Alteration in the cytosolic triacylglycerol biosynthetic machinery leads to decreased cell growth and triacylglycerol synthesis in oleaginous yeast

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Altered nutrient content (levels of glucose) caused a drastic reduction in cell growth and triacylglycerol (TAG) production in the wild-type (WT) *Rhodotorula glutinis*. This was due to the decreased level of synthesis of TAG biosynthetic enzymes, reflected by a reduction in enzyme activity. A similar observation was made in the case of non-lethal mutants of TAG-deficient oleaginous yeast, namely *TAG1* and *TAG2*, which were generated by ethyl methane sulphonate mutagenesis. Metabolic labelling of TAG-deficient cells with [¹⁴C]acetate, [³²P]orthophosphate and [¹⁴C]mevalonate showed a negligible TAG formation with minimal alterations in phospholipid and sterol compositions. Assays on the activities of cytosolic TAG biosynthetic enzymes revealed that lysophosphatidic acid and diacylglycerol acyltransferases (ATs) were defective in *TAG1* and *TAG2* respectively. The activity of membrane-bound isoforms of TAG biosynthetic

enzymes remains unaltered in the mutants. Analysis of cytosolic TAG biosynthetic enzymes by immunoblotting and immunoprecipitation indicated that the defective ATs were a part of the TAG biosynthetic multienzyme complex. Quantitatively, the cytosolic lysophosphatidic acid-AT was comparable between *TAG1* and the WT. However, diacylglycerol-AT was relatively less in *TAG2* than the WT. These results demonstrated that either by decreasing the nutrient content or mutating the enzymes of the soluble TAG biosynthetic pathway, TAG production was decreased with concomitant reduction in the cell growth.

Key words: acyltransferases, diacylglycerol, fatty acid activation, oil mutants, *Rhodotorula glutinis*, triacylglycerol biosynthetic complex.

INTRODUCTION

Triacylglycerol (TAG) is the major form of storage lipid in many yeasts, oilseeds, adipocytes, etc. TAGs are synthesized by the progressive acylation of glycerol 3-phosphate by three acyltransferases (ATs), whereas the phosphate moiety is removed by a phosphatase [1,2]. Together, these reactions constitute the Kennedy pathway [3] for TAG biosynthesis. Endoplasmic reticulum is generally accepted as the main site of these enzymes [4–7]. Previous studies have suggested that some of these enzymes are also localized in the lipid bodies in yeast [8] and higher plants [9–11]. We have recently reported the presence of a TAG biosynthetic complex (TBC) in the cytosol of *Rhodotorula glutinis* [12]. Apart from the involvement of these enzymes in the generation of TAG, G3P-AT, lysophosphatidic acid (LPA)-AT and phosphatidic acid phosphatase (PAPase) are also involved in phospholipid biosynthesis. Hence, it is necessary to understand the mode of regulation between TAG and phospholipid biosynthesis. Diacylglycerol (DAG)-AT is the only enzyme that is committed to TAG biosynthesis. It has been suggested that DAG-AT may be one of the rate-limiting steps in storage lipid accumulation in plants and thus a potential target in the genetic modification of TAG biosynthesis. AS11, an ethyl methane sulphonate (EMS)-induced *Arabidopsis thaliana* mutant, was identified to have altered the fatty acid composition, low amounts of diacylglycerols and TAGs in developing seeds and delayed seed development [13]. The regulatory role of DAG-AT has also been reported for TAG biosynthesis in permeabilized rat hepatocytes [14–16]. In *Saccharomyces cerevisiae*, PAPase activity, corroborated with the increase in TAG content during the

stationary phase, suggested a role for PAPase in the regulation of TAG [17]. Intracellular translocation of PAPase from cytosol to endoplasmic reticulum during seed development in maturing safflower seeds could control the TAG biosynthesis in a feed-forward mechanism [18]. Yeast LPA-AT, when overexpressed in tobacco, caused an overall increase in the lipid content [19]. This indicated the absence of regulation downstream of LPA-AT. Thus, in different systems, various enzymes have been implicated in the regulation of TAG biosynthesis. To understand the regulation of TAG biosynthesis in oleaginous yeast, the present study was aimed at the isolation and characterization of the mutants that were defective in the soluble TAG biosynthesis. The physiological role of TAG synthesis was examined by analysing the growth of wild-type (WT) and mutant *R. glutinis* in various media. For the first time, evidence is provided in the present study to show that the cytosolic isoform of TAG biosynthetic enzymes is responsible for TAG accumulation in oleaginous yeast.

EXPERIMENTAL

Materials

R. glutinis (MTCC 1151) was obtained from the Institute of Microbial Technology (Chandigarh, India). [¹⁴C]Palmitoyl-CoA (51 mCi/mmol), [¹⁴C]fatty acids (55 mCi/mmol), [glycerol-¹⁴C(U)]phosphatidic acid (PA)-dipalmitoyl (100 mCi/mmol), [¹⁴C]acetate (51 mCi/mmol), [²⁻¹⁴C]mevalonic acid (53.9 mCi/mmol), [α -³²P]dATP (3000 mCi/mmol) and [³⁵S]-protein labelling mix were obtained from PerkinElmer Life

Abbreviations used: ACP, acyl carrier protein; AT, acyltransferase; DAG, diacylglycerol; EMS, ethyl methane sulphonate; PA, phosphatidic acid; LPA, lysophosphatidic acid; PAPase, phosphatidic acid phosphatase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SE, sterol esters; TAG, triacylglycerol; TBC, TAG biosynthetic complex; WT, wild-type.

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Sciences. [^{32}P]orthophosphate was from Board of Radiation and Isotope Technology (Mumbai, India). Silica-TLC plates were from Merck. All other reagents were from Sigma.

Growth conditions

Yeast cells were grown in malt-yeast extract medium (pH 7.0) containing 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.3 % (w/v) malt extract supplemented with 1 % (w/v) glucose (YPDM) with aeration at 30 °C. Alternatively, the cells were grown in 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone supplemented with 1 % dextrose (YPD) or minimal medium (MM) supplemented with 0.67 % (w/v) yeast nitrogen base, 0.2 % (w/v) amino acids and 1 % glucose. Yeast cells were also grown on high glucose (2 %) or low glucose (0.2 %) containing minimal medium. Cell density was determined by monitoring the turbidity or light scattering at 600 nm (1 unit = 8×10^7 cells/ml).

Chemical mutagenesis

Cells in the exponential phase of culture (100 ml) were made into pellets and suspended in 2.5 ml of 50 mM potassium phosphate buffer (pH 7.0). The cells were layered on to a 50 % (w/v) sucrose cushion (7.5 ml) and centrifuged at 3000 *g* for 20 min. Owing to the property of TAG accumulation, WT cells float on a 50 % sucrose cushion. The floating layer was collected and washed twice with potassium phosphate buffer. The washed cells were resuspended in 5 ml of the buffer, sonicated for 1 min at 50 % duty cycle and a power setting of 8 (Vibra cell) to dissociate the aggregated cells. EMS was added to a final concentration of 3 % (v/v). The cells were vortexed vigorously and incubated for 30 min at 30 °C. The reaction was terminated by the addition of 10 ml of 10 % (w/v) sodium thiosulphate. The cells were collected by centrifugation, washed twice with sterile water and resuspended in 2.5 ml of potassium phosphate buffer. The mutagenized cells were grown for 21 h in 50 ml of growth medium. The cells were harvested by centrifugation, and the cell pellet was suspended in 1 ml of buffer, sonicated for 1 min to dissociate the aggregated cells, layered on to a 50 % sucrose cushion (7.5 ml) and centrifuged. The TAG-deficient cells did not float but pelleted. The pellet thus obtained was washed twice with phosphate buffer. Cells were serially diluted and plated on YPDM containing 1 % (w/v) agar.

The mutants, *TAG1* and *TAG2*, stored at -80 °C as glycerol stock were revived after a few freeze-thaw cycles and grown for 21 h and the cells were layered on top of the 50 % sucrose cushion and centrifuged. The floating layer obtained after the centrifugation was collected and washed with buffer. The cells obtained were used for another round of enrichment of revertants.

Quantitative analysis of TAG

The analyses were performed on silica gel G TLC plates, prewashed with chloroform/methanol (1:1, v/v) and activated at 120 °C. To obtain a linear densitometric response, the upper limit of the sample weight was determined to be 15 μg /spot. All the spots in the present study were kept between 10 and 15 μg [20]. WT and mutant cells (100 ml of culture) were grown in different media as described above, and the cultures were centrifuged and cell pellets were dried by freeze-thawing individually. Dried cells (25 mg) were used for lipid extraction as described previously [21]. Appropriate amounts (equal aliquots from each set) of lipid samples along with standards were spotted on TLC plates and developed with light petrol/diethylether/acetic acid (70:30:1, by vol.). The plates were sprayed with 50 %

(v/v) sulphuric acid and charred on an oven. The intensity of the charred TAG spots was analysed densitometrically using image master VDS software.

Nile Blue A staining

A smear of cells grown for 21 h was prepared on a glass slide and the heat fixed. The slides were immersed in 1 % (w/v) aqueous solution of Nile Blue A stain for 10 min at 55 °C. The slides were washed with water to remove the excess stain followed by washing for 1 min in 8 % (v/v) acetic acid. The slides were then rinsed in water, air-dried and visualized under fluorescence microscope (magnification $\times 60$).

Metabolic labelling with [$1\text{-}^{14}\text{C}$]acetate, [^{32}P]orthophosphate and [$2\text{-}^{14}\text{C}$]mevalonic acid

Yeast cells were grown in various media (as described in the Experimental section and the Figure legends) up to late exponential phase (21 h) and an equal number of cells (10^7) were pelleted in each case. The pellet was resuspended in 0.4 ml of Tris buffer (pH 7.5) and 2.5 μCi of [^{14}C]acetate was added and grown for 5 h with shaking at 30 °C. The yeast cells, during this labelling, cross two generations, since 2.5 h is the doubling time. For [^{32}P]orthophosphate and [^{14}C]mevalonate labelling, cells were treated in the same way and 2.5 μCi of each label was added and incubated for 1 and 3 h respectively. After the incubation period, the cell pellet was washed with ice-cold PBS. To the pellet, 0.5 ml of 10 % acetic acid in propan-2-ol (v/v) was added and boiled for 5 min. To the mixture, 1 ml of hexane was added and vortexed thoroughly [20]. The hexane layer was removed and concentrated, and lipids were separated on silica gel G TLC plates developed with light petroleum/diethyl ether/acetic acid (70:30:1, 80:20:1, by vol.) to separate the neutral lipids or with chloroform/methanol/acetone/acetic acid/water (10:2:4:3:1, by vol.) solvent system to resolve phospholipids. Lipids were identified by comparing their migration with the standards and the TLC plates were subjected to autoradiography. The individual spots were scraped off from the plate and the radioactivity was measured in a liquid-scintillation counter.

Preparation of subcellular fractions

Late-exponential phase cells were resuspended in 10 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl_2 , 1 mM PMSF, 100 μM of leupeptin and 5 % (w/v) sucrose. The cells were lysed using glass beads (0.45–0.6 μm) in the absence of detergent. Equal volumes of acid-treated glass beads were added to the cell suspension and vortexed for 1 min followed by incubation on ice for 1 min. This treatment was repeated approx. 12 times. The lysate was precleared by centrifugation at 300 *g* for 15 min. The supernatant was subjected to centrifugation at 10000 *g* for 15 min. The supernatant thus obtained was further centrifuged at 240000 *g* for 60 min to obtain the cytosol (soluble fraction). The pellet was washed and resuspended in the lysis buffer and centrifuged at 240000 *g* for 60 min to obtain the pellet (membranes). All operations were performed at 4 °C.

Purification of TBC

All operations were performed at 4 °C. The soluble fraction from the exponentially growing cells was used for purification. Cytosol was loaded on a 7 % native polyacrylamide gel and electrophoresed under constant current at 4 °C. After the run, the resolving gel was cut into 0.5 cm slices and the protein was eluted by finely crushing the gel pieces in 10 mM Tris/HCl buffer (pH

7.5), containing 0.1 M NaCl, 5 mM MgCl₂ and 5% sucrose, and incubated overnight at 4 °C. The gel-eluted protein was used for further studies and was estimated by the method of Bradford [22].

Enzyme assays

LPA-AT and DAG-AT activities were measured as described previously [12]. For the PAPase assay, PA was suspended in 1% (v/v) Triton X-100 and sonicated for 5 min in the sonic bath. The substrate stock solution was diluted ten times in the assay. PAPase assay mixture consisted of 50 mM Tris/HCl (pH 7.5), 50 µM [¹⁴C]PA (220 000 d.p.m.) and 5–25 µg of TBC or 20–45 µg of cytosol in a total volume of 100 µl. The assay was linear for 15 min even at the highest protein concentration used in the assay (45 µg). The incubation was performed at 30 °C for 10 min and stopped by the addition of 50 µl of 3 M HCl followed by 400 µl of chloroform/methanol (1:2, v/v). After lipid extraction, the lower chloroform-soluble materials were separated by TLC on 250 µm silica gel G plates using chloroform/methanol/water (196:4:1, by vol.) as the solvent system [21]. The lipids were visualized with iodine vapour and spots of DAG were scraped off for determination of radioactivity by liquid-scintillation counting. Other assays [LPA-AT, DAG-AT, acyl-carrier protein (ACP) and acyl-ACP synthetase] were performed as described previously [12,23]. Boiled enzyme and zero time were used as controls. Average control values were subtracted from the actual assay value and the specific activity was calculated after the correction. A time course of the assays for all three enzymes was performed using 20 µg of TBC as the enzyme source.

Antisera production

The peptide CY-VVGAQPFGGARGS corresponding to DAG-AT was conjugated to BSA using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester [24]. Antibodies were raised against the synthetic peptides of LPA-AT, PAPase and DAG-AT as described previously [12].

Western blotting and immunoprecipitations

For immunoblotting, proteins (60–75 µg of total protein was loaded in each case) were separated by either native PAGE or SDS/PAGE and transferred on to a nitrocellulose membrane for immunoblotting. Peptide-specific antibodies were used at a dilution of 1:600 and ACP antibody at a dilution of 1:3000. Acyl-ACP synthetase antibody was used at a dilution of 1:400. All the antibodies were diluted in Tris-buffered saline containing 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA. Immunoprecipitations were performed as described previously [12].

RESULTS

TAG formation in various growth media

R. glutinis was grown on various media to determine the effect of nutrient content on TAG formation. WT cells were grown on minimal medium in the presence of decreasing concentrations of dextrose and in the absence of malt extract and the growth rate was determined by measuring the light scattering at 600 nm (Figure 1a). With decreasing concentrations of sugar in the growth medium, there was a significant decrease in the cell density, which was observed initially during the exponential phase and continued till the stationary phase. When the logarithm of light scattering was plotted as a function of growth time (Figure 1b), no significant change in the growth pattern was

observed either in the minimal medium or in the rich medium. Under limiting dextrose concentrations, cells were stained at various time intervals with Nile Blue A and viewed under fluorescence microscope. Results indicated that both the YPD and 0.2% of glucose-supplemented cells accumulate lower amounts of TAG as compared with cells grown in YPDM (results not shown). This was measured by quantification of lipids extracted from cells grown for 21 and 36 h. Whereas WT cells grown for 36 and 21 h in YPDM accumulated 34 ± 3.9 and 26 ± 2.1 mg of TAG/100 mg of dried cells respectively, the same cells grown for 36 h in 0.2% glucose-containing medium accumulated only 12.16 ± 0.9 mg of TAG/100 mg of dried cells. Studies on the incorporation of [¹⁴C]acetate into neutral lipids revealed that the amounts of free fatty acids, TAG and sterol esters (SEs) were reduced in cells grown in YPD and low-glucose containing media (Figure 1c). A similar pattern of SE and SE labelling was observed with [¹⁴C]mevalonic acid (Figure 1e). There was a drastic reduction in the phospholipids when the cells were grown in YPD and low-glucose containing medium as seen in the level of incorporation of [³²P]orthophosphate in the total phospholipid (Figure 1d).

Time-course analyses of different enzymes of TBC

R. glutinis cells were grown in YPDM media for 21 h, and the cytosol and membranes were prepared. The purified TBC (20 µg) was used in the time course studies for LPA-AT, PAPase and DAG-AT (Figure 2a). In brief, reaction was stopped at the indicated time points and the respective activities were calculated. We observed that LPA-AT and DAG-AT activities were linear until 30 min and PAPase showed linearity until 15 min. Similar experiments were performed with total membranes and the results are presented in Figure 2(b).

Analysis of TAG biosynthetic enzyme from *R. glutinis* grown on various media

The cells grown on sugar-limiting media had significantly low levels of neutral lipids and phospholipids. In order to examine the cause for the decreased TAG biosynthetic capacity, we measured the LPA-AT, PAPase and DAG-AT activities from the cytosolic and the membrane fractions of yeast grown in various media (Table 1). When *R. glutinis* was grown on low sugar, the cytosolic LPA-AT, PAPase and DAG-AT activities decreased by 53, 47 and 66% respectively, as compared with the activities of the cells grown in rich medium. The microsomal LPA-AT, PAPase and DAG-AT showed only 13–19% reduction in the activity when the cells were grown in 0.2% glucose-containing medium (Table 1).

Immunoblot analyses were performed on the cytosolic fractions (75 µg of total protein) from the cells grown in various media using LPA-AT and DAG-AT, PAPase and ACP-specific antibodies. The immunoblots revealed a severe decrease in the LPA-AT (Figure 2c), PAPase (Figure 2d) and DAG-AT (Figure 2e) amounts (42, 62 and 64% respectively) in the cells grown on YPD or low-glucose containing media. ACP profile did not show any significant change (Figure 2f). These studies indicated that the decrease in TAG production in cells grown on YPD or low-glucose media could be due to decreased level of expression of the genes (Figure 2) that resulted in decreased TAG biosynthetic enzyme activities (Table 1).

Generation and screening of TAG-deficient *R. glutinis*

To address further the role of TAG biosynthesis with respect to its production and regulation, WT cells were subjected to EMS

Figure 1 Effect of various media on the growth and lipid accumulation of WT *R. glutinis*

(a) Exponentially growing culture of WT *R. glutinis* was added to a final concentration of 1% in YPDM (●) or YPD (▲) or MM (◆) or high-glucose (△) or low-glucose (□) containing fresh medium and incubated at 30 °C under constant shaking. At regular time intervals, turbidity was measured at 600 nm. (b) The results from (a) were replotted on a exponential scale. (c) The WT cells grown for 21 h in various media (YPDM, lane 1; MM, lane 2; high-glucose medium, lane 3; low-glucose medium, lane 4; YPD, lane 5) were metabolically labelled for 5 h with [^{14}C]acetate ($2.5 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$). The total lipids were extracted and the lipid was resolved on silica-TLC using light petrol/diethyl ether/acetic acid (70:30:1, by vol.) solvent system followed by autoradiography. (d) The labelling was done with [^{32}P]orthophosphate ($1 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$) for 1 h and phospholipids were resolved on TLC using the chloroform/methanol/acetone/acetic acid/water (10:2:4:3:1, by vol.) solvent system followed by autoradiography. (e) The metabolic labelling was done with [^{14}C]mevalonate ($2 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$) for 5 h and the total lipids were extracted and resolved on silica-TLC using light petrol/diethyl ether/acetic acid (70:30:1, by vol.) solvent system followed by autoradiography. The TLC experiments were performed three times and a typical autoradiograph is shown in each case. In all the labelling experiments, cell number was normalized to 10^7 cells and total lipid extracted from the cells was resolved on silica-TLC. FFA, free (non-esterified) fatty acid; NL, neutral lipids; LPC, lysophosphatidylcholine.

mutagenesis to generate TAG biosynthesis mutants. Subjecting the mutagenized cells to a simple screen enhanced the TAG-deficient cell population. Owing to accumulation of lipids, the WT cells floated on 50% sucrose cushion, whereas the cells defective

in TAG biosynthesis had lost the property to accumulate TAG and thus pellet under the same conditions. The TAG-deficient cells thus obtained were stained with Nile Blue A to determine the extent of lipid formation. In addition, total lipid was extracted

Figure 2 Time-course analyses and immunoblot of the cytosolic TAG biosynthetic enzymes

The cytosol (**a**) and membrane (**b**) prepared from the culture grown for 21 h were used to determine the activities of LPA-AT (●), DAG-AT (○) and PAPase (▼) at different time points. The activity was represented graphically as a function of time. The experiments were performed four times and the values represent means $\pm$ S.E.M. The cytosol from 21 h grown *R. glutinis* in YPDM (lane 2) or YPD (lane 3) or MM (lane 4) or high- (lane 5) or low- (lane 6) glucose-containing medium were resolved on SDS/polyacrylamide gels and probed with anti-LPA-AT (**c**) or anti-PAPase (**d**) or anti-DAG-AT (**e**) or anti-ACP (**f**) antibodies in immunoblots. An equal number of cells was used to prepare cytosol and 75 μ g of total protein was loaded in each lane. Molecular-mass markers in kDa are shown on the left-hand side of each panel.

from all the mutants and the lipid profile was analysed by silica-TLC using neutral and phospholipid solvent systems. Analysis of the neutral lipid profile of the mutagenized cells demonstrated that the mutants were defective in TAG production, as there was no visible TAG spot on the TLC. From the total pool of 610 mutants, about 20 mutants showed negligible TAG formation.

We chose two mutants, *TAG1* and *TAG2* having unaltered non-esterified fatty acid and low TAG content for further studies.

Growth studies on TAG biosynthetic mutants

The growth rates of WT oleaginous yeast cells and the mutants,

Table 1 TAG biosynthetic enzyme activities (pmol · min⁻¹ · mg protein⁻¹) from cells grown in various media

The exponential phase oleaginous yeast cells from 1 litre of culture, grown on various media were lysed with glass beads and fractions were obtained by differential centrifugation. Enzyme activities (specific activity) were measured as described in the Experimental section. Values are the means ± S.E.M. for three separate experiments, each performed in duplicate.

	YPDM	MM	2% Glc	YPD	0.2% Glc
LPA-AT					
Cytosol	1.55 ± 0.013	1.52 ± 0.014	1.45 ± 0.012	0.82 ± 0.006	0.73 ± 0.005
Membranes	1.89 ± 0.017	1.89 ± 0.019	1.90 ± 0.015	1.61 ± 0.014	1.54 ± 0.010
PAPase					
Cytosol	1.15 ± 0.009	1.12 ± 0.010	1.15 ± 0.012	0.68 ± 0.006	0.63 ± 0.005
Membranes	1.49 ± 0.010	1.47 ± 0.011	1.45 ± 0.010	1.21 ± 0.011	1.29 ± 0.009
DAG-AT					
Cytosol	0.86 ± 0.005	0.88 ± 0.003	0.85 ± 0.005	0.37 ± 0.001	0.29 ± 0.001
Membranes	1.97 ± 0.013	1.94 ± 0.017	1.87 ± 0.015	1.68 ± 0.013	1.65 ± 0.014

Figure 3 Growth pattern of TAG-deficient *R. glutinis*

Exponentially growing cultures of *R. glutinis* (●, WT; □, TAG1; △, TAG2) in malt-yeast extract medium were added to a final concentration of 1% to fresh medium and incubated at 30 °C under constant shaking. At regular time intervals, growth was measured as the turbidity at 600 nm. The values were plotted on a linear scale graph (a) and a semi-log scale graph (b). TAG1 (c) and TAG2 (d) were grown in YPDM (●) or MM (◇) or YPD (▲) or high-glucose (△) or low-glucose (□) containing medium and turbidity was measured.

grown at 30 °C in rich medium, minimal medium, YPD, high- and low-glucose-containing media were monitored by measuring the turbidity or light scattering at 600 nm. When the turbidity was plotted on a linear scale for TAG1 and TAG2 as compared with WT a decrease in the cell density was evident in YPDM medium from late-exponential to stationary phase. TAG2 cell

number was nearly half of that of the WT cells in rich medium at the stationary phase of growth (Figure 3a). The TAG1 and TAG2 mutants had almost similar growth pattern in the early exponential phase of growth as compared with WT (Figure 3b). The growth of TAG1 (Figure 3c) and TAG2 (Figure 3d) decreased further when grown in YPD or 0.2% glucose-

Figure 4 [^{14}C]Acetate labelling of TAG-deficient oleaginous yeast cells

(a) WT, *TAG1* and *TAG2* grown in YPDM or MM or high- or low-glucose-containing medium were labelled with [^{14}C]acetate ($2.5 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$) for 5 h followed by lipid extraction. Lanes 1, 4, 7, 10 represent WT, lanes 2, 5, 8, 11 represent *TAG1* and lanes 3, 6, 9, 12 represent *TAG2*. The lipids were resolved on silica-TLC using light petrol/diethyl ether/acetic acid (70:30:1, by vol.) followed by autoradiography. The labelling experiments were repeated three times and a typical autoradiogram is shown. The spots were identified with the help of standards and individual spots were scraped and the associated radioactivity was counted in a liquid scintillation counter. The incorporation of [^{14}C]acetate in various lipids of WT (□), *TAG1* (■) and *TAG2* (■) cells grown in different media was measured in (b) (YPDM), (c) (MM), (d) (2% glucose) and (e) (0.2% glucose). In all the labelling experiments, cell number was normalized to 10^7 cells and the total lipid extracted from the cells was resolved on TLC. The values are means $\pm$ S.E.M. of three independent experiments. PL, phospholipids.

Figure 5 Metabolic labelling of WT, TAG1 and TAG2 with $[^{14}\text{C}]$ sodium acetate, $[^{14}\text{C}]$ mevalonate and $[^{32}\text{P}]$ orthophosphate

(a) WT, TAG1 and TAG2 were grown in YPDM medium and labelled with $[^{14}\text{C}]$ acetate for 5 h and the extracted lipids were resolved on TLC by chloroform/methanol/acetone/acetic acid/water (10:2:4:3:1, by vol.) solvent system followed by autoradiography. The TLC experiments were performed four times and a typical autoradiograph is shown. (b) WT, TAG1 and TAG2 grown in YPDM were labelled with $[^{14}\text{C}]$ mevalonate ($2 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$) for 5 h followed by lipid extraction. For two independent sets of experiments, the lipids were resolved on silica-TLC using light petrol/diethyl ether/acetic acid (70:30:1, by vol.) solvent system followed by autoradiography. A typical autoradiograph is shown. (c) $[^{32}\text{P}]$ Orthophosphate ($2.5 \mu\text{Ci} \cdot \text{ml}^{-1} \cdot 10^7 \text{ cells}^{-1}$) was used for labelling the WT, TAG1 and TAG2 grown in YPDM and the radiolabelled lipids were resolved on TLC by employing chloroform/methanol/acetone/acetic acid/water (10:2:4:3:1, by vol.) solvent system followed by autoradiography. (d) The incorporation of $[^{32}\text{P}]$ orthophosphate into phosphatidic acid of WT ($\square$), TAG1 ($\blacksquare$) and TAG2 ($\blacksquare$) was measured by scraping the respective spots. Silica-TLC experiments were performed four times and a typical autoradiograph is shown. Cell number was normalized to 10^7 cells and the total lipid extracted from the cells was resolved on TLC.

Figure 6 Enzymic characterization of *TAG1* and *TAG2*

WT (a) *TAG1* (b) and *TAG2* (c) cytosolic (240 000 *g* supernatant) LPA-AT (○), PAP (●) and DAG-AT (■) total activities were determined for 3×10^8 cells at various stages of growth in YPDM medium. Values are the means $\pm$ S.E.M. of five separate experiments, each performed in duplicate.

containing media. No accumulation of TAG was found in 21 h-grown TAG-deficient cells upon staining with Nile Blue A (results not shown). The amount of TAG was quantified using the silica-TLC and it was observed that the *TAG1* and *TAG2* accumulated 2.28 ± 0.2 and 3.06 ± 0.4 mg TAG/100 mg of dried cells at 36 h respectively.

Labelling studies on TAG mutants

TAG1 and *TAG2* were characterized to determine the nature of defect in TAG biosynthesis. Time course experiments on the incorporation of [14 C]acetate into lipids of WT *R. glutinis* indicated that the incorporation of acetate into TAG and phosphatidylcholine (PC) reached the maximum at 7 h of label-

Figure 7 Soluble and membrane-bound isoforms of TAG biosynthetic activities from *TAG1* and *TAG2*

(a) *R. glutinis* WT (□), *TAG1* (▒) and *TAG2* (■) cytosolic LPA-AT, PAPase (PAP) and DAG-AT activities were determined at the 21 h of growth. (b) The total membranes were used for the determination of LPA-AT, PAPase and DAG-AT activities of the WT and mutants. (c) The TBC was purified from native PAGE from the 21 h grown cultures of WT, *TAG1* and *TAG2* cells and used for the determination of activities of LPA-AT, PAPase and DAG-AT. The activities were measured as described in the Experimental section. The values are the means $\pm$ S.E.M. of three separate sets of experiments, each performed in duplicate.

Figure 8 Components of analyses of TBC in *TAG1* and *TAG2* by Western blots

LPA-AT antiserum (a), DAG-AT antiserum (b), anti-PAPase 2 (anti-PAP 2) (c), and acyl-ACP synthetase antiserum (d) were used to probe the TBC of the WT, *TAG1*, and *TAG2* after SDS/PAGE (12%) and subsequent transfer to nitrocellulose membrane. Cell number was normalized prior to cytosol preparation and 60 μ g of the total protein was loaded on to each lane. Normal rabbit serum was used as the negative control. (e) The spontaneous 'revertants' of the *TAG1* and *TAG2* were purified from the floating layers on 50% sucrose. After two rounds of purification, revertants were allowed to grow for 21 h and then they were metabolically labelled with [¹⁴C]acetate (2.5 μ Ci $\cdot$ ml⁻¹ $\cdot$ 10⁷ cells⁻¹) for 2 h 30 min. Lipids were extracted from an equal number of cells and analysed using light petrol/diethyl ether/acetic acid (70:30:1, by vol.) solvent system to resolve the neutral lipids, and then autoradiography was performed. (f) The TAG spots were identified, scraped and the associated radioactivity was determined. The experiment was repeated for three times and a typical autoradiogram is shown.

ling and we could observe reasonable incorporation of label in other storage lipids like sterol and SE only at 4 h (results not shown). The pattern of incorporation of [¹⁴C]acetate into various lipids of WT, *TAG1* and *TAG2* was compared. Analysis of the neutral lipid profile of the *TAG1* and *TAG2* indicated that both the mutants were defective in TAG production under various growth media. *TAG1* and *TAG2* accumulated minimum amounts of TAG (8–13%) as compared with WT cells grown in YPDM or 2%-glucose-containing medium (Figure 4a). The TAG formation was only 24% in *TAG1* and 33% in *TAG2* as compared with WT in minimal medium. In low glucose medium, there was no detectable amount of TAG even in WT and the mutants. No significant change was observed in the sterol content in all cell types under various growth conditions.

However, there was no detectable amount of SE in the cells grown in low glucose medium. When the ratio of sterol against other neutral lipids was considered, it was found that the ratio was nearly constant with WT as well as the mutants in all the media, except in low glucose where the ratio was 8–10 times lower.

When [¹⁴C]acetate-labelled lipids were resolved on TLC using the phospholipid solvent system (Figure 5a), *TAG1* showed a 64% reduction in PA as compared with WT. *TAG2* showed an increase (18%) in PC content as compared with WT and there was a considerable increase (31–39%) in phosphatidylethanolamine (PE) content as well. The profiles of other phospholipids were similar to that of the WT. Sterol and SE formations were monitored by labelling the cells with [¹⁴C]mevalonic acid

and it was found that the labelling pattern was comparable with that of WT (Figure 5b). Hence, a decrease in TAG accumulation is not compensated by a concomitant increase in SEs (storage lipid).

To understand the regulation of phospholipid biosynthesis, the mutants and the WT cells were labelled with [32 P]-orthophosphate and total lipids were extracted and resolved on TLC using a phospholipid solvent system (Figure 5c). *TAG2* showed marginal increase in PC levels than that of WT *R. glutinis*. A similar observation was also made with [14 C]acetate labelling (Figure 5a). On the other hand, *TAG1* phospholipid profile indicated a 77% reduction in the PA content (Figure 5d). The phospholipid profile obtained by labelling with [14 C]acetate (Figure 5a) or [32 P]orthophosphate (Figure 5c) showed an increase in the PE content in the mutants as compared with WT. Differences in the labelling of PE with [14 C]acetate and [32 P]-orthophosphate could be due to the independent turnover of the head group and acyl moiety in the membrane lipid.

Determination of the mutation in *TAG1* and *TAG2*

To determine the defect in the TAG biosynthetic mutants, the individual TAG biosynthetic enzyme activities from the cytosol were measured as a function of time. The DAG-AT and PAPase activities of *TAG1* were comparable with that of WT (Figure 6a) but it had < 5% total LPA-AT activity in the cytosol (Figure 6b). Nevertheless, *TAG2* had only approx. 12% DAG-AT activity that led to the reduced TAG formation (Figure 6c). These results suggested that the *TAG1* and *TAG2* had a defective LPA-AT and DAG-AT respectively.

Activities of the isoforms of TAG biosynthetic enzymes

To get an insight into the isoforms of enzymes of the TAG biosynthetic pathway, the cytosol (240000 g supernatant) and the membrane-bound TAG biosynthetic enzymes were isolated from the mutants and WT and their activities were measured. The cytosolic LPA and DAG-AT activities from *TAG1* and *TAG2* respectively, were compromised (5–15%), as compared with WT in cells grown in YPDM (Figure 7a). It was surprising to observe that the membrane-bound TAG biosynthetic enzyme activities were unaltered in the total membrane fraction from the mutants (Figure 7b). Native polyacrylamide gel-purified cytosolic TBC from WT and mutants were used for assaying individual enzyme activities (Figure 7c). A severe reduction in specific activity was observed in LPA-AT in the case of *TAG1* and DAG-AT in the case of *TAG2*. The PAPase activity remained almost the same.

Cytosolic TAG biosynthetic enzyme profiles of *TAG1* and *TAG2*

To establish the apparent difference observed in the LPA-AT and DAG-AT activities, immunoblot analyses were performed with the purified TBC. There was no apparent change in the levels of LPA-AT and PAPase in Western blot from the TBC of *TAG1* and *TAG2* as compared with the WT (Figures 8a and 8c). The amount of DAG-AT was comparable with WT and *TAG1* cells, whereas there was a considerable decrease in the amount of cytosolic DAG-AT in the *TAG2* mutant (Figure 8b).

Western blotting was performed using anti-acyl-ACP synthetase antibodies to detect the amount of synthetase in the TBC, and it was found that both *TAG1* and *TAG2* had comparable amounts of the enzyme to the WT (Figure 8d). The formation of acyl-ACP by the TBC from the WT and mutants was measured and the amounts of acyl-ACP formed were almost the same, indicating the presence of a functional acyl-ACP

Table 2 TAG biosynthesizing capacity (pmol · min⁻¹ · mg protein⁻¹) of the immunoprecipitates

The enzyme activities were measured using [14 C]16:0-CoA as an acyl donor. Values are means ± S.E.M. of three separate experiments each performed in duplicate.

	LPA-AT	PAPase	DAG-AT
Anti-LPA-AT			
WT	1.52 ± 0.06	0.88 ± 0.03	0.28 ± 0.02
<i>TAG1</i>	1.15 ± 0.01	0.78 ± 0.04	0.26 ± 0.01
<i>TAG2</i>	1.45 ± 0.05	0.81 ± 0.01	0.04 ± 0.00
Anti-PAPase (major)			
WT	1.38 ± 0.06	0.86 ± 0.04	0.22 ± 0.01
<i>TAG1</i>	0.07 ± 0.02	0.81 ± 0.03	0.25 ± 0.00
<i>TAG2</i>	1.34 ± 0.05	0.84 ± 0.01	0.06 ± 0.00
Anti-acyl ACP synthetase			
WT	1.96 ± 0.01	0.88 ± 0.01	0.75 ± 0.06
<i>TAG1</i>	0.05 ± 0.00	0.76 ± 0.02	0.69 ± 0.04
<i>TAG2</i>	1.90 ± 0.02	0.81 ± 0.05	0.07 ± 0.00

synthetase (results not shown). Finally, the individual TBC enzyme activities were measured in the immunoprecipitates of anti-LPA-AT, PAPase, acyl-ACP synthetase and ACP antibodies. The LPA and DAG-AT activities were negligible in the *TAG1* and *TAG2* mutants respectively (Table 2). These results demonstrated that *TAG1* and *TAG2* had a defective LPA-AT and DAG-AT respectively.

Isolation and characterization of revertants

The mutants were stored as 50% (v/v) glycerol stock at 80 °C. The cells which had undergone 3–5 freeze–thaw cycles were used for isolation of the revertants using the same technique as isolation of the mutants from sucrose cushion. The floating layers found after the centrifugation were considered as spontaneous revertants and grown. The incorporation of [14 C]acetate into the neutral lipids was observed by metabolic labelling of the cells as described above. Both the reverse mutants behaved like the WT cells (Figures 8e and 8f). These results suggested a possible point mutation in the isolated *TAG1* and *TAG2*.

DISCUSSION

TAG biosynthetic enzymes in oleaginous yeast (*R. glutinis*) are organized as a multi-enzyme complex in the cytosol [12]. The present study focuses on the role of cytosolic machinery in TAG synthesis and accumulation. TAG is the major form of storage lipid in eukaryotes. In addition, unsaturated TAGs have also been shown to be essential for the penetration of the pollen tubes into the stigma of tobacco plants [25]. EMS-induced mutation in the DAG-AT in *A. thaliana* showed a reduction in seed TAG and delayed cell growth [13]. EMS generally causes point mutations, but one cannot rule out the possibility of induced deletions and insertions [26,27]. When *R. glutinis* was grown on sugar-limiting media, the WT cells failed to accumulate TAG and SE and there was a significant reduction in cell growth, suggesting the role of TAG in cell growth. The availability of the precursors for TAG and phospholipid biosynthesis may modulate the expression level of the enzymes involved in the respective pathway at the transcriptional level, which may in turn control cell growth [28]. Fatty acids and their derivatives could also modulate the amount of transcription factors, thereby potentially acting as regulators of expression of these genes [29].

The following lines of evidence indicate that cytosolic TAG biosynthetic machinery is involved in the accumulation of

TAG. First, WT cells grown under low carbon source showed a significantly low level of TAG accumulation. The cytosolic fractions from the WT cells grown under low carbon source also showed a decreased TAG biosynthetic activity and transcriptional level of proteins involved in TAG biosynthesis. Total membrane extract from the cells, however, did not show an appreciable reduction in the activity. Secondly, TAG biosynthetic mutants showed a drastic reduction in TAG accumulation with a corresponding decrease in LPA-AT and DAG-AT activities in the cytosol. The membrane fraction showed an unaltered TAG biosynthetic activity in the mutants. These results differ from those obtained with other eukaryotic systems, where TAG biosynthesis is associated solely with membrane fraction [1–4].

The mutant studies have increased our understanding of the regulation of lipid biosynthesis [30,31]. The isolation of non-lethal mutants in LPA-AT and DAG-AT confirms the assumption that the segregated pools of lipids are generated in different regions of the cell by different isoforms of the enzymes and are used for different functions. In WT *E. coli*, it is proposed that fatty acid and phospholipid synthesis is coupled [32]. The mutations in the enzymes of the TAG biosynthetic pathway in the cytosol have no effect on fatty acid biosynthesis, indicating that fatty acid and glycerolipid biosynthesis are not coupled in oleaginous yeast. Physiological analysis of these mutants establishes the role of cytosolic isoforms in TAG accumulation. It was reported earlier in *S. cerevisiae* that a deletion mutation in oxysterol-binding protein (YDL109c) caused an elevation in TAG levels [33]. By contrast, the isolated TAG-deficient *R. glutinis* cells did not show an elevated level of SEs as a compensatory mechanism of fatty acid storage.

In the present study, we observed that there was a marginal increase in the phosphatidylserine (PS), PE and PC levels in the mutants. PS is shown to be an important intermediate in the fresh synthesis of the two main yeast phospholipids PE and PC [28]. Thus in the mutants there may be a change in the membrane phospholipid composition due to increased phospholipid levels.

TAG biosynthetic isoform activities from WT and the isolated mutants revealed that the membrane-bound enzymes were unaltered whereas the cytosolic enzymes were defective in oleaginous yeast. Although TAG biosynthetic activities (40%) are associated with the total membrane fraction, it could not compensate for the TAG accumulation in oleaginous yeast grown in all media except in minimal medium. Our studies revealed that the membrane-bound isoform had no direct involvement in the TAG biosynthesis for storage purposes. However, these could be involved in TAG biosynthesis that may be required for other cellular processes. Several mutants of the glycerolipid biosynthetic pathway have been generated in various systems. The JC201 strain of *E. coli* contains a temperature-sensitive lesion in LPA-AT [34], and a similar mutant was also isolated from *S. cerevisiae* [35]. Alteration of this enzyme in the mutant is detrimental as it is involved in membrane lipid synthesis [30,34]. On the other hand, mutation in LPA-AT from *Nisseria meningitidis* [36] and the plastidial LPA-AT [37] did not cause any significant changes in the glycerolipid profile, indicating the presence of multiple pathways for PA biosynthesis. In our studies, the isolated non-lethal *TAG1* from oleaginous yeast was defective in the soluble LPA-AT. Analysis of TAG biosynthetic enzymes revealed that the *TAG2* mutant was defective in the cytosolic DAG-AT and showed a similar effect to the plant system [13].

The molecular structure of oil bodies in yeast is similar to that of plant oil bodies [38,39]. The formation of oil bodies is currently a subject of much controversy. Some groups favour the development of oil bodies from the endoplasmic reticulum,

whereas others propose several alternative mechanisms [40–42]. It is possible that the oil bodies in oleaginous yeast are derived from the cytosolic pool of TAG, and this is consistent with experimental evidence obtained during our studies. In conclusion, our results suggest that the cytosolic TBC is responsible for the TAG accumulation in oleaginous yeast. The relationship between TAG biosynthesis and cell growth needs to be elucidated in detail in oleaginous yeast.

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