovered.

In summary, our results highlight the importance of TAG synthesis in Saccharomyces cerevisiae to protect cells from unsaturated FA-induced lipotoxicity. In the absence of TAG formation, excess FA are channeled into phospholipids, causing massive membrane proliferations but only moderate induction of the unfolded protein response, which is dispensable for this process altogether. Triacylglycerol synthesis is thus a crucial determinant and regulator of cellular fatty acid and membrane homeostasis. Garbarino et al. found similar results (74).

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