Isolation and Localization of a Cytosolic 10 S Triacylglycerol Biosynthetic Multienzyme Complex from Oleaginous Yeast*

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Triacylglycerol is one of the major storage forms of metabolic energy in eukaryotic cells. Biosynthesis of triacylglycerol is known to occur in membranes. We report here the isolation, purification, and characterization of a catalytically active cytosolic 10 S multienzyme complex for triacylglycerol biosynthesis from *Rhodotorula glutinis* during exponential growth. The complex was characterized and was found to contain lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein synthetase, and acyl carrier protein. The 10 S triacylglycerol biosynthetic complex rapidly incorporates free fatty acids as well as fatty acylcoenzyme A into triacylglycerol and its biosynthetic intermediates. Lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, and diacylglycerol acyltransferase from the complex were microsequenced. Antibodies were raised against the synthetic peptides corresponding to lysophosphatidic acid acyltransferase and phosphatidic acid phosphatase sequences. Immunoprecipitation and immunolocalization studies show the presence of a cytosolic multienzyme complex for triacylglycerol biosynthesis. Chemical cross-linking studies revealed that the 10 S multienzyme complex was held together by protein-protein interactions. These results demonstrate that the cytosol is one of the sites for triacylglycerol biosynthesis in oleaginous yeast.

The *de novo* biosynthesis of triacylglycerol occurs by the sequential acylation of glycerol-3-phosphate (1–3). Glycerol-3-phosphate acyltransferase catalyzes the first step in glycerolipid synthesis (4), generating lysophosphatidic acid (LPA). Alternatively, LPA is formed by acylation followed by reduction of dihydroxyacetone phosphate. Dihydroxyacetone phosphate acyltransferase (5) and acyl-dihydroxyacetone phosphate reductase (6, 7) catalyze the formation of LPA from dihydroxyacetone phosphate, respectively. The acylation of LPA by LPA acyltransferase to form phosphatidic acid (PA), which is the branch point for the synthesis of diacylglycerol (DAG) and phospholipids. PA phosphatase catalyzes the dephosphorylation of PA to DAG that is an immediate precursor of triacylglycerol (TAG), phosphatidylcholine, and phosphatidylethanolamine. DAG, an important signal molecule leading to protein kinase C activation (8), can also be derived from phospholipids by the action of phospholipase C (9). DAG acyltransferase catalyzes the acylation of DAG, which is a committed step in TAG biosynthesis. Recently, an acyl-CoA-independent enzyme for TAG synthesis has been reported in plants and yeast cells that uses phospholipid as acyl donor and DAG as acyl acceptor. This reaction is catalyzed by phospholipid-DAG acyltransferase (10). The same reaction can also be catalyzed by lecithin-cholesterol acyltransferase in yeast (11). All of the enzymes in these pathways are membrane-bound in eukaryotic systems (1–4, 12, 13). In *Saccharomyces cerevisiae*, mitochondrial membranes and endoplasmic reticulum have been identified as the major sites for phospholipid and TAG synthesis (3, 6, 14).

*Rhodotorula glutinis*, a pink budding yeast, accumulates about 50% dry weight per cell as lipid (15). Due to the large accumulation of TAG, the biosynthetic enzymes are active in *R. glutinis*. Here we present for the first time the isolation, characterization, polypeptide composition, and immunolocalization of a cytosolic multienzyme complex for TAG biosynthesis in oleaginous yeast cells during exponential growth phase. This study provides direct evidence for the cytosol to be one of the sites for TAG biosynthesis. Understanding the lipid biosynthesis would enable one to genetically engineer fungi and plants with desired fatty acid composition and the altered oil content (16).

**EXPERIMENTAL PROCEDURES**

*MATERIALS—* *R. glutinis* (MTCC 1151) was obtained from the Institute of Microbial Technology (Chandigarh, India). [1-14C]Palmitoyl-CoA (51 mCi/mmol), [1-oleoyl-9,10-H3]LPA (60 Ci/mmol), [glycerol-U-14C]LPA (100 mCi/mmol), [2-14H]G3P (12 Ci/mmol), [1,3-14C]fatty acid (55 mCi/mmol), and [35S]protein labeling mix were obtained from NEN Life Sciences. [1,4C]sodium acetate (56.4 mCi/mmol) was from the Board of Radiation and Isotope Technology (Mumbai, India). Superose 12 (10/30) FPLC column, gel filtration molecular mass standards, and ampholines (pH 3–10) were from Amersham Pharmacia Biotech. Protein assay reagents were obtained from Pierce. Thin layer chromatography plates were from Merck. All other reagents were obtained from Sigma.

*Yeast Growth Conditions—* Yeast cells were grown in malt-yeast extract medium (pH 7.0) containing 0.3% yeast extract, 0.5% peptone, 0.3% malt extract supplemented with 1% glucose with aeration at 30 °C. Cell density was determined by colony-forming units (one A600 = 9 × 10^7 cells).

*Nile Blue A Staining—* A smear of cells at various stages of growth was prepared on a glass slide and heat-fixed. The slides were immersed in a 1% 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 a 1-min wash in 8% acetic acid. The slides were then rinsed in water, air-dried, and visualized under fluorescence microscope (× 100 magnification).

*Incorporation of [1-14C]Acetate into TAG—* Yeast cells (8 × 10^7 cells/ml of phosphate-buffered saline, pH 7.4) were labeled with 2.5 μCi of [1-14C]acetate for 3 h. Cells were harvested by centrifugation, and the cell pellet was washed with ice-cold phosphate-buffered saline. To the

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1 The abbreviations used are: LPA, lysophosphatidic acid; ACP, acyl carrier protein; DAG, diacylglycerol; G3F, glycerol 3-phosphate; PA, phosphatidic acid; TAG, triacylglycerol; TBC, triacylglycerol biosynthetic complex; FPLC, fast protein liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PL, phospholipid; FFA, free fatty acids.
Lipids were extracted and separated by silica-TLC using a neutral lipid solvent system as described under “Experimental Procedures.” Lipid bands were visualized by iodine vapor, and spots corresponding to LPA, PA, DAG, and TAG were identified by their migration with standards and then scraped off for measurement of radioactivity. In addition, acyl-acceptor synthetases were also assayed using labeled acyl acceptors [2-3H]G3P (50 μM, 100,000 dpm) for G3P acyltransferase or [1-oleoyl-9,10-3H]LPA (50 μM; 150,000 dpm) for LPA acyltransferase along with 20 μM palmitoyl-CoA. PA phosphatase activity was measured by monitoring the formation of DAG from [glycerol-U-14C]PA/dipalmitoyl (1.1 × 10^5 dpm).

ACP and acyl-ACP synthetase assays were carried out as described (18). The reaction mixture consisted of 0.1 M Tris-HCl (pH 8.0), 0.4 M LiCl, 5 mM MgCl2, 5 mM ATP, 0.2% Triton X-100, [1-14C]palmitoyl-CoA (1.1 × 10^5 dpm) followed by liquid scintillation counting.

Size Exclusion Chromatography—The soluble fraction was concentrated (2 mg of protein) and was applied to a Superose 12 FPLC column fitted with the Bio-Rad BioLogic low pressure chromatography system. The column was equilibrated with 10 mM Tris-HCl, pH 7.5 containing 0.1% NaCl, and the elution was with the same buffer at a flow rate of 0.25 ml/min. Fractions (1 ml) were collected and assayed for TAG biosynthetic enzyme activities (LPA acyltransferase, PA phosphatase, and DAG acyltransferase).

Sucrose Density Gradient—The soluble fraction (75 mg) or the purified complex (25 μg) was layered onto a 10–30% linear sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, and 0.1 M NaCl. The tubes were centrifuged for 18 h at 200,000 × g (Beckman SW 41 rotor), and fractions (1 ml) were collected and assayed for enzyme activities.

Purification of TAG Biosynthetic Enzyme Complex—All operations were conducted at 4 °C except for the FPLC purification step, which was conducted at ambient temperature. The soluble fraction from the exponentially growing cells was used for the 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 progressively cut into 0.5-cm slices, and the protein was eluted by finely crushing the gel pieces in 10 ml Tris-HCl (pH 7.5) buffer containing 0.1% NaCl, 5 mM MgCl2, and 5% sucrose and incubated overnight at 4 °C. The gel-eluted protein was used for further studies.

SDS-polyacrylamide gel electrophoresis was performed based on the method of Laemmli (19). Isoelectric focusing of proteins was performed as described under “Experimental Procedures.”

Fig. 1. Growth, cell viability, TAG synthesis, and accumulation. A, exponentially growing cultures of R. glutinis in malt-yeast extract medium were added to a final concentration of 1% to fresh medium and incubated at 30 °C. At regular time intervals, A600 was measured. B, culture aliquots were taken at regular time intervals and diluted, and the cell count was taken. Viable cells were expressed as colony-forming units/ml. C, profile of TAG accumulation in R. glutinis at the indicated time points was determined. Cells were isolated at different growth times, and the cell number was adjusted to 2.0 A600.

pellet, 0.5 ml of 10% acetic acid in isopropyl alcohol was added and boiled for 5 min. To the mixture, 1 ml of hexane was added and vortexed thoroughly (17). The hexane layer was removed and concentrated, and the lipids were separated on silica gel G thin layer plates developed with petroleum ether/diethylether/acidic acid (70:30:1, v/v/v). Lipids were identified by their migration with standards and then scraped from the plate and counted in liquid scintillation counter.

Preparation of Subcellular Fractions—Logarithmic phase cells (21 h) were suspended in 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, and 5% sucrose. The cells were lysed using glass beads (0.45–0.6 mm) in the absence of detergent. Differential centrifugation was used to fractionate intracellular components. The supernatant (10,000 × g) thus obtained was centrifuged at 240,000 × g for 60 min to obtain the soluble fraction (cytosol). The pellet was washed with the lysis buffer and centrifuged again at 240,000 × g for 60 min to obtain membranes. All the operations were carried out at 4 °C.

Enzyme Assays—The assay mixtures consisted of all of the components of lysis buffer except protease inhibitors with labeled acyl donor, 20 μM [1-14C]palmitoyl-CoA (100,000 dpm), 5–25 μg of enzyme, and 0.1 mM G3P for G3P acyltransferase or 50 μM LPA (1-oleoyl) for LPA acyltransferases or 50 μM 1,2-diolein for DAG acyltransferase in a total volume of 100 μl. The incubation was carried out at 30 °C for 30 min and stopped by extracting lipids as described above. Lipids were separated on silica-TLC plates using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) and chloroform/methanol/acetic acid/water (170:25:25:4, v/v/v/v) as the solvent systems for separating neutral lipids and phospholipids, respectively. The lipids were visualized by staining with iodine vapor, and spots corresponding to LPA, PA, DAG, and TAG were scraped off for measurement of radioactivity. In addition, acyl-acceptor synthetases were also assayed using labeled acyl acceptors [2-3H]G3P (50 μM, 100,000 dpm) for G3P acyltransferase or [1-oleoyl-9,10-3H]LPA (50 μM; 150,000 dpm) for LPA acyltransferase along with 20 μM palmitoyl-CoA. PA phosphatase activity was measured by monitoring the formation of DAG from [glycerol-U-14C]PA/dipalmitoyl (1.1 × 10^5 dpm).

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Lipids were extracted and separated by silica-TLC using a neutral lipid solvent system as described under “Experimental Procedures.” Nile blue A staining of R. glutinis. Phase-contrast and fluorescence micrographs are shown of R. glutinis cells grown at the indicated growth time intervals. E, metabolic labeling of yeast cells with [14C]acetate and its incorporation into TAG, DAG, free fatty acids, and PL at various time intervals was performed. Incorporation and analysis of labeled lipids was carried out as described under “Experimental Procedures.”
using a gradient of pH 3–10 according to the instructions of Amersham Pharmacia Biotech. Protein was measured by the Bradford method (20).

Protein Sequencing—The 10 S complex was resolved on a 15% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The five proteins were subjected to N terminus microsequencing by the Edman method. The proteins that did not yield N terminus sequences were subjected to tryptic digestion, and microsequencing of the tryptic peptides was performed. Protein sequencing was performed at the protein sequencing facility at Rockefeller University.

Antiserum Production—Rabbits were immunized by subcutaneous injection of 250 μg of purified ACP emulsified in Freund’s complete adjuvant. Three booster doses of 125 μg of protein emulsified in Freund’s incomplete adjuvant were administered at three weekly intervals. Ten days after the last injection, blood was collected, and serum was separated and stored at −20 °C.

The major peptide CY-ALELQADDFNK corresponding to LPA acyltransferase and phosphatidic acid phosphatase peptides (major peptide CY-NALTGLHMGGGK, and minor peptide C-YVEGARP) were conjugated to bovine serum albumin using N-maleimidobenzoyl-N-hydroxysuccinimide ester (21). The conjugated peptides (300 μg) were emulsified and injected into rabbits. The antibody production, specificity, and titer were analyzed by enzyme-linked immunosorbent assay (22).

Western Blotting—Proteins were separated by either native or SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane for immunoblotting as described (23). Peptide-specific antibodies were used at a dilution of 1:600, ACP antibody at 1:3000, and acyl-ACP synthetase antibody was at 1:400 in 0.1% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20.

TABLE I

Distribution of triacylglycerol biosynthetic enzyme activities in soluble and particulate fractions of R. glutinis

The logarithmic phase oleaginous yeast cells from 1 liter of culture were lysed with glass beads, and fractions were obtained by differential centrifugation. Enzyme activities were measured as described under “Experimental Procedures.” Values are the mean ± S.E. of nine separate experiments, each performed in duplicate. Sup, supernatant.

| Fraction | LPA acyltransferase | PA phosphatase | DAG acyltransferase |
|----------|---------------------|----------------|--------------------|
|          | Specific activity   | Total activity | Specific activity   | Total activity | Specific activity   | Total activity |
|          | pmol/min/mg         | pmol/min       | pmol/min/mg         | pmol/min       | pmol/min/mg         | pmol/min       |
| 10,000 × g Sup | 1.59              | 32.42 ± 4.54  | 1.14              | 27.35 ± 0.75  | 0.98              | 23.62 ± 1.65  |
| 240,000 × g Sup | 1.60              | 27.00 ± 3.78  | 1.16              | 19.50 ± 0.58  | 0.83              | 13.95 ± 1.12  |
| 240,000 × g Pellet | 1.88             | 12.29 ± 0.31  | 1.49              | 9.70 ± 0.20   | 1.96              | 14.75 ± 0.74  |

FIG. 2. Identification of a soluble triacylglycerol biosynthetic enzyme complex. A, cytosol was applied onto a Superose 12 gel filtration column, and the elution profile of the TAG biosynthetic enzyme activities was determined. B, the cytosol was subjected to a 10–30% linear sucrose density gradient centrifugation. LPA acyltransferase, PA phosphatase, and DAG acyltransferase activities were estimated in 1-ml fractions and found to be colocalized in both the gel filtration and density gradient studies.

FIG. 3. TAG biosynthetic enzymes exist as a multienzyme complex in the cytosol. A, native PAGE (7%) profile of R. glutinis cytosol is indicated in lane 1. The mobility of electrophoretic markers is shown on the right (lane 2). B, cytosol was electrophoresed on 7% native gel, and the proteins were eluted from the gel pieces. LPA acyltransferase, PA phosphatase, and DAG acyltransferase activities were measured from the eluted proteins. The protein eluted from the 2nd cm of the native gel showed highest TAG synthase activity. C, a single band was observed upon silver staining when the active fraction containing the TAG biosynthetic enzymes was re-electrophoresed on a 7% native polyacrylamide gel. D, native PAGE-eluted active fraction was analyzed by 12% SDS-PAGE, and five proteins were visualized upon silver staining (lane 2), indicating the presence of a multienzyme complex. E, the purity of the multienzyme complex was confirmed by isoelectric focusing.

CY-NALTGLHMGGGK, and minor peptide C-YVEGARP) were conjugated to bovine serum albumin using N-maleimidobenzoyl-N-hydroxysuccinimide ester (21). The conjugated peptides (300 μg) were emulsified and injected into rabbits. The antibody production, specificity, and titer were analyzed by enzyme-linked immunosorbent assay (22).
The particulate fractions were capable of synthesizing TAG. Enzyme with 1.2 M sorbitol for 6 h at 30 °C to obtain spheroplasts. The cells were incubated with lysate for 1 h, and the immunocomplex was sequenced with protein A beads. The bound proteins were analyzed by gel electrophoresis followed by fluorography.

Cross-linking of Proteins—Cross-linking was carried out as described (24). In short, preparations of the purified complex (5 µg) were mixed with disuccinimidyl suberate to a final concentration of 0.5 mM in a total volume of 50 µL. The cross-linking was performed for 60 min at 4 °C. The reaction was stopped by the addition of 5 µL of 0.25 x Tris-HCl, pH 7.5. The cross-linked products were resolved by 8% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was probed with anti-ACP and anti-LPA acyltransferase antibodies for the detection of the cross-linked products.

Indirect Immunofluorescence—Logarithmic phase (21 h) R. glutinis cells were fixed with 4% paraformaldehyde for 20 min followed by 4% formaldehyde for 60 min. The fixed cells were washed three times with 0.1 x phosphate buffer (pH 5.9) and resuspended in 50 mg of lytic enzyme with 1.2 x sorbitol for 6 h at 30 °C to obtain spheroplasts. The cells were washed, resuspended (10^7 cells/mL), and plated on 12-mm coverslips, which were pretreated with poly-L-lysine. The coverslips smeared with R. glutinis spheroplasts were treated with ice-cold methanol for 6 min and acetone for 30 s. The primary antibodies were added at a dilution of 1:20 except for anti-ACP antibody, which was used at a dilution of 1:200. Secondary antibody fluorescein isothiocyanate/tetramethylrhodamine isothiocyanate conjugates were used for localization. The slides were viewed in a confocal laser-scanning microscope (Leica TCS SP, Heidelberg, Germany) to locate the TBC.

**RESULTS**

**Growth and TAG Synthesis in R. glutinis**—The growth of the oleaginous yeast cells was monitored by both A600 and colony-forming units at 30 °C (Fig. 1, A and B). Both A600 and viable cell count increased proportionally with time. Analysis of TAG profiles at various growth periods indicated that TAG accumulation was found even at the early logarithmic phase (Fig. 1C). Cells stained at various time intervals with Nile blue A and viewed under a fluorescence microscope, revealed that 21-h grown cells accumulate low amounts of TAG, whereas stationary phase cells showed an intense Nile blue A staining (Fig. 1D), indicating large accumulation of TAG. At 21 h, TAG is present as small oil droplets, which is evident by the fluorescence staining, whereas in the stationary phase cells, TAG is present as large oil bodies. To determine the rate of TAG biosynthesis in R. glutinis, cells were metabolically labeled with [3H]acetate, and its incorporation into free fatty acids, DAG, TAG, and PL was measured at various growth intervals (Fig. 1E). These results indicated that exponentially growing (21-h) cultures are active in synthesizing TAG. Therefore, all subsequent experiments were carried out on 21-h cultures.

**Distribution of TAG Biosynthetic Activity**—We investigated TAG formation in the exponentially growing R. glutinis cells and found that both the soluble (240,000 × g supernatant) and the particulate fractions were capable of synthesizing TAG. Table I summarizes the enrichment of TAG biosynthetic enzyme activities in the soluble (cytosol) fraction. Of the various TAG biosynthetic enzymes assayed, the soluble fraction exhibited high amounts (49–69% total activity) of LPA and DAG acyltransferases and PA phosphatase activities as compared with the corresponding enzymes in the particulate fraction (Table I). On the other hand, a negligible amount of glycerol-3-phosphate acyltransferase activity could be detected in the soluble fraction. The pattern of distribution of the enzyme activities remained the same under different lysis procedures such as French press and sonication (data not shown). In the 21-h grown culture, no discrete fraction of lipid body was observed upon centrifugation, thus eliminating the possibility of contamination of TAG biosynthetic enzymes from lipid particles. These results indicated that an additional TAG biosynthetic pathway could exist in the soluble fraction. LPA and DAG acyltransferases and PA phosphatase activities are collectively represented as “triacylglycerol synthase” (TAG synthase).

To demonstrate that the soluble fraction had TAG synthase activity, this fraction was loaded onto a gel exclusion column (Superose 12). Most of the TAG synthase activity eluted between 158 and 200 kDa (Fig. 2A). TAG synthase activity (5%) tagged with the corresponding enzymes in the particulate fraction was also found in the void volume fraction that could be due to the nonsedimentable membrane fragments and lipid particles generated during the fractionation procedure. This experiment confirmed that the TAG biosynthetic activity in R. glutinis is also present in the particulate fractions.

The sedimentation value of the purified complex was estimated by loading the cytosol onto a 10–30% linear sucrose gradient, and the various fractions were analyzed for TAG synthase activity. The analysis revealed that the LPA acyltransferase, PA phosphatase, and DAG acyltransferase activities were associated with one fraction. The sedimentation value of the active fraction was calculated to be 10 S (Fig. 2B). Purification of TAG Synthase—TAG biosynthetic activity was found to be high in the soluble fraction and this fraction was electrophoresed on a 7% native polyacrylamide gel at 4 °C. To determine the region of the gel corresponding to TAG synthase, the eluted proteins were assayed for various enzyme activities. LPA acyltransferase, PA phosphatase, and DAG acyltransferase were detected in the same region of the gel (Fig. 3A). By identifying the number of bands present in the active gel eluted fraction, the same fraction was reloaded onto a native gel and a single band was visualized upon silver staining (Fig. 3C). An overall summary of the purification procedure is shown in Table II. The native polyacrylamide gel electrophoresis step was effective and resulted in 469-, 426-, and 409-fold purification of LPA acyltransferase, PA phosphatase, and DAG acyltransferase, respectively, with a recovery of 39–56%. The ratio of acyltransferases to PA phosphatase activity remained constant during purification. Upon loading this fraction onto a Superose 12 column, the TAG synthase activity was reduced at 21 h, whereas in the stationary phase cells, TAG is presented as large oil bodies.
eluted as a single peak with the native molecular size of 180 kDa. The active fraction from the gel filtration column contained LPA acyltransferase, PA phosphatase, and DAG acyltransferase activities (data not shown). These results suggested the possibility of a soluble enzyme complex for TAG biosynthesis in *R. glutinis.*

**Identification of a 10 S Multienzyme Complex**—We wanted to examine if the enzymes were present as a multifunctional protein or multienzyme complex. The gel-eluted active fraction containing TAG synthase was resolved under denaturing and reducing conditions on a polyacrylamide gel that showed five polypeptides upon silver staining (Fig. 3D). The purified complex was subjected to isoelectric focusing (IEF) followed by silver staining, and the profile showed the presence of five polypeptides, of which four were basic (pI > 8.0) and one was acidic (pI 4.0) proteins (Fig. 3E). These data, in conjunction with the native PAGE of the purified complex and sucrose density gradient, indicated the presence of a 10 S multienzyme complex for TAG biosynthesis in the cytosol of *R. glutinis.*

**10 S Complex and Its Polypeptide Composition**—To identify the nature of polypeptides in the complex, the purified 10 S multienzyme complex was loaded onto a 12% SDS-polyacrylamide gel in the presence of 0.1% SDS without boiling the sample and electrophoresed at 4 °C. The gel was cut into 0.5-cm sections, and proteins were eluted from the gel and assayed for TAG synthase activity. As shown in Figs. 4A and 5A, LPA acyltransferase and PA phosphatase activities were predominantly found at the 5th and 4th cm, respectively, and the yield was 7–10%. LPA acyltransferase and PA phosphatase migrated separately with molecular masses of 32 and 48 kDa, respectively. DAG acyltransferase activity could not be localized in the gel. These results indicated that two of the polypeptides in the 10 S TBC were LPA acyltransferase and PA phosphatase. The electrophoresed proteins were blotted onto polyvinylidene difluoride membrane; the polypeptides corresponding to molecular sizes of 32 and 48 kDa were excised and digested with trypsin; and the tryptic peptide sequences were determined. The internal sequences (major peptide XALELQADDFNK and minor peptide XXVNNXPGXIEQ) of LPA acyltransferase (32 kDa) did not match with any known sequences in the data base. Tryptic peptide sequences (major peptide NALTGLHMGGGK and minor peptide YVEGARPXXK) of PA phosphatase (48 kDa) showed 40–100% identity with *Homo sapiens* PA phosphatase 2a and 2b isoforms and with *Musculus domesticus* kidney PA phosphatase.

To confirm that 10 S TBC contained LPA acyltransferase, immunoblots of native and SDS-polyacrylamide gels of proteins from purified complex, cytosol, and membranes were probed with polyclonal antibodies raised against the major internal peptide of the cytosolic LPA acyltransferase. The antibody recognized the complex in the native immunoblot (Fig. 4B) and a single band of 32 kDa from the complex and the cytosol in the SDS-polyacrylamide gel immunoblot (Fig. 4B). The same antibody was used for probing the 10 mM CHAPS-solubilized *R. glutinis* microsomal membranes and was found to recognize a polypeptide in SDS gel with a molecular size of 28 kDa (Fig. 4B). Similarly, immunoblots were carried out on purified complex, cytosol, and solubilized membranes with polyclonal antibodies raised against two peptides of cytosolic PA phosphatase (Fig. 5, B and C). The antibodies for the major and minor peptides of PA phosphatase recognized a single protein in the cytosol, which had a molecular mass of 48 and 45 kDa in the microsomal membranes.

To determine whether the five different proteins identified in the TBC were held together by physical interactions, logarithmic phase-grown *R. glutinis* cells were metabolically labeled with [35S]methionine followed by immunoprecipitation with antibodies raised to the three peptides. All three peptide-specific antisera, one to LPA acyltransferase and two to PA phos-
zyme complex was treated with 0.1% SDS and 50 mM dithiothreitol and progressively cut into 0.5-cm slices, and the eluted protein (20 amide gel, and proteins were visualized by fluorography. Native and precipitates were analyzed on 7% native gel as well as 12% SDS-polyacrylamide gels were identical in all three cases. The presence of LPA acyltransferase, PA phosphatase, and DAG acyltransferase was further confirmed by assaying for their activities in the immunoprecipitate (Table III).

DAG acyltransferase (56 kDa) polypeptide was microsequenced, and the internal sequence (XLWAVGQAQPFG-GARGS) showed 40–80% identity to the known DAG acyltransferase sequences available in the data base. The presence of DAG acyltransferase in the TBC was confirmed by assaying for its activity in the immunoprecipitate (Table III). DAG acyltransferase was found to be the most labile enzyme of the 10 S multienzyme TBC.

To study the formation of TAG, the purified complex was incubated with either [14C]palmitic acid in the presence of ATP, MgCl₂, and LPA or [14C]palmitoyl-CoA in the presence of LPA. Surprisingly, the rate of TAG synthesis was comparable with palmitic acid or palmitoyl-CoA, indicating that the complex was capable of activating the fatty acid. The TBC preferred unsaturated long chain fatty acids over saturated short chain fatty acids. The order of preference for free fatty acid as substrate by the TBC was as follows: linoleic > oleic > stearic > palmitic > myristic acids (data not shown). During fatty acid synthesis, activation of fatty acids was shown to be via the formation of acyl-ACP in the cytosol (25). Fatty acyl-CoA were the substrates for TAG biosynthesis, and this activation was established in the microsomes. To examine the nature of fatty acid activation by the TBC, ACP was purified to homogeneity from R. glutinis as described (26), and polyclonal antibodies were raised to the purified ACP. To ensure that the 10 S complex contained ACP, immunoblots of native and SDS-polyacrylamide gels of purified TBC were probed with antibodies to ACP purified from R. glutinis. The antibodies recognized a 21-kDa protein under denaturing conditions and the complex under native conditions. The membrane fraction was devoid of ACP (Fig. 6A).

**Cross-linking of the 10 S Complex**—To rule out the possibility of any lipid-protein interactions, the TBC was subjected to cross-linking using a homobifunctional cross-linker, disuccinimidyl suberate. Upon probing the cross-linked product with LPA acyltransferase and ACP antibodies, it was observed that the cross-linked product migrated at ~200 kDa (Fig. 6B), confirming that the complex was held together by protein-protein interactions and not by lipid-protein interactions.

**Immunolocalization of TAG Biosynthetic Complex in the Cytosol**—To determine the subcellular localization of the TBC, R. glutinis spheroplasts were probed with anti-LPA acyltransferase, anti-PA phosphatase major and minor peptide, and anti-ACP antibodies for indirect immunofluorescence. The staining pattern revealed the cytosolic nature of TBC (Fig. 7A). PA phosphatase major peptide antibodies were found to have lower affinity than the antibodies to the minor peptide. This was also evident upon immunostaining. The staining pattern for LPA acyltransferase and PA phosphatase was similar to ACP, which was used as the cytosolic marker. These results SDS-gel fluorographs showed the presence of TBC and five polypeptides corresponding to the proteins of the TBC, respectively. Anti-PAPase 1 represents the antiserum to the PA phosphatase major peptide and anti-PAPase 2 to PA phosphatase minor peptide. Normal rabbit serum was used as the negative control in immunoprecipitations.

**Fig. 5. PA phosphatase is a part of the TBC.** A, purified multienzyme complex was treated with 0.1% SDS and 50 mM dithiothreitol and electrophoresed on a 12% polyacrylamide gel. The resolving gel was progressively cut into 0.5-cm slices, and the eluted protein (~20 µg) was assayed for TAG synthase. The 4th cm of the gel exhibited PA phosphatase activity whose molecular size corresponded to 48 kDa. B and C, peptides were synthesized based on the internal sequences of PA phosphatase and polyclonal antibodies raised to these peptides. TBC, cytosol, and 10 mM CHAPS-solubilized membrane fraction was electrophoresed on native/SDS gels and transferred to nitrocellulose membranes, which were probed with PA phosphatase antibodies. Both of the antibodies recognized a 48-kDa protein under denaturing and TBC in nondenaturing conditions. PA phosphatase was found to be present in the membrane fraction as a 45-kDa protein (lane 4). D, 35S-labeled R. glutinis lyase was immunoprecipitated with anti-PA phosphatase (major and minor peptides) antibodies. The immunoprecipitates were analyzed on 7% native gel as well as 12% SDS-polyacrylamide gel, and proteins were visualized by fluorography. Native and SDS-gel fluorographs showed the presence of TBC and five polypeptides corresponding to the proteins of the TBC, respectively.
R. glutinis for ACP from in the TBC and in the cytosol was observed by probing with antibodies (B). lane 4 blotted. Ining with antibodies to LPA acyltransferase and ACP. With both of theuct was analyzed by 6% SDS-PAGE followed by visualization by prob-

R. glutinis confirmed the cytosolic nature of the TBC. We have proposed a model for the TAG biosynthesis in R. glutinis (Fig. 7B).

**DISCUSSION**

Where does triacylglycerol biosynthesis occur in oleaginous yeast? Our data demonstrate that TAG biosynthesis occurs in both the cytosol and the membrane. The cytosolic fraction has higher total activity than the membranes (Table I), and that may account for the increased TAG accumulation in vivo. While there is no direct evidence to support the possibility that membranes are the only sites for TAG synthesis, there is also no evidence for the absence of this biosynthetic pathway in the cytosol. The presence of soluble enzymes that provide important precursors for triacylglycerol biosynthesis is well documented. A soluble G3P acyltransferase has been isolated from cocoa seed (27). PA phosphatase, responsible for dephosphorylation of PA, is located in the cytosol of S. cerevisiae (28, 29) and higher plants (30). In developing rapeseed, the presence of a soluble DAG biosynthetic activity has been demonstrated (31). LPA phosphatase (32), DAG kinase (33, 34), inactive choline cytidylyltransferase (35), and active ethanolaminephosphate cytidylyltransferase (36) have also been found in the cytosol in animal systems.

The following observations reveal the presence of the cytosolic TAG pathway in R. glutinis. First, subcellular distribution studies indicate that more than 60% of the total activity is associated with the soluble fraction (Table I). Second, the enzymes involved in TAG synthesis are included in the gel filtration column (Superose 12) as well as 7% native polyacrylamide gel (Fig. 3A). Third, the cytosolic TAG biosynthetic enzymes are immunolocalized with specific antibodies to LPA acyltransferase and PA phosphatase (Figs. 4B and 5, B and C). This leads to the question of how cytosolic TAG biosynthesizing enzymes function in a hydrophilic environment. The most likely explanation is that these enzymes exist as a complex for efficient substrate channeling and to achieve increased accumulation of TAG. Immunoprecipitation (Figs. 4C and 5D) and immunolocalization (Fig. 7A) studies carried out with antibodies raised against the enzymes of the TAG biosynthetic pathway indicated that these enzymes are indeed present as a complex. Protein cross-linking studies confirmed the presence of a multienzyme complex. This also ruled out the possibility that the proteins of the complex could be held together by lipid protein interactions in the cytosol of R. glutinis. The isoforms of TAG biosynthetic enzymes that catalyze the same reactions in different subcellular locations may provide independent regulation at the level of enzyme and gene expression (37). The segregated pools of lipids may be generated in different regions of the cell and used for different functions. For example, PA phosphatase in the endoplasmic reticulum has been shown to be involved in TAG synthesis (38), whereas the same enzyme in the plasma membrane is involved in signal transduction (39).

The unexpected finding of this study is that the TBC accepts free fatty acids to form TAG, and the complex consists of ACP, acyl-ACP synthetase, LPA acyltransferase, PA phosphatase, and DAG acyltransferase. Acyl-ACP synthetase in prokaryotes has been implicated in fatty acid and lysophospholipid metabolism but does not provide acyl-ACP for other intracellular enzymes (40). This enzyme has not been reported previously in any eukaryotic system.

The demonstration of a cytosolic pathway for TAG synthesis in oleaginous yeast by sucrose gradient velocity sedimentation, purification, specific antibody interaction, immunoprecipitation, cross-linking, and immunolocalization raises several fundamental questions concerning the currently accepted view of assembly and biogenesis of oil bodies from the endoplasmic reticulum. One possibility is that oil bodies are synthesized in the cytosol and that the extent of assembly depends on the local concentration of the newly synthesized TAG (41). The molecular structure of oil bodies in yeast may be similar to that of plant oil bodies (42, 43).

Finally, is the cytosolic pathway responsible for TAG accumulation? Preliminary studies on the isolation of mutants (defective in TAG accumulation) and characterization indicate that it is the cytosolic pathway that is responsible for TAG accumulation in oleaginous yeast.2 It may be advantageous for the cell to produce TAG near the site of fatty acid

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2 A. Gangar, S. Raychaudhuri, and R. Rajasekharan, unpublished data.
synthesis. Our results provide the first direct evidence for the existence of a soluble 10 S multienzyme triacylglycerol biosynthetic complex in oleaginous yeast. The isolation of a cytosolic multienzyme TBC has significant implications in understanding the biosynthesis and regulation of triacylglycerol accumulation.

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