Lipid dynamics in yeast under haem-induced unsaturated fatty acid and/or sterol depletion

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In the yeast *Saccharomyces cerevisiae*, UFA (unsaturated fatty acids) and ergosterol syntheses are aerobic processes that require haem. We took advantage of a strain affected in haem synthesis (*hem1Δ*) to starve specifically for one or the other of these essential lipids in order to examine the consequences on the overall lipid composition. Our results demonstrate that reserve lipids (i.e. triacylglycerols and steryl esters) are depleted independently of haem availability and that their UFA and sterol content is not crucial to sustain residual growth under lipid depletion. In parallel to UFA starvation, a net accumulation of SFA (saturated fatty

acids) is observed as a consequence of haem biosynthesis preclusion. Interestingly, the excess SFA are not mainly stored within triacylglycerols and steryl esters but rather within specific phospholipid species, with a marked preference for PtdIns. This results in an increase in the cellular PtdIns content. However, neutral lipid homeostasis is perturbed under haem starvation. The contribution of two lipid particle-associated proteins (namely Tgl1p and Dga1p) to this process is described.

Key words: *DGA1*, phospholipid, sterol, *TGL1*, triglyceride, yeast.

INTRODUCTION

Most organisms are exposed to constant changes in their environment. Survival depends therefore on their capacity to adapt to the new conditions. One major challenge of biology is to understand the mechanisms of these complex adaptative responses. Recent advances in genome sequencing have been accompanied by the development of techniques such as DNA or cDNA microarrays, which allow monitoring of the global transcriptional response to environmental changes.

The budding yeast *Saccharomyces cerevisiae* has been the subject of a large number of studies aimed at understanding the mechanisms that allow it to adapt to a wide variety of growth conditions. For example, the adaptative response of this microorganism to oxygen-limited conditions has been the subject of extensive studies, including the most recent via microarrays [1–4]. Respiration deficiency resulting from oxygen limitation has several consequences, among which UFA (unsaturated fatty acids) and ergosterol deprivation are potentially sufficient to compromise growth. Indeed the biosynthetic routes of these essential lipids are strictly aerobic [5].

Therefore, upon oxygen limitation, yeast growth relies on its capacity to induce hypoxic genes encoding key enzymes of these pathways (e.g. *OLE1* for UFA biosynthesis, *ERG11* for the sterol pathway), enabling compensation for intermediate substrate scarcity and induction of an efficient exogenous sterol-import machinery which is shut down in aerobiosis (aerobic sterol exclusion [6]). Given that up-regulation of key enzymes is not sufficient to restore growth upon strict anaerobiosis, long-term survival under these conditions relies only on sterol import whose molecular basis remains to be elucidated. Some genes, whose involvement in this phenomenon was recently highlighted, belong to the hypoxic gene family [7,8].

Comparison of various transcription profiling experiments under anaerobiosis identified genes and open reading frames

that are not directly associated with oxygen availability. For example, genes involved in phospholipid metabolism like *PIS1* (PtdIns synthase) or *EK11* (ethanolamine kinase) were shown to be induced upon oxygen deprivation [1,3], suggesting that the adaptative response has a much wider consequence on lipid homeostasis than previously suspected. The up-regulation of the potential ester hydrolase encoded by *TGL1* [9,10] under oxygen limitation [1] has also been observed. This is of particular interest since it suggests that mobilization of fatty acids, which accumulate in TG (triacylglycerols) and SE (steryl esters) within cytoplasmic lipid droplets [11,12], may be used to maintain, at least transiently, membrane lipid composition under anaerobiosis.

In yeast, haem depletion is sufficient to induce both UFA and sterol biosynthesis preclusion, since it is used as the cofactor of several key enzymes of their biosynthesis pathways (for a review, see [13]). Moreover, haem is also known to induce transcriptional regulation of many hypoxic genes via the transcriptional factors *ROX1* and *HAP1* or *HAP2/3/4* [5,14]. For example, haem depletion alone can relieve aerobic sterol exclusion [6]. Since true anaerobiosis is difficult to achieve in the laboratory, we therefore used a *hem1Δ* strain to study the consequences of UFA and/or sterol depletion on lipid homeostasis in response to haem limitation. More specifically, we focused on the role of TG and SE as potential suppliers for UFA and sterols, but also on the consequences of UFA starvation on the acyl chain content of phospholipids. In the light of recent microarrays studies [1], the contribution of hypoxic genes to this process was also investigated.

EXPERIMENTAL

Strains and culture conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. The *hem1Δ* strain is isogenic to FY1679 [15]. Cells were grown

Abbreviations used: ALA, δ -aminolevulinic acid; DAG, diacylglycerol; ER, endoplasmic reticulum; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; SE, steryl esters; SFA, saturated fatty acids; TG, triacylglycerols; UFA, unsaturated fatty acids; CDP-DAG, cytidine diphosphate diacylglycerol.

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Table 1 Yeast strains used in this study

Strain	Characteristics	Source
FY1679 a	<i>MATa leu2 his3 trp1 ura3</i>	Progeny of diploid FY1679
FY1679 α	<i>MATα leu2 his3 trp1 ura3</i>	Progeny of diploid FY1679
FYH04	<i>MATa his3 leu2 trp1 ura3 hem1::LEU2</i>	[15]
<i>hem1Δa</i>	<i>MATa his3 leu2 trp1 ura3 hem1::LEU2</i>	Progeny of FY1679 α $\times$ FYH04
<i>hem1$\Delta$$\alpha$</i>	<i>MATα his3 leu2 trp1 ura3 hem1::LEU2</i>	Progeny of FY1679 α $\times$ FYH04
<i>tgl1Δ</i>	<i>MATα his3 leu2 lys2 ura3 YKL140w::kanMX4</i>	Y14990 (EUROSCARF)
<i>hem1Δ tgl1Δ</i>	<i>MATα his3 leu2 lys2 ura3 YKL140w::kanMX4 hem1::LEU2</i>	This study, progeny of Y14990 $\times$ <i>hem1Δa</i>
<i>lro1Δ</i>	<i>MATa his3 leu2 met15 ura3 YNR008w::kanMX4</i>	Y05383 (EUROSCARF)
<i>hem1Δlro1Δ</i>	<i>MATα his3 leu2 met15 ura3 YNR008w::kanMX4 hem1::LEU2</i>	This study, progeny of Y05383 $\times$ <i>hem1Δa</i>
<i>dga1Δ</i>	<i>MATa his3 leu2 met15 ura3 YOR245c::kanMX4</i>	Y02501 (EUROSCARF)
<i>hem1Δ dga1Δ</i>	<i>MATα his3 leu2 ura3 trp1 YOR245c::kanMX4 hem1::LEU2</i>	This study, progeny of Y02501 $\times$ <i>hem1$\Delta$$\alpha$</i>

aerobically at 28 °C in standard YPD medium [1 % yeast extract (w/v), 1 % peptone (w/v) and 2 % dextrose (w/v)] supplemented with 80 $\mu\text{g} \cdot \text{ml}^{-1}$ ALA (δ -aminolevulinic acid). Haem-induced lipid starved conditions were obtained by cultivating the cells in YPD medium. Alternatively, YPD medium was supplemented with 80 $\mu\text{g} \cdot \text{ml}^{-1}$ ergosterol and/or 1 % Tween 80 (v/v), used as the source of UFA.

β -Galactosidase assays

β -Galactosidase assays were performed as described previously [16] on *hem1 Δ* cells containing a plasmid bearing the 1 kb DNA sequence immediately upstream of the *DAN1* open reading frame cloned 5' to the reporter gene *lacZ* (plasmid pMR278 [17]), grown in the appropriate medium. β -Galactosidase activity (A), given as nmol of *O*-naphthol (measured at A_{420}) $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$, was calculated as follows: $A = (A_{420} \times 100 \times 1700) / (4.5 \times V \times c \times t)$, where V is the volume of the protein extract (ml), c is the protein concentration ($\text{mg} \cdot \text{ml}^{-1}$) and t is the incubation time at 30 °C (min).

Lipid analysis

Lipid extracts were obtained from $\approx 2 \times 10^9$ shock-frozen yeast cells grown as indicated. Cells were harvested, washed with distilled water and taken up to 1 ml with cold water. Cells were then broken by vigorous shaking with a mini-beadbeaterTM (Biospec Products) for 1 min at 5000 rev./min in the presence of 500 μl of glass beads (diameter 0.3–0.4 mm; Sigma). Cellular lipids were extracted using chloroform/methanol (2:1, v/v) as described by Folch et al. [18]. The final organic phase was evaporated and lipids were dissolved in either 100 μl of hexane (neutral lipids) or 100 μl of chloroform/methanol (1:1; phospholipids).

Neutral lipids [TG, DAG (diacylglycerol), SE and non-esterified fatty acids] were separated by TLC in hexane/diethyl ether/acetic acid (80:20:1, by vol.) on precoated Silica gel 60 F₂₅₄ plates (Merck). Phospholipids were resolved on a precoated LK5 silica gel plates (Whatmann) using chloroform/ethanol/water/triethylamine (30:35:7:35, by vol.) as the mobile phase. Lipids were visualized under UV after vaporization of a primuline solution [0.05 mg/ml in acetone/water (80:20, v/v)] and identified by using appropriate standards. Neutral lipids (TG, DAG, SE and non-esterified fatty acids), free sterols and phospholipids were scraped from the plate.

Fatty acid methyl esters were obtained from each lipid class as described by Dahlqvist et al. [19], with slight modifications. Briefly, the various lipid species were eluted from the silica with hexane (neutral lipids) or chloroform/methanol/water (5:5:1, by vol.; phospholipids) and transesterification was done by heat-

ing the eluate at 50 °C for 16 h in 2 % (v/v) H₂SO₄ in dry methanol. The resulting fatty acid methyl esters were extracted in hexane and analysed by GC using a 25 m $\times$ 0.32 mm AT-1 capillary column (Alltech), with methylheptadecanoic acid as the standard. Fatty acids were quantified and used to calculate the amount of lipid in each class. The lipid content was finally expressed as nmol of fatty acid/10⁹ (broken) cells.

For specific sterol identification and quantification, free sterols were separated from SE by TLC in dichloromethane on precoated Silica gel 60 F₂₅₄ plates and SE spots were subjected to saponification as reported previously [8]. The different sterol species were then separated by GC on the column described above, and identified by the means of their retention times relative to dihydrocholesterol, used as a standard. The results are expressed as nmol of sterol/10⁹ cells.

Cell radiolabelling with glycerol

Yeast cells were grown in YPD + ALA to a cell density of 4×10^7 cells $\cdot \text{ml}^{-1}$, after which they were incubated with 2 $\mu\text{Ci}/\text{ml}$ [³H]glycerol (specific activity, 1.0 mCi $\cdot \text{mol}^{-1}$; Sigma). After 16 h cells were harvested, washed with distilled water and chased for 4 h in fresh, unlabelled YPD medium containing ALA or not. At the end of each incubation, lipids were extracted from 2×10^9 cells as described above. The ³H-labelled spots associated to TG were scraped from the silica gel plates, transferred into vials and the ³H content was determined in a liquid scintillation counter.

RESULTS

hem1 Δ cells as a model to study UFA and/or sterol depletion

In order to study lipid redistributions under haem depletion, we used a strain mutated for the δ -aminolevulinic acid synthase (*hem1 Δ*) as an experimental model [20]. Haem is the prosthetic group of several enzymes of the ergosterol pathway and of Ole1p, which catalyses fatty acid desaturation (see [13] for a review). Another biological function of haem is its direct involvement in the down-regulation of hypoxic genes via the *ROX1* transcription repressor [5,21,22]. Consequently, an *hem1 Δ* strain can mimic most anaerobiosis-induced effects in standard, oxygenated culture conditions in the absence of ALA, provided that the medium is supplemented with ergosterol and oleic acid (as the UFA source). On the other hand, aerobic growth can be achieved if ALA is added to the medium to restore haem synthesis. Thus, in our experimental system designed to study lipid dynamics, *hem1 Δ* cells were cultivated in ALA (aerobiosis) to stationary phase, and subsequently shifted to fresh YPD medium either containing ALA (aerobiosis to aerobiosis) or without supplementation (aerobiosis

Table 2 Fatty acid composition of neutral lipids from haem-competent cells

hem1Δ cells were grown to stationary phase in YPD + ALA. Results are expressed as the means $\pm$ S.E.M. from at least three independent experiments. Total quantities are given as nmol of fatty acid/10⁹ cells. NEFA, non-esterified fatty acids.

	Fatty acid composition (%)			
	TG	DAG	SE	NEFA
C _{16:0}	18.2 $\pm$ 0.0	21.9 $\pm$ 3.9	9.8 $\pm$ 2.3	31.5 $\pm$ 7.4
C _{16:1Δ⁹}	47.6 $\pm$ 3.4	30.8 $\pm$ 3.4	52.5 $\pm$ 1.5	17.2 $\pm$ 2.9
C _{18:0}	5.5 $\pm$ 0.6	9.9 $\pm$ 2.7	4.5 $\pm$ 0.8	23.0 $\pm$ 3.1
C _{18:1Δ⁹}	26.1 $\pm$ 2.8	33.8 $\pm$ 4.5	31.0 $\pm$ 1.9	24.7 $\pm$ 0.2
C _{18:1Δ¹¹}	2.5 $\pm$ 0.0	3.5 $\pm$ 0.1	2.2 $\pm$ 0.4	3.6 $\pm$ 1.2
% of UFA	76.2 $\pm$ 0.6	68.2 $\pm$ 1.2	85.8 $\pm$ 3.1	45.5 $\pm$ 4.4
Total quantities (nmol/10 ⁹ cells)	132 $\pm$ 6	41.8 $\pm$ 4.3	39.9 $\pm$ 4.2	18.2 $\pm$ 1.2

to haem-induced lipid starvation). Alternatively, ergosterol or oleate was added independently to specifically starve the strain of UFA or sterol respectively.

In a first step, we analysed the standard lipid composition of the *hem1Δ* strain grown in aerobiosis. For this purpose, we quantified the acyl chain composition of each lipid species. This allowed us to determine the total amount of each lipid class, but also to address the degree of saturation of their acyl chains. In the experiments presented in Tables 2 and 3, the amount of each lipid class (neutral lipids and phospholipids) is expressed as nmol of fatty acid/10⁹ cells. Despite some variations depending on the lipid class considered, the most abundant fatty acids in aerobiosis are the UFA palmitoleic and oleic acids (47.6 and 26.1 % of the fatty acid content of TG, respectively; Table 2). The SFA (saturated fatty acids) palmitic acid (C_{16:0}) and stearic acid (C_{18:0}) represent 18.2 and 5.5 % of total fatty acids in aerobically built TG, respectively (Table 2). In contrast, SFA are enriched in the non-esterified fatty acid population, as compared with other neutral lipids (Table 2).

The relative amounts of the various phospholipid species in aerobiosis are given in Table 3. PtdCho (phosphatidylcholine; $\approx$ 44 %), PtdEtn (phosphatidylethanolamine; $\approx$ 22 %) and PtdIns ($\approx$ 23 %) are the three major phospholipids, followed by PtdSer (phosphatidylserine; $\approx$ 6 %) and PtdOH (phosphatidic acid; $\approx$ 5 %). Even though phospholipid acyl chains are mainly unsaturated, noticeable differences in saturation levels could be observed within the various species. Acyl chain unsaturation is higher in PtdCho (76.2 % UFA) and PtdEtn (77.7 %), followed by PtdSer (70.4 %), and lower in PtdOH (64 %) and PtdIns (56.3 %; Table 3). These results are in good agreement with previous studies on standard yeast strains grown in aerobic conditions [13,23–25].

Table 3 Fatty acid composition of phospholipids from haem-competent cells

hem1Δ cells were grown to the stationary phase in YPD + ALA. Results are expressed as the means $\pm$ S.E.M. from at least three independent experiments. Total quantities are given as nmol of fatty acid/10⁹ cells. PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; ND, not detectable.

	Fatty acid composition (%)				
	PtdCho	PtdEtn	PtdIns	PtdOH	PtdSer
C _{16:0}	15.7 $\pm$ 0.5	19.8 $\pm$ 1.4	32.7 $\pm$ 1.8	27.2 $\pm$ 1.7	30.4 $\pm$ 1.9
C _{16:1Δ⁹}	44.2 $\pm$ 0.5	41.7 $\pm$ 7.8	23.7 $\pm$ 0.2	23.7 $\pm$ 3.5	19.4 $\pm$ 1.2
C _{18:0}	7.2 $\pm$ 0.7	2.4 $\pm$ 0.7	10.9 $\pm$ 0.6	5.6 $\pm$ 1.7	2.5 $\pm$ 0.6
C _{18:1Δ⁹}	29.3 $\pm$ 1.2	36.0 $\pm$ 7.0	32.6 $\pm$ 1.4	43.4 $\pm$ 0.6	47.8 $\pm$ 2.3
C _{18:1Δ¹¹}	3.5 $\pm$ 0.5	ND	ND	ND	ND
% of UFA	76.2 $\pm$ 0.5	77.7 $\pm$ 0.8	56.3 $\pm$ 1.2	64.0 $\pm$ 1.0	70.4 $\pm$ 2.5
Total quantities (nmol/10 ⁹ cells)	202.7 $\pm$ 2.2	100.3 $\pm$ 1.0	106.3 $\pm$ 8.7	22.5 $\pm$ 1.4	28.5 $\pm$ 2.1

As shown in Figure 1(A), the transfer of cells to fresh YPD medium without addition (haem-induced lipid starvation) resulted in growth arrest after about five generations. Under these conditions, hypoxic gene expression, monitored by using the *lacZ* reporter gene fused to the promoter of the canonical hypoxic gene *DAN1* [17], was observed 4 h after shift. In parallel, the acyl chain content of the various lipid classes was also determined. As a control, the same measurements were carried out on cells transferred to fresh YPD medium supplemented with ALA (Figure 1B) that would allow for discrimination between variations related to cell growth or more specifically to lipid starvation. As shown, haem starvation resulted in UFA depletion in as little as 6 h after shift to haem-induced lipid starvation. SFA then accumulated within the cells after 8 h, reaching a maximum after 24 h under these conditions (Figure 1B).

TG and SE pools are depleted in a haem-independent manner

Residual growth was observed when *hem1Δ* cells were starved of lipids (Figure 1A). To address the question of the putative role of TG and SE in this process, TG and SE amounts were determined as a function of time after shift to YPD or YPD + ALA medium (Figure 2A). Unexpectedly, the TG and SE levels dropped very rapidly within the lag period following the shift to fresh medium, even in the presence of ALA, indicating that this phenomenon is not specific to the lipid starvation induced by haem depletion.

We then questioned the relative contribution of hydrolysis to the observed drop in TG amount. Since *hem1Δ* cells only grew for 1.6 generations within 4 h following the shift (Figure 1A), a simple dilution of TG stores due to cell division and TG synthesis arrest could not fully account for the observed 7-fold decrease of TG pools (Figure 2A). Therefore, the fate of aerobically built TG was examined after prior labelling of glycerolipids with [³H]glycerol. As displayed in Figure 2(B), the label incorporated into TG decreased 12-fold after a 4 h chase (one would expect a 3-fold decrease in the case of simple dilution; see above), demonstrating that TG are hydrolysed during the lag period. Moreover, since the same results were obtained upon haem depletion (Figure 2B), we conclude that TG mobilization is not dependent on the haem status, but is rather a more general process related to growth resumption.

Deletion of *TGL1* affects sterol homeostasis in haem-competent and non-haem-synthesizing cells

Tgl1p has recently been shown to display a steryl-esterase activity [9]. Since *TGL1* belongs to the hypoxic gene family [1], we investigated whether deletion of this gene may affect free sterol

Figure 1 Consequences of haem depletion for cell growth and fatty acid content

(A) *hem1 Δ* cells were grown to stationary phase in YPD medium containing ALA (aerobic-like conditions) and shifted to YPD medium (haem-induced lipid starvation; $\blacksquare$, $\bullet$) or YPD + ALA ($\square$). Attenuance of the cultures (solid lines; $\blacksquare$, $\square$) and β -galactosidase activity (dashed line; $\bullet$) were determined at the indicated time points following the shift. β -Galactosidase values are means from two independent experiments. The error bars (S.D.) are indicated. (B) In parallel, the UFA (upper panel) and SFA content (lower panel) of neutral lipids (TG, DAG, non-esterified fatty acids and SE) and phospholipids was determined for the *hem1 Δ* cells shifted to YPD + ALA (open bars) or YPD without addition (black bars), as described in the Experimental section. UFA and SFA amounts are expressed as nmol of fatty acid/10⁹ cells. Values are means $\pm$ S.E.M. from at least three independent experiments.

and/or SE homeostasis upon haem depletion. As shown in Figure 3(A), and as already described by Athenstaedt et al. [26], the amount of aerobically built SE was increased in the *TGL1*-deleted strain. A closer examination of the sterol and the acyl chain content of SE revealed that *TGL1* deletion also induced redistribution of

the SE pattern, with a specific enrichment of ergosterol moieties and UFA (Figure 3A).

When the cells were shifted for 6 h to fresh YPD, SE pools were fully depleted even in the absence of Tgl1p, showing that this protein is not a major contributor to the reduction in SE

Figure 2 TG and SE depletion during the lag period

(A) *hem1Δα* cells grown to the stationary phase in aerobic-like conditions were transferred at a cell density of 4×10^6 cells $\cdot$ ml⁻¹ to YPD + ALA (open bars), or to YPD without addition (black bars). TG (upper panel) and SE (lower panel) were extracted and quantified at the indicated time points following the shift, as described in the Experimental section. Total amounts of TG and SE are given as nmol of fatty acid (FA) per 10⁹ cells. The arrows indicate the approximate time of haem-induced hypoxic gene up-regulation, determined as described in the text. (B) *hem1Δα* cells grown in YPD + ALA were incubated with [³H]glycerol as described in the Experimental section. The stationary-phase cells (open bar) were then transferred at a cell density of 4×10^6 cells $\cdot$ ml⁻¹ to unlabelled YPD + ALA (black bar) or YPD without addition (grey bar), and were allowed to grow for 4 h. Lipids were extracted from 2×10^9 cells and separated as described in the Experimental section. The [³H] labelling associated with TG was determined for each condition. Values are means $\pm$ S.E.M. from at least three independent experiments.

content observed after resumption of growth (Figure 3B, upper panel). Despite the release of ergosterol contained in SE, free ergosterol amounts decreased, both in the wild-type and in the *TGL1*-deleted strain, as a consequence of haem-induced sterol biosynthesis preclusion (Figure 3B, lower panel). Interestingly, free ergosterol amounts were reduced in the absence of Tgl1p

Figure 3 Tgl1p influences sterol homeostasis

hem1Δα (open bars) and *hem1Δtgl1Δ* (black bars) cells were grown to the stationary phase in YPD + ALA. (A) SE were extracted from 2×10^9 cells and the sterol (upper panel) and acyl chain (lower panel) compositions were determined as described in the Experimental section. Sterols are listed from the left to the right according to their appearance in the ergosterol biosynthesis pathway: LAN, lanosterol; ZYM, zymosterol; FEC, fecosterol; ERG 5-7, ergosta-5,7-dienol; ERG, ergosterol. (B) These stationary-phase cells (ALA) were then transferred to YPD medium at a cell density of 4×10^6 cells $\cdot$ ml⁻¹ and were allowed to grow for 6 h under these conditions (YPD). The levels of SE (upper panel) and free ergosterol (lower panel) were determined in both conditions. Values are means $\pm$ S.E.M. from at least three independent experiments.

under this condition (2-fold decrease; Figure 3B), while the amounts of other free sterol species were not affected (results not shown). This suggests that Tgl1p may be involved in free ergosterol homeostasis under conditions of sterol depletion.

Figure 4 TG and SE dynamics upon haem depletion

hem1Δα cells were cultivated in aerobic-like conditions as described in the legend of Figure 2 and transferred to YPD + ALA (open bars), YPD without addition (black bars) or YPD + oleate (Ole; grey bars). TG (A, C and E) and SE (B, D and F) were extracted from cells at the indicated time points following the shift. Their total fatty acid composition (A and B) and their $C_{18:1} \Delta^9$ (C and D) and SFA (E and F) contents were determined. Values are means $\pm$ S.E.M. from at least three independent experiments.

Haem-depleted cells cannot replenish their TG and SE stocks because of substrate limitation

Lipid redistributions were then analysed once haem depletion was obtained, i.e. 4 h after the shift to fresh YPD medium. As previously mentioned, SFA accumulate within the cells under these conditions (Figure 1B). We therefore questioned whether reserve lipids may serve as an efficient storage form for the accumulating saturated fatty acyl chains. As shown in Figures 4(A) and 4(B), non-haem-synthesizing cells did not fully reconstitute their TG and SE pools upon reaching the stationary phase, unlike aerobically grown cells. This could be related to an almost complete lack of UFA storage within these species (see the $C_{18:1} \Delta^9$ content, Figures 4C and 4D), accompanied by a limited accumulation of SFA (Figures 4E and 4F). Under these conditions of SFA accumulation, TG and SE did not contain more saturated fatty acyl chains in comparison with growth under aerobic conditions. Since reserve lipids in haem-competent cells are mainly composed of UFA (Table 2), we therefore tested whether the failure to reconstitute lipid stores might be directly related to UFA limitation. For this purpose, oleate was exogenously supplied

to the medium. As shown in Figure 4, $C_{18:1} \Delta^9$ accumulated in TG, whose pools were fully reconstituted (Figures 4A and 4C). These observations indicated that haem-depleted cells are capable of efficient TG synthesis in the presence of an exogenous source of UFA.

In contrast to TG, exogenous oleate was not incorporated in SE at the end of the time course (Figure 4D). A likely explanation is a limitation in free sterol moieties as co-substrates for SE synthesis, due to haem-induced sterol biosynthesis preclusion. This hypothesis was confirmed when exogenously supplied cholesterol was efficiently esterified in the same conditions (results not shown).

From the above observations, it appears that haem-starved cells cannot replenish their SE and TG pools because of substrate limitation (UFA for TG and free sterol moieties for SE).

Dga1p is a major contributor to TG synthesis under haem depletion

DGA1 [27,28] and *LRO1* [19,29] are the main contributors to TG synthesis in yeast. It has been shown that Dga1p can mediate

Figure 5 Relative contributions of Dga1p and Lro1p to TG synthesis

hem1Δα (open bars), *hem1Δdga1Δ* (black bars) and *hem1Δlro1Δ* cells (grey bars) were inoculated to a cell density of 4×10^6 cells in YPD + ALA (ALA), YPD without addition (YPD) or YPD + oleate (Ole). Cells were allowed to grow under these conditions for 24 h and TG were extracted from 2×10^9 cells as described in the Experimental section. The acyl chain composition of TG was determined for each condition. The total acyl chain levels (A) and the specific SFA (B) or C_{18:1} Δ⁹ (C) contents of TG are given. Values are means $\pm$ S.E.M. from at least three independent experiments.

most TG synthesis in stationary phase, since deletion of *DGA1* causes an $\approx 50\%$ reduction in TG synthesis in the late stationary phase whereas deletion of *LRO1* mainly affects TG synthesis in exponential growth [27]. The same observations were made when the steady state levels of TG in *hem1Δ*, *hem1Δdga1Δ* and *hem1Δlro1Δ* strains were determined under aerobiosis-like conditions (Figure 5A). The deletion of *DGA1* resulted in a 2.3-fold decrease in TG levels, affecting both their SFA (ALA, Figure 5B) and UFA (see C_{18:1} Δ⁹; ALA, Figure 5C) content. Deletion of *DGA1* also resulted in a reduction of TG content upon haem depletion (2.3-fold decrease; YPD, Figure 5A) as opposed to a deletion of *LRO1*. This phenomenon was not corrected by oleate supplementation (Ole, Figure 5A).

These results indicate that Dga1p is a major contributor to TG synthesis in non-haem-synthesizing cells, and that it catalyses the incorporation of both saturated and unsaturated acyl chains within TG.

Haem depletion induces a redistribution in the phospholipid pattern

In contrast to storage lipids, the total amount of phospholipids remained unchanged under haem depletion (Figure 6A). A closer examination however revealed redistribution among the various species, with, most notably, an increase of PtdIns (about 3-fold) at the expense of PtdSer (about 5-fold decrease; Figure 6B).

The lipid redistributions specifically related to haem depletion were analysed as a function of cell growth, by comparing the lipid content of haem-competent and haem-depleted cells (Figure 7). Dramatic changes could be observed, even in the presence of ALA (Figure 7A). Under these conditions, the main change was a decrease of PtdSer and PtdCho contents after 8 h in fresh medium (3.9- and 2.2-decrease, respectively; Figure 7A). The original levels of both PtdSer and PtdCho were then re-attained when the culture reached the stationary phase (see the 24 h point; Figure 7A). On the contrary, PtdIns levels remained relatively constant during growth (Figure 7A).

Upon haem depletion, SFA accumulation could be observed in PtdIns after 8 h (2.7-fold increase as compared with time 0), DAG (3.8-fold increase) and, to lesser extent, in PtdCho (1.4-fold increase; compare Figures 7B and 7D). At this time, free SFA content remained unchanged (Figure 7D). An increase of free SFA was finally observed after 24 h and saturated fatty acyl chains continued to accumulate in PtdCho and PtdIns (Figure 7D). As a consequence, the total amount of PtdIns increased within haem-starved cells after 24 h (Figure 7C). Moreover, PtdSer remained at low levels throughout the experiment upon haem depletion (Figure 7C) and this phospholipid did not reach its original levels after 24 h.

Altogether, these results suggest that haem-depleted cells favour PtdIns rather than PtdSer synthesis.

DISCUSSION

Reserve lipid dynamics under haem-induced lipid depletion

Our working hypothesis was that, even though reserve lipids are not essential to yeast grown under aerobiosis [23], they may play a role in physiological conditions when the cells have to face natural lipid-depletion, i.e. under haem or oxygen limitation. However, both TG and SE levels dropped in yeast cells when shifted from stationary phase to fresh medium, irrespective of haem availability (Figure 2A). This decrease in TG was, at least in part, due to hydrolysis, as was demonstrated by the [³H]glycerol labelling experiment (Figure 2B). Moreover, the growth of an *lro1Δdga1ΔarelΔare2Δ* quadruple mutant (strain H1246 [23]) was tested in anaerobiosis-type conditions and in the absence of both exogenous UFA and sterols. This strain, which does not contain any lipid reserves, was capable of residual growth (equivalent to a wild-type strain) in these conditions (results not shown). These observations indicate that TG and SE do not represent an efficient UFA and sterol storage to support growth upon lipid starvation and that their presence alone cannot account for the observed residual growth.

Alternatively, one may assume that reserve lipids could aid growth resumption by providing fatty acids and sterols required for the formation of membrane lipids. However, the lack of reserves was not detrimental in the early logarithmic phase either, since no delay in growth resumption could be observed with the *lro1Δdga1ΔarelΔare2Δ* quadruple mutant (results not shown). Thus the physiological relevance of lipid storage within TG and SE still has to be elucidated. A possible explanation comes from an observation from Sandager and co-workers [23], who demonstrated that the lack of lipid reserves was detrimental for long-term survival of stationary phase cells upon nitrogen

Figure 6 Consequences of haem-induced lipid depletion on the phospholipid composition

hem1Δ cells were cultivated in aerobiosis as described in the legend of Figure 2 and transferred for 24 h to YPD containing ALA (open bars) or to YPD without addition (black bars). Phospholipids were extracted, analysed and quantified as described in the Experimental section. The total amount of phospholipids (A) and the levels of each phospholipid species (B) are expressed as nmol of fatty acid (FA) per 10⁹ cells. Values are means ± S.E.M. from at least three independent experiments.

Figure 7 Time-course of SFA accumulation within lipid species

hem1Δ cells, cultivated in aerobiosis as described in the legend of Figure 2, were transferred to YPD medium supplemented with ALA (A and B) or to YPD medium without addition (C and D). Phospholipids and neutral lipids were extracted from cells after 0 h (open bars), 6 h (black bars), 8 h (grey bars) and 24 h (hatched bars) following the shift to fresh medium. The total amount of acyl chains (A and C) and the saturated acyl chain content (i.e. C_{16:0} and C_{18:0}; B and D) of the various lipid species were obtained as described in the Experimental section. Values are means ± S.E.M. from at least three independent experiments. FFA, non-esterified fatty acids.

starvation. This may lead one to assume that the constitutive lipid mobilization we observe may well be a crucial 'starter' for lipid-membrane synthesis when cells have been subjected previously to restrictive conditions which lead to the shut down of lipid metabolism. These aspects, which should highlight the physiological meaning of lipid reserve dynamics, will deserve deeper investigations.

However, once haem depletion is definitely attained, lipid starvation results in SFA accumulation (Figure 1B). Taking into consideration that the degree of acyl chain saturation of membrane lipids is a major regulator of membrane fluidity [30], the question arises as to how yeast can handle this huge increase in intracellular SFA.

We showed that free SFA were not stored at high levels within TG. Surprisingly, SFA levels in TG under haem depletion were strictly similar to those of cells grown under aerobiosis-like conditions (Figure 4E). However, TG synthesized under these conditions were specifically enriched in C_{16:0} (56% of TG acyl chains), which is the main SFA that accumulates within lipid species under haem-induced lipid starvation (results not shown). This observation suggests that TG display a limited capacity for the storage of SFA, even under conditions of SFA accumulation. This was confirmed when oleate was added to the medium. In this case, TG were mainly composed of C_{18:1} Δ⁹ (Figure 4C) but again, they displayed the same levels of saturated fatty acyl chains (Figure 4E).

The main contributors to TG synthesis in yeast are Dgalp [23,27,28] and Lro1p; [19,23,29]. Dgalp is located in the lipid particles and is an acyl-CoA:diacylglycerol acyltransferase [27,28], whereas Lro1p, which is associated with yeast microsome, is a phospholipid:diacylglycerol acyltransferase [19,29]. Therefore, the contributions of *DGAI* and *LRO1* to TG synthesis under conditions of haem-induced UFA starvation were investigated. It was confirmed that Dgalp is a major contributor to TG synthesis in aerobiosis-like conditions [27], but also under haem depletion, regardless of exogenous UFA availability (Figure 5). In contrast, deletion of *LRO1* did not affect TG levels in any conditions tested (Figure 5). It should be noted that *DGAI* has not been reported to be an hypoxic gene [1].

A deletion of *DGAI* affected both the saturated and unsaturated acyl chain content of TG, showing that this acyl-CoA:diacylglycerol acyltransferase displays a relatively wide specificity for the acyl-chains. Although oleyl-CoA ($C_{18:1} \Delta^9$) is the preferred Dgalp substrate *in vitro*, Dgalp can efficiently use palmitoyl CoA ($C_{16:0}$) as a substrate [27]. This is consistent with the fact that $C_{16:0}$ or, alternatively, $C_{18:1} \Delta^9$ (in the case of oleate supplementation) can be efficiently stored within TG, in a *DGAI*-dependent manner, when haem synthesis is precluded (Figure 5).

SFA cannot be efficiently stored within SE either (Figure 4). The absence of sterol moieties as SFA counterparts for SE synthesis can be accounted for by ergosterol biosynthesis shutdown (Figure 3B). However, haem-starved cells are capable of efficient SE synthesis, since it has been shown that exogenous supplied cholesterol can be efficiently esterified ([31]; T. Ferreira, unpublished work). This observation is in good agreement with the fact that an isoform of acyl-CoA:sterol acyltransferase, encoded by *ARE1*, is a hypoxic gene induced under haem depletion [32].

The fact that haem-starved cells display an efficient SE synthesis machinery could be seen as a biological paradox, since it is not expected that cells would favour storage under conditions of sterol starvation. However, one may find some physiological relevance in this phenomenon. First, it has been shown that Are1p displays a better specificity for sterol intermediates, with a preference for lanosterol [33]. Accordingly, we found that the major component of SE under sterol starvation is lanosterol (results not shown). Therefore, one may assume that Are1p may prevent the accumulation of potentially harmful sterol intermediates, such as lanosterol [34]. Secondly, one may also consider the specificity of steryl ester hydrolases. Indeed, we could show that deletion of *TGL1*, which encodes a potential steryl esterase [9] associated with lipid particles [26], results in the specific enrichment of ergosterol moieties and of unsaturated fatty acyl chains within SE (Figure 3A). These observations suggest that Tgl1p may display specificity for the hydrolysis of ergosterol esters and UFA containing SE. This is consistent with the fact that *TGL1* is up-regulated under conditions of both UFA and ergosterol depletion, i.e. under oxygen limitation [1]. Also consistent with this hypothesis is the observation that deletion of *TGL1* results in a decrease of free ergosterol (≈ 2 -fold decrease as compared with the corresponding wild-type strain; Figure 3B) when cells are shifted to sterol-starved conditions. Therefore one may consider that sterol homeostasis under haem depletion is as a result of a complex balance between the storage of specific toxic intermediates in SE and the release of free ergosterol to sustain, at least transiently, membrane proliferation and residual growth.

Altogether, these results highlight the involvement and the possible functions of two lipid particle-associated proteins, namely Dgalp and Tgl1p, in neutral lipid dynamics upon haem deprivation.

Diversion of phospholipid metabolism towards PtdIns in haem-starved cells

Since SFA levels increased within the cells and considering that they were not efficiently stored within reserve lipids, we examined the putative consequences on the saturation level of phospholipid acyl chains.

As shown in Figure 7(D), SFA accumulate preferentially in PtdIns. After 24 h under haem starvation, PtdIns contains as much as $\approx 80\%$ of SFA (Figure 7), which indicates that $\approx 65\%$ of the PtdIns molecules display two saturated fatty acyl chains. Accordingly, within the phospholipid population, disaturated fatty acid-containing species (two $C_{16:0}$ or one $C_{16:0}$ and one $C_{18:0}$) are only observed for PtdIns in normal aerobic conditions [24]. Since cells grown for 8 h under haem starvation can be rescued if shifted back to aerobiosis-like conditions (results not shown), this suggests that the observed SFA accumulation within PtdIns is not detrimental to growth. Therefore one may assume that PtdIns, due to its greater tolerance to saturated fatty acyl chains, may be a good candidate to minimize the putative deleterious effect of SFA incorporation into other phospholipids (Figure 7D).

CDP-DAG (cytidine diphosphate diacylglycerol) is a common precursor for PtdIns and PtdSer biosynthesis in the endoplasmic reticulum. PtdIns synthase (encoded by *PIS1*) catalyses PtdIns biosynthesis from CDP-DAG and inositol, whereas PtdSer synthase (encoded by *PSSI/CHO1*) is responsible for the biosynthesis of PtdSer from CDP-DAG and serine (for review, see [13]). Therefore, one may assume that the observed PtdIns increase, which occurs at the expense of PtdSer (Figure 7), is due to the competition between PtdIns synthase and PtdSer synthase for CDP-DAG. To account for the remarkable differences in the unsaturation level of PtdIns and PtdSer under oxygenated conditions (Table 3), Schneider and co-workers [24] proposed that PtdIns synthase and PtdSer synthase may possess distinct preferences for different molecular species of their common substrate, CDP-DAG. According to this hypothesis, PtdIns synthase would display a greater affinity towards CDP-DAG with higher acyl chain saturation, whereas PtdSer synthase would preferentially utilize the unsaturated CDP-DAG species. This hypothesis would fully account for our results under conditions of SFA accumulation.

Since *PIS1* has been shown to be moderately up-regulated upon oxygen limitation (1.8-fold increase [1]), an alternative explanation for the observed increase in PtdIns and low PtdSer levels in haem-depleted cells may be a general re-routing of phospholipid metabolism in response to *PIS1* transcriptional activation. However, we observed that *PIS1* induction is marginal in haem-depleted cells (results not shown).

To conclude, the increase in the level of PtdIns under haem depletion is probably the consequence of a complex mechanism, including biosynthesis re-routing and/or selective phospholipase activities. Since PtdIns is involved in many cellular functions, including signalling (as a source of polyphosphoinositides), protein trafficking (as the core of protein glycosylphosphatidylinositol anchors) and complex lipid synthesis (as a sphingolipid precursor), the putative effects of the observed PtdIns variations are presently under investigation. Identification of the genes involved in this process and the consequences of their deletion could bring new insights on the mechanisms that sustain yeast adaptation to haem/oxygen deprivation.

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