Coordination of Storage Lipid Synthesis and Membrane Biogenesis

EVIDENCE FOR CROSS-TALK BETWEEN TRIACYLGLYCEROL METABOLISM AND PHOSPHATIDYLINOSITOL SYNTHESIS*

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Despite the importance of triacylglycerols (TAG) and steryl esters (SE) in phospholipid synthesis in cells transitioning from stationary-phase into active growth, there is no direct evidence for their requirement in synthesis of phosphatidylinositol (PI) or other membrane phospholipids in logarithmically growing yeast cells. We report that the dga1Δ lro1Δ are1Δ are2Δ strain, which lacks the ability to synthesize both TAG and SE, is not able to sustain normal growth in the absence of inositol (Ino− phenotype) at 37 °C especially when choline is present. Unlike many other strains exhibiting an Ino− phenotype, the dga1Δ lro1Δ are1Δ are2Δ strain does not display a defect in INO1 expression. However, the mutant exhibits slow recovery of PI content compared with wild type cells upon reintroduction of inositol into logarithmically growing cultures. The tgl3Δ tgl4Δ tgl5Δ strain, which is able to synthesize TAG but unable to mobilize it, also exhibits attenuated PI formation under these conditions. However, unlike dga1Δ lro1Δ are1Δ are2Δ, the tgl3Δ tgl4Δ tgl5Δ strain does not display an Ino− phenotype, indicating that failure to mobilize TAG is not fully responsible for the growth defect of the dga1Δ lro1Δ are1Δ are2Δ strain in the absence of inositol. Moreover, synthesis of phospholipids, especially PI, is dramatically reduced in the dga1Δ lro1Δ are1Δ are2Δ strain even when it is grown continuously in the presence of inositol. The mutant also utilizes a greater proportion of newly synthesized PI than wild type for the synthesis of inositol-containing sphingolipids, especially in the absence of inositol. Thus, we conclude that storage lipid synthesis actively influences membrane phospholipid metabolism in logarithmically growing cells.

Eukaryotic organisms store excess energy as triacylglycerols (TAG)3 and steryl esters (SE) for later use during times of deprivation. TAG and SE are hydrophobic compounds separated from the aqueous cellular environment of the cytoplasm in specialized structures called lipid droplets (1). Lipid droplets are dynamic organelles that play important roles in the biosynthesis, mobilization, and trafficking of intracellular neutral lipids. They function in close apposition with other organelles, particularly the endoplasmic reticulum (ER), endosomes, mitochondria, and peroxisomes (2, 3).

In the budding yeast, Saccharomyces cerevisiae, the formation of lipid droplets is tightly linked to the synthesis of TAG and SE. The diacylglycerol acyltransferases encoded by the DGA1 and LRO1 genes (Fig. 1) are the main enzymes involved in the biosynthesis of TAG (4–6), whereas ARE1 and ARE2 (Fig. 1) encode the enzymes that primarily mediate the esterification of ergosterol and its precursors leading to SE (7, 8). These four enzymes account for all TAG and SE biosynthesis in yeast, which begins during exponential growth and reaches its peak as cells enter stationary phase (9). During times of energy scarcity or upon recovery from stationary phase when exposed to glucose, TAG degradation occurs via the activity of lipid hydrolases encoded by the TGL3, TGL4, and TGL5 genes (10–12). The products of TAG degradation, diacylglycerols (DAG) and free fatty acids, also serve as precursors for membrane lipid synthesis (13) as well as for energy production when free fatty acids are the only carbon source available in the growth medium (14). At the cellular level, TAG degradation is up-regulated by Cdc28p/Cdk1p-dependent phosphorylation of the Tgl4p lipase (12). Lipolysis contributes to bud formation, presumably by providing precursors for synthesis of lipids involved in membrane biogenesis or signaling (12). Conversely, impairment in membrane trafficking leads to a block in phospholipid synthesis and concomitant TAG accumulation (15).

However, a yeast quadruple mutant strain (dga1Δ lro1Δ are1Δ are2Δ) lacking the ability to store fatty acids in either TAG or SE is viable (4, 16). The sole growth phenotypes relative to wild type that have been reported for this strain are unsaturated fatty acid-induced toxicity and a prolonged lag phase after transfer to fresh YPD media (17, 18). No significant change in growth of this strain was observed during exponential or stationary phase (4, 18). These

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data suggest that the reserve of TAG and SE is not essential to sustain growth in yeast, at least when cells grow in rich media.

Certain mutants, defective in membrane trafficking (Sec13-1), were shown to exhibit increased TAG synthesis at the expense of phospholipid synthesis when shifted to a temperature that restricted membrane trafficking and cell growth (15). That study specifically showed that deleting the DAG acyltransferases, Dga1p and Lro1p, in the sec13-1 mutant, defective in membrane and protein transport from the ER, led to a lowering of the temperature at which the mutant could grow (i.e., its restrictive temperature). The lowering of the restrictive temperature in the sec13-1 dga1Δlro1Δ strain, was especially pronounced when it was grown in the absence of the phospholipid precursor, inositol. In wild type cells, lack of inositol supplementation results in a substantial reduction in the synthesis of phosphatidylinositol (PI) (19, 20).

The above-summarized evidence suggests that the cell coordinates the synthesis and breakdown of storage lipids with its demand for membrane lipid synthesis. Consistent with this idea, we showed in a previous study that the fatty acids required for the rapid burst of PI synthesis after inositol supplementation to cells deprived of inositol are derived in part from both de novo fatty acid synthesis and phosphatidylcholine (PC) turnover (19, 21). However, these two sources of fatty acids do not fully account for the burst in PI synthesis (19), suggesting that additional fatty acids might be derived from hydrolysis of TAG. In the current study we tested the ability of the cells to grow in the absence of inositol and to rapidly restore PI content in response to inositol reintroduction when they are unable to mobilize TAG. We report that upon inositol reintroduction, the dga1Δlro1Δare1Δare2Δ strain, unable to synthesize TAG or SE, and the tgl3Δtgl4Δtgl5Δ strain, lacking the TAG lipases, both exhibit slow recovery of PI content in comparison to wild type cells. However, only the dga1Δlro1Δare1Δare2Δ strain failed to grow in the absence of inositol. Thus, failure to hydrolyze TAG does not fully explain the lno− phenotype of the dga1Δlro1Δare1Δare2Δ strain. We present evidence that this mutant exhibits reduced synthesis of PI even when grown continuously in the presence of exogenous inositol. The mutant also devotes a larger percentage of newly synthesized PI to the synthesis of inositol-containing sphingolipids for which PI serves as a precursor.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The S. cerevisiae strains used in this study are listed in Table 1. All the strains were derived from the S288C genetic background. Cultures were maintained on 1% yeast extract, 2% peptone, 2% glucose, 2% agar media plates. All experiments were conducted using cultures grown to mid-logarithmic phase at 30 or 37 °C on a rotary shaker (New Brunswick Scientific Co., Inc.) at 200 rpm using chemically defined synthetic media as described by Ješch et al. (22). Cells were grown in 50-ml batches of complete synthetic media with (+) or without (−) inositol (75 μM) with (C+) or without (C−) choline (1 mM) as indicated. Solid media had the same composition plus 2% agar.

Analysis of lnos Pheno type—For the analysis of inositol auxotrophy phenotype (lno− phenotype) (23), yeast strains were grown overnight in 1+ medium at 30 °C. After 16 h, the cultures were diluted back to A600 = 0.15 in 50 ml of the same medium and allowed to grow to mid-logarithmic phase at 30 °C.

Cell Growth and Calculation of Doubling Time—To determine doubling times, cultures of wild type and dga1Δlro1Δare1Δare2Δ strains were grown overnight in 1− medium at 30 or 37 °C and in 1+ medium at 37 °C. After 16 h, cultures were diluted back to A600 = 0.15 in 50 ml of 1− medium at 30 or 37 °C and in 1+ medium at 37 °C and allowed to reach A600 = 0.5. At this cell density each culture was divided in half. One-half of each culture (25 ml) was filtered, washed with pre-warmed medium containing inositol, and resuspended in 25 ml of 1− medium at its original ini-
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cubation temperature of 30 or 37 °C or in 1°C medium at 37 °C. The other half was also filtered, washed with prewarmed medium lacking inositol, and resuspended in 1°C medium at its original incubation temperature of 30 or 37 °C or in 1°C medium at 37 °C. The samples were harvested for absorbance measurements at 1.5, 3, 4.5, and 6 h after the shift.

The A600 of the cultures was used to calculate the doubling time according to the formula log2(Td) = (t2 − t1) × log2(log(q2/q1)), in which Td is the doubling time, t2 is a time point, t1 is zero time or an earlier time point, q2 is the A600 at a given time point, q1 is the A600 at zero time. Generation times were calculated using the above formula for the intervals from 0–3 h and from 3 to 6 h after the media shift for all the growth conditions. The doubling time for yeast in complete synthetic medium at its original incubation temperature of 30 or 37 °C varied from 3 to 6 h after the media shift for all the growth conditions.

RNA Isolation and RT-PCR Analysis—Wild type and dga1Δlro1Δare1Δare2Δ strains were grown under the growth conditions identical to those described above for the calculation of the generation time. Total RNA was isolated using RNeasy® Mini kit including a DNA digestion with RNase-free DNase Set (both from Qiagen). 1 μg of RNA was transcribed into cDNA using oligo(dT) 12–18 primer (0.5 μg), PCR grade dNTP mix (0.5 μM), First Strand Buffer (1X), DTT (10 mM), and 100 units of SuperScript® III reverse transcriptase (Invitrogen). Real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems), and the following TaqMan® probes and primers: INO1, TaqMan® probe, 5’-6-carboxyfluorescein (Fam)-CTG TTG CCC ATG GTT AGC CCA AAC G-6-carboxytetramethylrhodamine (Tamra)-3’; forward primer (5’-9 GGA ATG ACG TTT ATG CTC CTT TTA A-3’) and reverse primer (5’-GTC CCA ACC AGA GAC GAC AAA-3’; OPI3, TaqMan® probe, 5’-Fam-AGC AGT CTG CAT TGC GAT AAC AGC CTA C-Tamra-3’; forward primer (5’-TCC CTA GGT ATC GTC AGA GAC ATG-3’) and reverse primer (5’-TCC CCC GTG ATC AGA GAA C-3’); ACTI, TaqMan® probe, 5’-Fam-TGC AAA CCG CTG CTC AAT CCT TCT CAA T-Tamra-3’; forward primer (5’-CGC CGT GGA TTA CGA ACA AG-3’) and reverse primer (5’-GAC CAT CTG GAA GTT CGT AGG ATT-Tamra-3’). ACTI gene served as an internal standard for normalization.

In brief, the reaction mix in a volume of 25 μl consisted of 0.5 μM primers, 0.2 μM TaqMan® probe, 1× Master Mix, and 5 ng of cDNA. All reactions were performed in technical duplicate. Non-template control (5 ng of RNA) and non-reaction control (diethylpyrocarbonate water) were routinely performed. The thermal program for the PCR included 95 °C for 10 min (Stage 1), 95 °C for 0.5 min and 60 °C for 1 min for a total of 40 cycles (Stage 2), and hold at 4 °C (Stage 3). Relative quantitation was done using the ΔΔCt method (see StepOnePlus™ user manual of Applied Biosystems). The ΔΔCt represents the change in mRNA expression after ACTI normalization relative to the wild type control calculated as 2−ΔΔCt = 2−(Gene Ct1 − ACTI Ct1) − (Gene Ct2 − ACTI Ct2), where “gene” represents the mRNA under study (INO1 or OPI3), x refers to the strain from which the mRNA tested was derived (i.e. wild type or quadruple mutant), and “cr” refers to the control mRNA. The value cr (for control mRNA) was derived from level of mRNA in the wild type strain, pre-grown as described above in 1°C medium at 30 or 37 °C or in 1°C medium at 37 °C but shifted to fresh medium of the same composition (i.e. containing inositol) at the same temperature for 1.5 h. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. to exceed background level). Each RT-PCR experiment was performed at least in triplicate.

Pulse Labeling of Phospholipids—To analyze de novo synthesis of phospholipids after the shift to fresh media, wild type and dga1Δlro1Δare1Δare2Δ cells were pre-grown to an A600 = 0.5 in 1°C medium at 37 °C, and the culture was divided in half as described above. One-half of the culture was filtered and washed in prewarmed 1°C medium and then resuspended in a fresh aliquot of 1°C medium, whereas the other was washed and filtered in prewarmed 1°C medium and then resuspended in fresh, prewarmed 1°C medium. After the shift to fresh medium with or without inositol, both cultures were incubated at 37 °C, and 5-ml samples were collected at 1.5 and 3 h and pulse-labeled for 20 min with 100 μCi/ml [32P]orthophosphate (specific activity of 32P in the medium was 13.51 mCi/mmol of phosphate). Labeled lipids were extracted as previously described (24). The individual phospholipid species were resolved by two-dimensional thin layer chromatography (25). Phospholipid identity was based on the mobility of known standards and quantified on a STORM 860 PhosphorImager (Amersham Biosciences).

Analysis of Sphingolipid Synthesis—Wild type and dga1Δlro1Δare1Δare2Δ mutant were grown and labeled under identical conditions described above for the phospholipid synthesis except that the cells were pulse-labeled for 30 min instead of 20 min to allow sufficient time for 32P label to enter through PI and be transferred to the inositol-containing sphingolipids. Growth of both strains was terminated by the addition of TCA to a final concentration of 5%. Cells were collected by centrifugation and washed once with water. Labeled lipids were extracted as previously described (24). The lipids were deacylated according to the method of Stock et al. (26). By this method, the dried lipid extracts were redissolved in 1 ml of chloroform:methanol:water (16:16:5, v/v/v). An equal volume of 0.2 N NaOH in methanol was added to each sample, and the mixture was incubated at 30 °C for 45 min. To each sample, 1.1 ml of 0.5% (w/v) EDTA was added, and the mixtures were neutralized by the addition of 0.2 ml of 1 N acetic acid. The nondeacylated lipids containing the complex sphingolipids were extracted with 0.5 ml of chloroform, dried under N2, and resuspended in 0.5 ml of chloroform:methanol:water (16:16:5, v/v/v). Labeled sphingolipids were separated by two-dimensional thin layer chromatography as described by Stock et al. (26). The amounts of the labeled sphingolipids on the chromatograms were quantified on a STORM 860 PhosphorImager.

Analysis of Total Lipid Composition Assessed by Labeling of Lipids of Logarithmically Growing Cultures to Steady State with [14C]Acetate— Cultures of wild type, dga1Δlro1Δare1Δare2Δ, and tgl3Δgdl1Δgls5Δ were grown in 1°C medium at 30 or 37 °C and in 1°C medium at 37 °C in
Moving inositol while adding a second phospholipid precursor, choline, to the growth medium and increasing the incubation temperature to 37 °C has detrimental consequences for cell growth and survival of mutants defective in certain aspects of lipid metabolism and stress response signaling (30).

Therefore, we tested the growth of the dga1Δbro1Δare1Δare2Δ mutant in four different media 1°C, 1°C, 1°C, and 1°C plates and allowed to grow at the designated temperatures for 3 days. Panel A, wild type and dga1Δbro1Δare1Δare2Δ strains grown at 30 °C. Panel B, wild type, dga1Δbro1Δare1Δare2Δ, and tgl3Δtag4Δtag5Δ strains grown at 37 °C. Figure 2. The dga1Δbro1Δare1Δare2Δ mutant exhibits inositol auxotrophy at 37 °C. Overnight cultures grown in 1°C or 1°C medium at 30 °C were diluted back to A660 = 0.15 in 50 ml of the same medium and allowed to grow to mid-logarithmic phase at 30 °C. Samples were harvested and resuspended at a concentration of 0.5 A660. Each sample was diluted in multi-well plates by 1:10 serial dilutions, and 10 μl of cells from each dilution were spotted on I°C , I°C , I°C , and I°C plates and allowed to grow at the designated temperatures for 3 days. Panel A, wild type, dga1Δbro1Δare1Δare2Δ, strains grown at 30 °C. Panel B, wild type, dga1Δbro1Δare1Δare2Δ, and tgl3Δtag4Δtag5Δ strains grown at 37 °C.

RESULTS

The Absence of TAG and SE Synthesis Leads to Growth Impairment in Medium Lacking Inositol at High Temperatures—We previously observed that deletion of the DGA1 and LRO1 genes in the sec13-1 genetic background resulted in a decrease in the permissive temperature at which the triple mutant would grow in the absence of inositol compared with the sec13-1 parent (15). This finding suggested that strains blocked in TAG synthesis might display additional phenotypes under conditions limiting phospholipid synthesis. We have also previously shown that the combined effects of re-

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FIGURE 3. The pattern of PI synthesis after reintroduction of inositol is reduced in mutant strains unable to mobilize or store TAG. Lipids in cultures of wild type, \(dga1\Delta-lo1\Delta-are1\Delta-are2\Delta\), and \(tgl3\Delta-tgl4\Delta-tgl5\Delta\) were labeled to steady state with \([1\text{C}^1\text{H}^1\text{C}]\text{acetate}\) \((1 \mu\text{Ci/ml})\) in \(1\text{C}^-\) medium at \(37^\circ\text{C}\) as described under “Experimental Procedures” for at least 7 generations and grown to mid-logarithmic phase and shifted to \(1\text{C}^-\) medium at \(37^\circ\text{C}\) for 180 min as described under “Experimental Procedures,” maintaining label at constant specific activity. At 180 min, inositol was reintroduced, and samples were taken at the time points depicted in the figure. Parallel cultures grown in the same fashion were treated with cerulenin at a final concentration of \(10 \mu\text{g/ml}\) at 90 min after the shift to medium lacking inositol. Cells were allowed to grow for additional 90 min, and inositol was reintroduced. Samples were collected at 0, 5, 15, and 30 min after inositol addition. Lipids were extracted and analyzed as described under “Experimental Procedures.”

Panel A, wild type. Panel B, \(dga1\Delta-lo1\Delta-are1\Delta-are2\Delta\). Panel C, \(tgl3\Delta-tgl4\Delta-tgl5\Δ\). Panel D, wild type + cerulenin. Panel E, \(dga1\Delta-lo1\Delta-are1\Delta-are2\Delta + \text{cerulenin}\). Panel F, \(tgl3\Delta-tgl4\Delta-tgl5\Δ + \text{cerulenin}\). The data represent the average of at least two independent experiments. Experimental error was less than 10% in all cases. Error bars are not shown for clarity of presentation. The lipids indicated are PI (solid squares) and PC (solid circles). ODU, optical density units.

turnover, and TAG lipolysis. We had previously observed that \(de novo\) fatty acid synthesis and turnover of PC contributed to the ability of wild type cells to rapidly restore PI content (19). To determine whether TAG metabolism plays a role in the burst of PI synthesis observed in the wild type strain after addition of inositol, we compared the changes in PI content in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) strain as well as the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Delta\) strain to those in the wild type strain. In the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Delta\) mutant, TAG synthesis is disabled altogether due to the lack of the respective acyltransferases that convert DAG to TAG, whereas in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) mutant, TAG lipolysis is blocked. Thus, fatty acids for PI synthesis in both mutants must come largely from PC turnover or \(de novo\) synthesis.

Because fatty acids generated from PC turnover are maximized in medium containing choline at \(37^\circ\text{C}\) \((34, 35)\), cells were grown under these conditions. To measure absolute phospholipid content, cells were pregrown and labeled with \([1\text{C}^1\text{H}^1\text{C}]\text{acetate}\) to steady state in medium containing both inositol and choline \(\text{(I}^-\text{C}^-\text{)}\) at \(37^\circ\text{C}\) and shifted to medium lacking inositol \(\text{(I}^-\text{C}^-\text{)}\) while maintaining the label constant as described under “Experimental Procedures.” After incubation for an additional 180 min, inositol was reintroduced, and samples were taken and analyzed for the rebound in PI content. The rebound in PI content under these conditions in the wild type strain was striking (Fig. 3A). In contrast, in the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) mutant, the rate of recovery of PI content after inositol supplementation was only about 30% that of the level in the wild type strain under the same conditions (Fig. 3B). PC content in the mutant strain, however, was slightly higher than in wild type and declined somewhat less sharply upon inositol reintroduction (Fig. 3B). Similarly, in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) mutant, the rate of recovery of PI content after inositol supplementation was very slow (compare Fig. 3, A and C). However, in this case, whereas PC content was somewhat lower than wild type before inositol re-addition, it declined in a fashion similar to that observed in wild type cells (Fig. 3, A and C). These data overall indicate that PC turnover is not markedly altered in \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) or \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) strains in comparison to wild type. However, a lack of fatty acids derived from TAG in both strains significantly attenuates the burst in PI synthesis.

To assess the contribution of \(de novo\) fatty acid synthesis to the burst of PI synthesis, lipids in all three strains were labeled to steady state with \([1\text{C}^1\text{H}^1\text{C}]\text{acetate}\) in \(1\text{C}^-\) medium at \(37^\circ\text{C}\) following the procedure described above. Cerulenin, an inhibitor of fatty acid synthesis (36), was added to the cultures at 90 min before inositol reintroduction. Wild type cells pretreated with cerulenin showed a much less dramatic rebound in PI content after inositol reintroduction than that observed in cells not treated with cerulenin (Fig. 3D), consistent with previously reported results (19). In the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) strain treated with cerulenin, re-synthesis of PI after inositol reintroduction was also attenuated (Fig. 3E), and the mutant also experienced a greater decline in PC content than wild type under these conditions (compare Fig. 3, D to F).

The results in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) cultures treated with cerulenin in the same fashion were strikingly different from either wild type or the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) cultures. The level of PI attained after inositol reintroduction in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) strain was lower than wild type or \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\), and PC metabolism was also dramatically altered in comparison to wild type or \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) strains (Fig. 3F). During the 90 min of treatment with cerulenin before the introduction of inositol, PC content in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) mutant dropped by 50% as compared with the untreated mutant cells (compare Fig. 3, C and F). This result suggests that \(de novo\) fatty acid synthesis is required in the \(tgl3\Delta-tgl4\Delta-tgl5\Δ\) mutant to maintain PC content, unlike wild type or the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) mutant.

These results support the notion that lack of the storage lipids, TAG and SE, and the inability to degrade TAG have major impacts on both PI and PC homeostasis in response to inositol supplementation and suggest that storage lipids may provide a source of fatty acids for these essential cellular phospholipids. The two mutant strains, however, exhibit different responses to the presence of cerulenin. These differences indicate that the inability to synthesize storage lipids has consequences independent of the ability to mobilize it. Moreover, although the reduction in the burst of PI synthesis was seen in both mutants, only the \(dga1\Delta-lo1\Delta-are1\Delta-are2\Δ\) mutant failed to grow in the absence of inositol. Thus, the slow rate of recovery of PI syn-
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thesis in the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} mutant does not fully account for its Ino\textsuperscript{−} phenotype.

The Kinetics of \textit{INO1} Derepression Is Altered in the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} Mutant as Compared with Wild Type—The Ino\textsuperscript{−} phenotype is frequently associated with reduced expression of \textit{INO1} and other phospholipid biosynthetic genes that are regulated by the Opi1p repressor (21, 23). \textit{INO1} expression is derepressed up to several hundred-fold in wild type cells grown in the absence of inositol as compared with expression levels in the presence of 75 \(\mu\text{M}\) inositol, and \textit{INO1} derepression is very rapid when cells are shifted to inositol-free medium (21, 22, 37). Because the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} mutant, which is unable to synthesize TAG, displays an Ino\textsuperscript{−} phenotype at 37 \(^\circ\text{C}\), we questioned whether this phenotype was due to a deficiency in \textit{INO1} expression.

First, we compared the growth of wild type and \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} strains in liquid medium as described under “Experimental Procedures” to ensure that analyses were performed at comparable growth rates, which are known to affect \textit{INO1} expression (23). The doubling time of both strains (Fig. 4) was calculated for the time intervals corresponding to 0–3 and 3–6 h after a shift of logarithmically growing cells from 1\(^\circ\text{C}\) medium to 1\(^\circ\text{C}\) medium at 30 or 37 \(^\circ\text{C}\) and from 1\(^\circ\text{C}\) + to 1\(^\circ\text{C}\) + at 37 \(^\circ\text{C}\). There was no change in the doubling time in either strain at 30 \(^\circ\text{C}\) for the first 6 h after the shift to 1\(^\circ\text{C}\) − media (Fig. 4A). At 37 \(^\circ\text{C}\), between 3–6 h after the shift to 1\(^\circ\text{C}\) −, both wild type and \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} mutant exhibited somewhat longer doubling times as compared with growth at 30 \(^\circ\text{C}\) (Fig. 4B). When shifted to 1\(^\circ\text{C}\) − medium at 37 \(^\circ\text{C}\), both strains showed a significant increase in doubling time in the interval from 3 to 6 h after the shift. However, the lengthening of doubling time was significantly greater in the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} strain (Fig. 4C). Growth of the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} mutant for an extended period of time in 1\(^\circ\text{C}\) + medium at 37 \(^\circ\text{C}\) resulted in a further gradual increase in doubling time, which ultimately exceeded 8 h before the culture reached stationary phase. This reduced increase in cell mass, however, was not due to a loss of viability (data not shown).

To ensure that the \textit{INO1} gene was fully repressed at the start of the experiment, the kinetics of \textit{INO1} derepression was measured in cells pregrown at 30 or 37 \(^\circ\text{C}\) in 1\(^\circ\text{C}\) − medium or in 1\(^\circ\text{C}\) + medium at 37 \(^\circ\text{C}\), identical to the conditions used in the experiments in which doubling times were measured (Fig. 4). To determine \textit{INO1} derepression in 1\(^\circ\text{C}\) − medium, each culture was shifted to prewarmed medium, which lacked inositol but was otherwise identical to the original culture medium, and samples were harvested at 1.5, 3, 4.5, and 6 h after the shift.

After the shift to 1\(^\circ\text{C}\) − medium either at 30 or 37 \(^\circ\text{C}\), no striking difference in the level of expression of \textit{INO1} was observed in the \textit{dga1\textdelta lro1\textdelta are1\textdelta are2\textdelta} mutant as compared with wild type (Fig. 5, \textit{A} and \textit{B}). In general, the extent of derepression of \textit{INO1} observed in both strains at 37 \(^\circ\text{C}\) upon a shift to 1\(^\circ\text{C}\) − medium was higher than that observed at 30 \(^\circ\text{C}\) in the same medium (Fig. 5, \textit{A} and \textit{B}). Moreover, there was oscillation in the level of \textit{INO1} mRNA over the time course of 6 h after the shift to 1\(^\circ\text{C}\) − medium at 37 \(^\circ\text{C}\) in the wild type strain, indicative of an auto-regulatory circuit controlling \textit{INO1} expression.\textsuperscript{5} At 1.5 h after the shift to 1\(^\circ\text{C}\) − medium at

\textsuperscript{5} H. F. Hofbauer, unpublished observation.
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30°C no Choline

![Graph A](image1)

37°C with Choline

![Graph C](image3)

37°C no Choline

![Graph B](image2)

FIGURE 5. Derepression of the INO1 gene in the wild type and quadrap
despite that it did not contain inositol. The samples were harvested at 1.5, 3, 4.5, and 6 h after the shift, and total RNA was isolated and analyzed by RT-PCR as described under "Experimental Procedures." A refers to the INO1 expression under repressing conditions defined as the level of INO1mRNA in each strain at the stated temperature and choline supplementa-
tion level. Data are the averages from three independent experi-
ments. Solid bars represent wild type cells, and open bars indicate the dga1Δlro1Δare1Δare2Δ strain.

37°C, the dga1Δlro1Δare1Δare2Δ mutant actually exhibited a higher level of INO1 expression than the wild type strain (Fig. 5C), whereas at 3 h in 1°C medium at 37°C, the wild type strain showed a spike in expression that was not seen in the dga1Δlro1Δare1Δare2Δ mutant (Fig. 5C). However, by 4.5 h after the shift, INO1 expression leveled off in both strains at fairly similar values (Fig. 5C).

Similar measurements were made of the patterns of dere-
pression of OP13, encoding a phospholipid methyltransferase involved in PC biosynthesis, which like INO1 is regulated by the Opi1p repressor but shows a much less dramatic dere-
pression ratio in wild type cells shifted to 1− medium (38). Derepression of OP13 in the dga1Δlro1Δare1Δare2Δ mutant was also similar to wild type (data not shown). Thus, the observed Ino− phenotype of the dga1Δlro1Δare1Δare2Δ mutant grown in the presence of choline at 37°C is not due to an in-
ability to derepress INO1 or coregulated lipid biosynthetic

genes.

The Rate of Phospholipid Synthesis in the dga1Δlro1Δare1Δare2Δ mutant is Significantly Decreased in Comparison to Wild Type at 37°C during Active Growth Especially in the Presence of Inositol—Because the growth de-
fect of the dga1Δlro1Δare1Δare2Δ mutant on 1− medium at 37°C cannot be explained simply by a defect in INO1 ex-
pression, we analyzed phospholipid synthesis by pulse-label-
ing with [32P]orthophosphate. The wild type and dga1Δlro1Δare1Δare2Δ strains were grown in the presence of inositol (1°C+ medium) at 37°C to mid-logarithmic phase and shifted by filtration to prewarmed 1− or 1°C+ medium as described under "Experimental Procedures" and previous ex-
periments involving derepression of INO1. At 1.5 and 3 h after the shift, aliquots were taken and pulse-labeled for 20 min followed by lipid extraction and analysis.

Wild type and dga1Δlro1Δare1Δare2Δ strains initially showed fairly similar patterns of incorporation of [32P] into PI at 1.5 h after the shift to 1− medium at 37°C (Fig. 6A). At this initial time point, however, labeling of phosphatidic acid (PA) in mutant cells was 30% higher than that observed in wild type cells (Fig. 6A), correlating well with the relative expression levels of INO1 in these two strains (Fig. 5C). These results are consistent with the model of regulation of INO1 proposed by Loewen et al. (21). It is plausible that the higher level of labeling of PA in the dga1Δlro1Δare1Δare2Δ mutant at this initial time point is due to its inability to synthesize TAG from DAG, thus, slowing the utilization of newly syn-
thesized PA during the period immediately after removal of inositol. However, in cultures grown in 1°C+ medium for 3 h, the level of PA in the wild type strain had increased by more than 3-fold over the levels observed in the same medium at 1.5 h, whereas labeling of PA in the mutant increased only modestly (Fig. 6B), again correlating to the lower level of INO1 expression observed in the mutant compared with wild type at this time point after the shift (Fig. 5). At 3 h in 1°C+ medium, labeling of PI had also increased substantially in the wild type strain over the levels observed in the same medium at 1.5 h (Fig. 6B). However, labeling of PI in the mutant was less than half that of wild type at 3 h and, unlike wild type, showed little change between 1.5 and 3 h (compare Fig. 6, A and B). Moreover, incorporation of [32P] into total phospholip-
ds was similar in both strains at both time points in 1°C+ medium (Fig. 6, Total PL).

When wild type cells acclimated to grow in 1°C+ medium were transferred to fresh 1− medium for 3 h at 37°C, overall labeling of phospholipids (Fig. 6, Total PL) was more than double in comparison to that observed in cells transferred for the same period of time from 1°C− medium to medium lacking inositol (1°C−) (Fig. 6C). Although the greatest propor-
tion of the relative increase in total phospholipid synthesis in the wild type strain in the presence of inositol as opposed to
its absence was accounted for by an increase in PI synthesis, labeling of PA, CDP-DAG, and PS were also increased (Fig. 6C). Labeling of total phospholipids and PI were also higher in the mutant in the presence of inositol, but the increase was less dramatic than in wild type cells (Fig. 6C). Thus, the lower rate of synthesis of PI in the mutant in comparison to wild type in \(1\)^\*\(^{13}\)C\(^{+}\) medium is clearly independent of \(INO1\) expression, as \(INO1\) is not expressed by either strain in the presence of inositol (Fig. 5). Also, the presence of exogenous inositol did not correct the relative reduction in the synthesis of PI in the mutant in comparison to wild type (Fig. 6). Because inositol is not limiting for PI production in inositol-containing medium, other factors must limit PI synthesis in the mutant relative to wild type. Because other phospholipids that derive label directly from PA all show reduced labeling in the mutant, it is likely that PA synthesis is among the limiting factors.

However, the reduced labeling of PA and PA-derived phospholipids in the \(dga1\Delta\lro1\Delta\lare1\Delta\lare2\Delta\) mutant could be due to either reduced PA synthesis and/or increased turnover of newly synthesized PA to produce DAG. In the presence of exogenous choline, PC is made largely via the CDP-choline pathway by the reaction of CDP-choline with DAG, which is derived from PA (Fig. 1). However, in the formation of DAG, the phosphate label from PA is lost, and the labeled phosphate in PC under these conditions is derived from CDP-choline. Consistent with the idea that a greater fraction of PA synthesis is being diverted to DAG, labeling of PC in the mutant strain was higher than in wild type after 3 h following the shift to either \(1\)^\*\(^{13}\)C\(^{+}\) or \(1\)^\*\(^{13}\)C\(^{−}\) medium (Fig. 6, B and C). However, the increase in synthesis of PC in the mutant is not sufficient to explain the extent of the reduction in the mutant in comparison to wild type in total labeling of all the lipids that derive phosphate from PA, including PS and PI. Thus, the \(dga1\Delta\lro1\Delta\lare1\Delta\lare2\Delta\) strain, which is completely unable to synthesize storage lipids, also experiences decreased synthesis of membrane phospholipids relative to wild type even when growing continuously in the presence of inositol.

**A Higher Proportion of Newly Synthesized PI Is Used to Support Inositol-containing Sphingolipid Synthesis in the \(dga1\Delta\lro1\Delta\lare1\Delta\lare2\Delta\) Mutant—Rapid use of PI as a precursor in the synthesis of inositol-containing sphingolipids (Fig. 1) could also contribute to the reduced labeling of PI observed in the \(dga1\Delta\lro1\Delta\lare1\Delta\lare2\Delta\) mutant as compared with the wild type strain (Fig. 6). During the synthesis of these lipids, inositol phosphate derived from PI is sequentially transferred to ceramide to form inositol-phosphorylceramide (IPC) and to mannosyl-inositol-phosphorylceramide (MIPC) to form mannosyl-dinositol-phosphorylceramide (M(IP)\(_2\)C), releasing DAG at each of the two steps that could also contribute to PC synthesis (Figs. 1 and 6). We analyzed the synthesis of inositol-containing sphingolipids by pulse labeling with \(^{32}\)P orthophosphate following the growth conditions described above for phospholipid labeling (Fig. 6), except that the cells were pulse-labeled for 30 min instead of 20 min to allow sufficient time for \(^{32}\)P label to enter through PI and be transferred to the inositol-containing sphingolipids. Because \(^{32}\)P label appearing in the inositol-containing sphingolipids must enter through PI (Fig. 1), the total amount of PI synthesized in a 30-min pulse is actually greater than the amount of label remaining in PI. This is reflected in the sum of the label retained in PI plus the label transferred to the three inositol-containing sphingolipids (see PI + SL). When inositol was present, the label appearing in PI was far greater in both strains, and the same was true of the label accounted for by the sum of PI plus the inositol-contain-
inositol-containing sphingolipids. In the absence of inositol, the label incorporated into PI labeling of PI in both I
tant was comparable or even slightly higher than in wild type individual inositol-containing sphingolipid species in the mu-
net. In Fig. 7A inset, compare PI + SL, I
t medium, labeling of PI alone in the mutant represented only 41% that in the wild type strain (Fig. 7A inset), whereas labeling of PI plus the inositol-containing sphingolipids (PI + SL) in the mutant represented 72% of that observed in the wild type (Fig. 7A inset). The cause of this discrepancy in labeling of PI versus PI + SL in the mutant versus wild type became apparent when labeling of the sphingolipids was examined. In the absence of inositol, the label incorporated into inositol-containing sphingolipids was reduced by 3-fold in both strains (Fig. 7A inset). However, in contrast to the wild type strain, the label incorporated into the sum of the three inositol-containing sphingolipids in I
t medium in the mutant actually exceeded the label retained in PI in I
t medium (Fig. 7A inset). Moreover, the rate of synthesis of the individual inositol-containing sphingolipid species in the mu-
tant was comparable or even slightly higher than in wild type in both I
t + C
t medium (Fig. 7A) despite the lower rate of PI synthesis in the mutant. In Fig. 7B, label incorporated into each individual lipid (i.e. PI, IPC, MIPC, and M(IP)2C) is presented as a percentage of the total label incorporated into PI + SL. When the data are displayed in this fashion it is evident that of the total label incorporated into PI + SL in the mutant, almost 60% was used in producing inositol-containing sphingolipids in I
t medium in a 30-min pulse. In con-
contrast, less than 40% of the total label in PI + SL was recovered in inositol-containing sphingolipids in the wild type under the same conditions (Fig. 7B). Indeed, the mutant incorporated a higher proportion of the total label entering PI (i.e. PI + SL) into sphingolipids, even in I
t medium (Fig. 7B).

Thus, the dga1Δtro1Δare1Δare2Δ mutant utilizes a higher proportion of newly synthesized PI to synthesize sphingolipids than the wild type strain. The net result is that the two strains synthesize similar amounts of inositol-containing sphingolipids (Fig. 7A) despite the reduced rate of synthesis of PI in the mutant. These data suggest that yeast cells have a mechanism(s) for ensuring adequate sphingolipid synthesis at the expense of PI. Furthermore, increased synthesis of inosi-
containing sphingolipids relative to PI synthesis is clearly a factor in the reduction in labeling of PI in the mutant in compa-
rison to wild type in I
t medium (Fig. 6). However, these considerations do not explain the fact that synthesis of both PI and PI + SL is greatly reduced in the mutant even in the presence of inositol. In fact, the proportionate decrease in PI + SL in the mutant versus wild type is actually greater in the presence of inositol. Nevertheless, reduced synthesis of PI coupled with increased demand for sphingolipid synthesis relative to total PI synthesis are likely contributing factors in the poor growth of the dga1Δtro1Δare1Δare2Δ mutant in the absence of inositol.

Exogenous Inositol Affects the Metabolism of Neutral Lipids in Wild Type and the dga1Δtro1Δare1Δare2Δ Mutant—We also assessed changes in the abundance of neutral lipid classes in wild type and the dga1Δtro1Δare1Δare2Δ strains after the shift from I
t to I
t medium. Cultures were allowed to reach mid-logarithmic phase in I
t medium (with or without choline at 30 or 37 °C) in the presence of [14C]acetate as described under “Experimental Procedures.” Cells were washed and transferred into medium lacking inositol at the same temper-
ature and choline concentration, maintaining constant specific activity of the label. After incubation at 30 or 37 °C, samples were taken at 0, 60, 120, and 180 min. After 180 min in I
t medium, inositol was reintroduced, and samples were collected at 5, 15, and 30 min (Fig. 8). Among the different neutral lipid classes, the most dramatic change in wild type cells after a shift from inositol-containing medium to I
t medium at either 30 or 37 °C was an increase in the abundance of TAG (Fig. 8A). At both temperatures, the increase in TAG levels was accompanied by an almost “mirror image” decrease in free fatty acid content (Fig. 8A). When inositol was added back to the wild type cultures, TAG content decreased with a concomitant increase in the level of DAG that is presumably derived from lipolysis (Fig. 8A). A similar but more accentu-
ad change in TAG levels in response to inositol re-addition occurred in wild type cells incubated at 37 °C in the absence of choline (Fig. 8A). In contrast, when wild type cells were shifted to I
t at 37 °C, only a marginal change was observed in the abundance of TAG (Fig. 8A), but free fatty acids decreased significantly after the media shift (Fig. 8A). However, when inositol was reintroduced, TAG levels declined by about 50% within 30 min, whereas fatty acids increased (Fig. 8A). SE
levels also decreased by about 30% after inositol re-addition, and free sterols increased (Fig. 8A). These data further demonstrate that TAG and SE degradation is stimulated upon the addition of inositol to actively growing cultures, increasing the availability of fatty acids for subsequent channeling into synthesis of PI and other membrane phospholipids derived from PA in wild type cells.

In comparison to wild type, the dga1Δlro1Δare1Δare2Δ mutant, which does not synthesize TAG or SE, contained 2–3-fold higher levels of free sterols under all the growth conditions tested (Fig. 8B). DAG and free fatty acid content were also higher under all three-growth conditions in the mutant compared with wild type, consistent with the metabolic block of the availability of fatty acids for subsequent channeling into synthesis of PI and other membrane phospholipids derived from PA in wild type cells.

The addition of inositol to cultures of the dga1Δlro1Δare1Δare2Δ mutant after 180 min growth in I−C− at 30 °C or in I−C+ medium at 37 °C had little impact on the neutral lipid composition of both strains shifted to inositol free media for 180 min. The graph on the right represents the neutral lipid composition of both strains when inositol was added back to cultures deprived of inositol. Panel A, wild type. Panel B, dga1Δlro1Δare1Δare2Δ. The data represent the average of three independent experiments. The magnitude of the error is similar to the data in Fig. 3, but error bars were omitted for clarity of the presentation. The lipids indicated are free fatty acids (open triangles), TAG (gray triangles), free sterols (solid circles), DAG (solid diamonds), and SE (open circles), ODU, optical density units.

FIGURE 8. The availability of inositol affects the abundance of neutral lipid classes in wild type. Lipids in all strains were labeled to steady state with [1-14C]acetate (1 μCi/ml) in I−C− medium at 30 or 37 °C and in I−C+ medium at 37 °C as described under “Experimental Procedures” for at least seven generations and grown to mid-logarithmic phase. They were shifted by filtration either in I−C− medium at 30 and 37 °C or to I−C+ medium at 37 °C as described under “Experimental Procedures” maintaining label at constant specific activity and cultured for 180 min. At this time point, inositol was reintroduced into the cultures, and aliquots were collected at 0, 5, 15, and 30 min. Lipids were extracted and analyzed as described under “Experimental Procedures.” Each panel of this figure contains two graphs. The graph on the left indicates the neutral lipid composition of both strains shifted to inositol free media for 180 min. The graph on the right represents the neutral lipid composition of both strains when inositol was added back to cultures deprived of inositol. Panel A, wild type. Panel B, dga1Δlro1Δare1Δare2Δ. The data represent the average of three independent experiments. The magnitude of the error is similar to the data in Fig. 3, but error bars were omitted for clarity of the presentation. The lipids indicated are free fatty acids (open triangles), TAG (gray triangles), free sterols (solid circles), DAG (solid diamonds), and SE (open circles), ODU, optical density units.

DISCUSSION

The Ino− phenotype is classically associated with a deficiency in expression of INO1, the structural gene for the inositol-3-phosphate synthase (23, 39). However, we report that the dga1Δlro1Δare1Δare2Δ mutant, which totally lacks lipid droplets (4, 16), exhibited no significant defect in INO1 expression at 37 °C in I−C− medium compared with wild type. Nevertheless, its ability to grow on agar plates under these conditions was greatly reduced (Fig. 2). Immediately after a shift of actively growing cells to I−C+ medium at 37 °C, the mutant actually exhibited higher INO1 expression than the wild type strain (Fig. 5), but within a few hours after the shift...
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were identified (10, 12, 32, 33). In this study we report that recently, the lipid hydrolases that catalyze TAG degradation of phospholipids upon resumption of growth. Most relevantly, fatty acids derived from TAG are utilized for the synthesis actively influences the metabolism of membrane lipids, in logarithmically growing cells. In addition, the reduced recovery of PI content immediately after inositol reintroduction displayed by both mutants in comparison to wild type suggests that a pool of fatty acids derived from TAG degradation may be used for rapid restoration of PI after inositol reintroduction. The data presented here also confirm the combined contribution of de novo fatty acid synthesis and PC turnover for the burst of PI content after inositol reintroduction, as previously reported (19). Thus, we conclude that there are at least three different metabolic pathways capable of providing fatty acids for the dramatic increase in PI upon inositol reintroduction in wild type cells.

The Absence of Lipid Droplets Affects Synthesis of Both PI and Inositol-containing Sphingolipids—The Ino^− phenotype of the dga1Δlro1Δare1Δare2Δ mutant to mobilize TAG for PI synthesis, as the tgl3Δtgl4Δtgl5Δ strain is able to sustain growth in 1−C^− medium at 37°C in the presence of choline. As we discuss below, the ability to synthesize TAG was shown to be protective under conditions of impaired membrane trafficking and declining phospholipid synthesis (15). However, the dga1Δlro1Δare1Δare2Δ strain also exhibits impaired growth in medium lacking inositol. This growth defect is not simply due to the inability of the dga1Δlro1Δare1Δare2Δ mutant to mobilize TAG for PI synthesis, as the tgl3Δtgl4Δtgl5Δ strain is able to sustain growth in 1−C^− medium at 37°C. This growth defect does not simply arise from the inability of the dga1Δlro1Δare1Δare2Δ mutant to mobilize TAG for PI synthesis, as previously reported (19). Thus, we conclude that there at least three different metabolic pathways capable of providing fatty acids for the dramatic increase in PI upon inositol reintroduction in wild type cells.

Lack of TAG Hydrolysis in Logarithmically Growing Cells Attenuates the Burst in PI Synthesis in Response to Inositol Reintroduction—In studies on stationary-phase cells reentering active growth, Taylor and Parks in 1979 (13) proposed that fatty acids derived from TAG are utilized for the synthesis of phospholipids upon resumption of growth. Most recently, the lipid hydrolases that catalyze TAG degradation were identified (10, 12, 32, 33). In this study we report that both the dga1Δlro1Δare1Δare2Δ mutant, which lacks the ability to produce TAG (4, 16) and the tgl3Δtgl4Δtgl5Δ mutant, which is unable to mobilize TAG (10, 12, 33), are compromised in rebuilding PI content to the levels seen in wild type cells immediately after the addition of inositol. Consistent with these results, TAG is consumed in proliferating wild type cells upon inositol reintroduction coincident with the rebuilding of PI content. We propose a new metabolic role for TAG as donor of fatty acids for the synthesis of PI in logarithmically growing yeast cells, particularly when inositol is reintroduced to cultures previously lacking this lipid precursor.

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The Absence of Lipid Droplets Affects Synthesis of Both PI and Inositol-containing Sphingolipids—The Ino^− phenotype of the dga1Δlro1Δare1Δare2Δ strain is clearly not due simply to its inability to mobilize TAG, and thus, the inability to store fatty acids in TAG and SE must contribute to its phenotype. Despite the fact that the dga1Δlro1Δare1Δare2Δ mutant is not able to store fatty acids in either TAG or SE, it is viable under the culture conditions employed in the studies by Oelkers et al. (4) and Sandager et al. (16). These two studies showed that storage lipids are not essential to sustain active logarithmic growth in yeast. However, TAG hydrolysis does play an important role in the recovery of cells from stationary phase into active growth (12, 13). Moreover, active TAG biosynthesis was shown to be protective under conditions of secretory stress (15). The sec13-1 strain, which has a defect in COP II vesicle trafficking from the ER when shifted to its restrictive temperature of 37°C, experienced a dramatic decrease in phospholipid synthesis relative to the wild type control, whereas synthesis of TAG increased. Thus, it appears that when membrane trafficking pathways are compromised, synthesis of phospholipids required for membrane proliferation declines in a fashion that is coordinated with the slowing of membrane trafficking. Under these conditions, excess fatty acids are channeled into TAG (15). Moreover, when the genes encoding the DAG acyltransferases, Dga1p and Lro1p, were deleted in the sec13-1 mutant, the restrictive temperature of the sec13-1 dga1Δ lro1Δ strain decreased by two degrees compared with the parental strain (sec13-1 DGA1 LRO1) in media lacking inositol (15). Thus, the increased synthesis of TAG appears to serve under these conditions to absorb the excess fatty acids not being used in membrane proliferation, providing a degree of protection under conditions of secretory stress (15).
The above evidence suggests that both TAG synthesis and breakdown are interdependent with ongoing membrane lipid synthesis. In agreement with these reports, we found that the lack of storage lipids in the \( \text{dga1}\Delta \text{iro1}\Delta \text{are1}\Delta \text{are2}\Delta \) mutant has a profound impact on the synthesis of membrane lipids. The inability of the mutant to synthesize TAG and SE affects the synthesis of all the phospholipids derived directly from PA (Fig. 6), and this effect is not specific to PI synthesis alone. These results indicate that the storage lipids in lipid droplets (Fig. 6), and this effect is not specific to PI synthesis alone. The synthesis of all the phospholipids derived directly from PA may be due in part to the absence of a pool of storage lipids (52). Because this mode of transport is not possible in the \( \text{dga1}\Delta \text{iro1}\Delta \text{are1}\Delta \text{are2}\Delta \) mutant, the delivery of free sterols from the ER to the plasma membrane by means of the membrane transport pathways.

In summary, we have found that the \( \text{dga1}\Delta \text{iro1}\Delta \text{are1}\Delta \text{are2}\Delta \) strain is not able to sustain growth in the absence of inositol at 37 °C. This is not due to a defect in \( \text{INO1} \) expression but may be due in part to the absence of a pool of storage lipids from which to draw fatty acids as precursors to sustain optimal synthesis of PI and other membrane phospholipids in logarithmically growing yeast cells. The need for a higher proportion of newly synthesized PI in producing spherolipids may also be a factor contributing to the growth defect experienced by the mutant in the absence of inositol. Together, these results suggest that synthesis of storage lipids and membrane-forming lipids, in particular PI and inositol-containing spherolipids, are interdependent and operate in a coordinated fashion to maintain lipid homeostasis in actively growing yeast cells.

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