A soluble diacylglycerol acyltransferase is involved in triacylglycerol biosynthesis in the oleaginous yeast Rhodotorula glutinis

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The biosynthesis of triacylglycerol (TAG) occurs in the microsomal membranes of eukaryotes. Here, we report the identification and functional characterization of diacylglycerol acyltransferase (DGAT), a member of the 10 S cytosolic TAG biosynthetic complex (TBC) in Rhodotorula glutinis. Both a full-length and an N-terminally truncated cDNA clone of a single gene were isolated from R. glutinis. The DGAT activity of the protein encoded by RgDGAT was confirmed in vivo by the heterologous expression of cDNA in a Saccharomyces cerevisiae quadruple mutant (H1246) that is defective in TAG synthesis. RgDGAT overexpression in yeast was found to be capable of acylating diacylglycerol (DAG) in an acyl-CoA-dependent manner. Quadruple mutant yeast cells exhibit growth defects in the presence of oleic acid, but wild-type yeast cells do not. In an in vivo fatty acid supplementation experiment, RgDGAT expression rescued quadruple mutant growth in an oleate-containing medium. We describe a soluble acyl-CoA-dependent DAG acyltransferase from R. glutinis that belongs to the DGAT3 class of enzymes. The study highlights the importance of an alternative TAG biosynthetic pathway in oleaginous yeasts.

INTRODUCTION

Oleaginous yeasts accumulate more than 25% of their dry weight of oil. Microbial oils can be useful substitutes for non-renewable energy sources as these oils contain fatty acids that are similar to plant oils. Microbial oils have an advantage over plant oils owing to the less expensive downstream process and the seasonally independent production. By identifying the enzymes involved in microbial oil biosynthesis, the pathway can be redesigned for greater yields. The main component of microbial oil is triacylglycerol (TAG). TAG is a non-polar, water-insoluble triester of glycerol with fatty acids. TAG molecules are stored in specialized organelles such as adiposomes in animal cells and oil bodies in plant seeds (Murphy, 1993). TAG is a pivotal component of lipid homeostasis, in addition to its role in storage in all eukaryotic cells. In animals, a disruption of the dynamic equilibrium between lipid synthesis and catabolism leads to metabolic diseases such as obesity and diabetes. In seeds, triacylglycerol is the major storage lipid and is important for seed development and embryo maturation. Cellular fatty acid uptake and utilization rates are tightly controlled to meet energy demands and maintain a lipid balance. TAG synthesis functions as a detoxification mechanism as excessive fatty acids in the cell are detrimental; thus, cells divert them into storage lipids to overcome free fatty acid toxicity (Connerth et al., 2010; Siloto et al., 2009).

A de novo biosynthesis of TAG occurs by the sequential acylation of glycerol-3-phosphate. Though there are well studied exceptions of non-membrane-bound (soluble) enzymes, most of the enzymes reported in this pathway are membrane-bound (Bishop & Bell, 1988; Kent et al., 1991; Raetz & Dowhan, 1990). Diacylglycerol acyltransferase (DGAT) catalyses the last and final committed step of the TAG biosynthesis. Various types of DGAT enzymes have been identified in microbes, plants and animal systems. The majority of the DGAT enzymes identified to date belong to the DGAT1 and DGAT2 classes (TurcettZolet et al., 2011; Yen et al., 2008). There is another class of DGAT enzymes known as the ‘PDAT family’. The enzymes of this family utilize the acyl group from phosphatidyl choline for the TAG biosynthesis (Dahlqvist et al., 2000). A novel bifunctional DGAT/wax ester synthase (ADP1) from Acinetobacter calcoaceticus was identified to be responsible for the synthesis of wax esters as the main lipid storage compound in addition to a minor amount of TAGs (Kalscheuer & Steinbüchel, 2003). The DGAT enzymes

Abbreviations: CTD, C-terminal domain; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; TAG, triacylglycerol; TBC, triacylglycerol biosynthetic complex.

The GenBank/EMBL/DDBJ accession number for the DGAT sequence from R. glutinis is DQ315417.

A supplementary figure is available with the online version of this paper.
belonging to the DGAT1, DGAT2 and PDAT families are well studied and are found to be membrane bound. In contrast with these enzyme families, a soluble form of DGAT has been identified in peanut cotyledons and was grouped into the DGAT3 family (Saha et al., 2006).

The existence of cytosolic enzymes that provide important precursors for TAG biosynthesis has been well documented in yeast, plant and animal systems. The dephosphorylation of phosphatidic acid (PA) has been observed in both the soluble and membrane fractions of Saccharomyces cerevisiae (Carman, 1997; Kohe & Satoshi, 1984). Cytosolic lysosphatidic acid (LPA) phosphatase (Reddy et al., 2010) isolated from S. cerevisiae; LPA acyltransferase (Ghosh et al., 2008a, b, 2009) isolated from S. cerevisiae, plants and humans; and DGAT (Rani et al., 2010) isolated from Arabidopsis thaliana are a few examples. The developmental role of the DGAT3 enzyme, a soluble DGAT homologue of Arachis hypogea in A. thaliana was recently reported (Hernández et al., 2012; Saha et al., 2006). However, the mechanism of action, substrate specificity and the physiological roles of soluble acyltransferases belonging to the DGAT3 family are poorly understood. Rhodotorula glutinis is an oleaginous, non-conventional pink budding yeast that accumulates around 66 % of its dry weight as oil (Ageitos et al., 2011). For the first time, in R. glutinis, the cytosol has been shown to be one of the sites of TAG biosynthesis (Gangar et al., 2001). The isolated 10 S soluble triacylglycerol biosynthetic complex (TBC) consisted of LPA acyltransferase, PA phosphatase, acyl carrier protein (Raychaudhuri & Rajasekharan, 2003), acyl–acyl carrier protein synthetase (Gangar et al., 2011) and superoxide dismutase (Gangar et al., 2002), as well as several DGATs (Raychaudhuri et al., 2003) and a possible DGAT. The presence of a cytosolic TBC in R. glutinis (Gangar et al., 2001) led us to study the soluble DGAT in detail. We have isolated a cDNA clone and its C-terminal domain encoding DGAT independently from the screening. In vivo studies, acetate labelling and genetic experiments such as fatty acid supplementation experiments proved the DGAT function of the protein. In vitro studies involving protein- and time-dependent DGAT assays established the activity and the specificity of the enzyme. The heterologous expression of RgDGAT in the H1246 strain (TAG-deficient mutant) resulted in the accumulation of TAG. The present study provides evidence for the existence of a cytosolic isoform of the TAG biosynthetic enzyme and an alternative TAG biosynthetic pathway that is responsible for TAG accumulation in oleaginous yeast.

**METHODS.** *R. glutinis* was obtained from the Institute of Microbial Technology, Chandigarh, India. [1-14C]-Labelled fatty acyl-CoAs (54 mCi mmol⁻¹) and [14C]Acetate (51 mCi mmol⁻¹) were obtained from Perkin-Elmer Biosciences. [14C]DAG was obtained from American Radiolabelled Chemicals. Peptone, yeast extract, tryptone, yeast nitrogen base and drop-out media were obtained from Difco. Silica gel 60F254; TLC plates were from Merck. Oligonucleotide primers, lipids, BODIPY 493/503 and solvents were purchased from Sigma-Aldrich and Avanti polar lipids. Protein was estimated with the Bradford reagent using BSA as the standard. Antibodies were raised against a putative DGAT protein of the *R. glutinis* TBC complex (Gangar et al., 2001; Ghosh et al., 2008b). Anti-acyl carrier protein and anti-superoxide dismutase antibodies were produced as described previously (Raychaudhuri & Rajasekharan, 2003; Raychaudhuri et al., 2003). Alkaline-phosphatase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG were obtained from Amersham Biosciences.

**Strains and growth conditions.** To serve as the phage infection host, *Escherichia coli* XL-1 blue MRF' cells were grown in Luria–Bertani (LB) containing 10 mM MgSO₄ and 0.2 % maltose. *E. coli* cells were grown in an LB broth containing 1 % tryptone, 0.5 % yeast extract and 1 % NaCl (pH 7.5) at 37 °C. The quadruple mutant S. cerevisiae strain (H1246) (MATα; ade2::HIS3::LEU2 pgi1::KanMX4 his3::TRP1 ade2) (Sundager et al., 2002) and the wild-type S. cerevisiae strain (BY4741; MATa; HIS3; LEU2 0; MET15 0; URA3 0) were used as heterologous hosts to study gene expression. Wild-type and quadruple mutant yeast cells were grown in a medium containing 2 % peptone, 1 % yeast extract and 2 % glucose at 30 °C. Transformed yeast cells were grown in a synthetic dropout medium that lacked uracil but included either 2 % glucose for growth or galactose for induction. For the fatty acid supplementation, synthetic medium, which contained either glucose and uracil or galactose without uracil, were used with specified amounts of oleic acid.

**Screening of cDNA library.** The expression library of *R. glutinis* (Raychaudhuri & Rajasekharan, 2003; Raychaudhuri et al., 2003) was screened using a degenerate oligonucleotide probe (3'- YNTGTGGCGNTGNNGGNCARCCNTTTYGGNGGNCM- GNGNWSN-3') that was designed using the internal amino acid sequence of the purified DGAT (Gangar et al., 2001). The oligonucleotide probe of the peptide was radiolabelled at the 3’ end with TdT and [α-32P]dATP. A total of 2 × 10⁶ plaques were screened and positive plaques were purified by three additional screenings with a homologous oligonucleotide probe. Plasmid DNA from the positive clones was sequenced on both strands using the Biotech Taq cycle sequencing kit and an automated sequencer (Applied Biosystems model 377; PE Applied Biosystems). Two cDNA clones, one full-length and one C-terminal domain (CTD) of the single gene, were independently isolated and cloned for further characterization.

**Bioinformatic analysis.** Multiple sequence alignment was obtained using a CLUSTAL X 1.83 Pedro’s Biomolecular Research Tool and ExpASy. The Molecular Biology Server was used for domain and motif identification. The protein hydropathy plot was generated based on a Kyte–Doolittle hydrophathy plot (http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm). A phylogenetic analysis was carried out using CLUSTAL W based on a neighbour-joining method. Conserved domains and motifs were identified using the Pfam database and also the conserved domain database of the National Centre for Biotechnology Information.

**Antibody production in rabbit.** Soluble 10 S TAG biosynthetic complex isolated from *R. glutinis* was separated on 12 % SDS-PAGE. A putative DGAT protein band from the SDS-PAGE was cut and ground using liquid nitrogen. The protein (250 µg of denatured putative DGAT protein) was then emulsified in Freund’s complete adjuvant and then injected subcutaneously into the rabbit for immunization. Three booster doses of 125 µg protein emulsified in Freund’s incomplete adjuvant were administered three times at weekly intervals. Ten days after the last injection, blood was collected, and serum was separated and stored at −20 °C. The antibody production, specificity and the titre were analysed by ELISA (Engvall, 1980).
Cloning, overexpression and purification of cDNA encoding DGAT from R. glutinis. RgDGAT and CTD genes were cloned into a yeast vector (pYES2/CT) with a His-tag at the C-terminal end. Positive clones obtained from the cDNA library screening were used as templates. The full-length RgDGAT was cloned using the following primers: 5′-GGTGAAAGCTTATGTTATGCTCGACGAAACGCTATC-3′ and 5′-ATAATAGATCTATGCTGGTGGTTGCGC-3′ between the HindIII–EcoRI sites. The CTD of RgDGAT was cloned at SacI–EcoRI sites using the following primers: 5′-ATTAGGACTTATGCGGAGGTCCGCTCTTC-3′ and 5′-ATAATGAATCTATGCGGCTGGTGGCTC-3′. Both of the constructs were transformed into yeast by using the lithium acetate method (Schiestl & Gietz, 1989). The yeast cells transformed with RgDGAT were resuspended in a lysis buffer, and the cells were then vortexed with glass beads, keeping them intermittently on ice (30 s vortex and 30 s on ice). The cells were spun at 12,000 r.p.m. for 30 min, and the supernatant was again subjected to ultracentrifugation at 100,000 g for 3 h. The ultracentrifugation supernatant was kept for binding with an Ni²⁺-NTA matrix. The proteins were separated on a 12 % SDS-PAGE and transferred onto a nitrocellulose membrane. The overexpression of RgDGAT was confirmed using anti-RgDGAT polyclonal antibodies raised against a putative DGAT protein of the R. glutinis TBC complex (Gangar et al., 2001; Ghosh et al., 2008b) at a dilution of 1:2000 (v/v).

In vivo metabolic labelling of neutral lipids. Yeast transformants (pYES2-RgDGAT, pYES2-CTD and pYES2 vectors) were grown to late exponential phase in synthetic media ([SM]-U containing 2 % glucose). Cell growth was continued until OD₆₀₀ 3.0. For neutral-lipid labelling, a sample of cells (OD₆₀₀ 0.4) was inoculated into a fresh medium that contained 2 % galactose and 0.5 μCi [¹⁴C]acetate ml⁻¹ and were grown for 24 h. The cells (OD₆₀₀ 20) were then harvested, and the lipids were extracted with glass beads and separated on a silica-TLC plate with a solvent system of petroleum ether/diethyl ether/acetic acid (70:30:1, v/v). The lipids were identified by their migrations relative to known standards. Individual spots were scraped off from the plate and the radioactivity was measured with a liquid-scintillation counter.

Cloning, overexpression and purification of RgDGAT-CTD in E. coli. The coding region of the RgDGAT-CTD was PCR amplified using the following primers: forward 5′-GGTGAAAGCTTATGTTATGCTCGACGAAACGCTATC-3′ and 5′-ATAATAGATCTATGCGGAGGTCCGCTCTTC-3′ and 5′-ATAATGAATCTATGCGGCTGGTGGCTC-3′. Positive clones obtained from the cDNA library screening were used as templates. The PCR mixture consisted of 100 ng template, 10 pmol each of sense and antisense primers, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1 unit PNK polymerase and a 1× reaction buffer. The PCR conditions used were as follows: initial denaturation of the template at 94 °C for 4 min, followed by 30 cycles of 1 min denaturations at 94 °C, 1 min annealing at 55 °C, and 1 min elongation at 72 °C. The final extension was performed at 72 °C for 10 min. The amplified PCR product was cloned in pSET B (Invitrogen), which has an N-terminal His-tag vector at the SacI and EcoRI sites. The construct was transformed into E. coliBL21(DE3) cells and induced with 0.5 mM IPTG for 3 h at 37 °C. Overexpression was confirmed by anti-DGAT as well as with an anti-his tag antibody. The recombinant protein was purified by Ni-NTA affinity column chromatography.

In vitro DGAT assay. The assay mixture consisted of a purified recombinant enzyme (1–5 μg), 10 μM [L-¹⁴C]oleoyl-CoA (100,000 d.p.m.) and 50 μM vesicles of dioleoyl-sn-glycerol in a total volume of 100 μl (Rani et al., 2010). The incubation was carried out at 30 °C for 20 min, and the reaction was stopped by addition of CHCl₃/CH₃OH (2:1, v/v). Control incubations were carried out for time zero and in the absence of the enzyme. Chloroform-soluble materials were separated using a silica-TLC plate with a solvent system of petroleum ether/diethyl ether/acetic acid (70:30:1, v/v). The lipids were visualized by iodine vapours and identified by their migrations relative to known standards.

In vivo fatty acid supplementation. Oleic acid was dissolved in ethanol at 0.5 M. Later the oleic acid was diluted in a warm medium containing 0.01 % (v/v) NP-40 immediately before yeast inoculation. NP-40 is a nonionic surfactant that was used to assure the dispersion of the fatty acid in the medium. Control experiments without oleic acid contained the same volume of ethanol and 0.01 % (v/v) NP-40. S. cerevisiae wild-type and quadruple mutant cells were allowed to grow on a YNB (URA⁺) containing 2 % glucose medium. Yeast transformants were grown in a YNB (URA⁻) medium containing 1 % raffinose and 2 % galactose for the overexpression of the genes (YNBG medium). The quadruple mutant yeast cells exhibit a growth defect in an oleic acid containing medium due to free fatty acid toxicity. This feature of the quadruple mutant was exploited to understand the DGAT function of the genes. Hence we carried out the fatty acid supplementation experiment by taking different dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵) of the yeast transformants (pYES2, pYES2+ RgDGAT and pYES2+ CTD) starting with 1 OD unit of cells. Plates of YNB (URA + or URA⁻) containing different concentrations of oleic acid (0.01 and 0.5 mM) dissolved in ethanol were prepared as described (Siloto et al., 2009).

BODIPY staining. S. cerevisiae quadruple mutant transformants (pYES2, pYES2–RgDGAT) grown in a synthetic medium (devoid of uracil) containing glucose as the carbon source, were washed and inoculated in a synthetic medium containing galactose for 24 h. One OD unit of induced yeast cells was centrifuged and the pellet was washed twice with 1× PBS. The pellet was then resuspended in BODIPY 493/503 (working concentration 1 mg ml⁻¹) and kept in darkness for 30 min. The cells were centrifuged and washed three times with water to remove the excess stain. The cells were resuspended in a residual amount of water, fixed onto slides and examined using confocal microscopy (Laser Scanning Confocal Microscopy, Olympus) at 40× magnification by using Fluoview FV1000 software.

RESULTS

Identification of the gene encoding RgDGAT

Previous work from our laboratory revealed the presence of a cytosolic TBC in R. glutinis (Gangar et al., 2001). Protein sequencing of the putative DGAT from the multienzyme complex resulted in a peptide sequence LWWAVGQPPFGGARGS. This information was used to screen the cDNA library of R. glutinis. Two positive clones were isolated (~894 bp and ~1.7 kbp) and sequenced. Surprisingly, those two genes were the same, and the clone with a short sequence had a truncation at the N-terminal end. We called the identified full-length gene RgDGAT, and the short sequence the C-terminal domain (RgDGAT-CTD) of the full-length sequence. A dendrogram representing the phylogenetic relationship of RgDGAT with genes belonging to known DGAT families, i.e., DGAT1, DGAT2, Bifunctional wax ester/TAG synthase family and DGAT3 from different organisms, was constructed. RgDGAT showed a similarity with At5g23940, a soluble DGAT recently reported in A. thaliana (Rani et al., 2010). The analysis of the dendrogram suggested that RgDGAT
belongs to the soluble DGAT3 class (Fig. 1). In support, a Kyte–Doolittle hydropathy plot of RgDGAT revealed the lack of any transmembrane domains in the protein structure in contrast to the DGAT1 and DGAT2 class of enzymes (Fig. S1, available with the online version of this paper).

**RgDGAT overexpression in *S. cerevisiae* enhances TAG in vivo**

Two different gene families, DGAT1 and DGAT2, encode the majority of cellular DGAT in all known systems (Cases et al., 2001; Lardizabal et al., 2001). In *S. cerevisiae*, four membrane-bound DGAT enzymes (*DGA1*, *LRO1*, *ARE1* and *ARE2*) have been identified (Czabany et al., 2007; Müller & Daum, 2004). To study the effect of RgDGAT overexpression on the neutral lipid content of cells, the gene was transformed into *S. cerevisiae* cells. The overexpression of RgDGAT in *S. cerevisiae* cells exhibited a higher accumulation of TAG compared with vector-transformed cells, a result revealed by $[^{14}C]$acetate labelling (Fig. 2a). A quadruple mutant of the *S. cerevisiae* strain H1246 in which all four genes were knocked out showed no evidence of storage lipid formation (Sandager et al., 2002). The yeast strain is devoid of inherent DGAT activity, and to overcome the background TAG produced in wild-type cells, the RgDGAT gene was also expressed in quadruple mutant cells (Sandager et al., 2002). To understand the fate of neutral lipid biosynthesis, a $[^{14}C]$acetate labelling study was performed with pYES2- and pYES2-RgDGAT-transformed mutant cells. As expected, the RgDGAT-expressing cells yielded TAG but cells having a vector alone did not

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**Fig. 1.** Phylogenetic analysis of homologue sequences of RgDGAT across the organisms. The tree was constructed using CLUSTAL W software by taking RgDGAT homologue sequences from the indicated organisms. Twenty-nine DGAT homologue sequences were taken from 15 different species and analysed based on the Neighbour-joining method. Membrane-bound DGATs, soluble DGATs, TAG/wax ester synthase and other homologue genes in *A. thaliana* are marked. The organisms and their GenBank/NCBI accession numbers (in parentheses) are below. *A. thaliana* DGAT1 (AF051849), *Brassica napus* DGAT1 (AF164434), *Perilla frutescens* DGAT1 (AAC23696), *Rattus norvegicus* DGAT1 (NP_445889), *Mus musculus* DGAT1 (NP_034176), *Bos taurus* DGAT1 (AAL49962) and *Caenorhabditis elegans* DGAT1 (AAF82410). *Saccharomyces cerevisiae* DGAT2 (YOR245C), *Mortierella ramanniana* 2A (AF391089), *Mortierella ramanniana* 2B (AF391090), *Acinetobacterspecies* (wax ester/TAG synthase), *Arabidopsis thaliana* DGAT3 (soluble DGATs), *R. glutinis* (RgDGAT) (AED93236.1), *Arachis hypogea* DGAT3 (AAX62735.1), *At5g23940* (DCR) (AED93236.1), *At1g48300* (AAP21223.1), *Bos taurus* DGAT2 (AAT78344.1), *Homo sapiens* DGAT1 (AAH06263.4), *Brassica napus* DGAT2 (AAD40881.1), *Rhodotorula glutinis* DGAT3 (ABC41546.1; this study, in bold type).
To characterize the enzyme in vitro, RgDGAT was overexpressed and purified from S. cerevisiae quadruple mutant cells. The overexpression of the RgDGAT protein in yeast (30 μg cell lysate) was confirmed by a Western blot using anti-DGAT antibodies (Fig. 3a). The cell lysate of the R. glutinis strain utilized in this work was used as a positive control. The RgDGAT protein was purified from yeast to near homogeneity (Fig. 3b) by Ni\(^{2+}\) -NTA affinity column chromatography as described in Methods. The purified protein was used for biochemical analyses. In yeast, TAG biosynthesis from DAG can be achieved by two mechanisms. While the enzyme Lro1p catalyses the transfer of the fatty acyl group from phosphatidyl choline to DAG, Dga1p, Are1p and Are2p utilize fatty acyl-CoA for TAG biosynthesis. When \(^{14}\text{C}\)-labelled oleoyl-CoA was given as an acyl donor and DAG as an acyl group acceptor, the enzyme catalysed the TAG formation in a protein- and time-dependent manner (Fig. 3c, d). The time-dependent DGAT assay was performed with 50 μM diolein, 10 μM \(^{14}\text{C}\)-labelled oleoyl-CoA and 5 μg enzyme in a total volume of 0.5 ml. An aliquot of 100 μl was withdrawn at several different time intervals (0, 10, 15, 30, 45 min), and lipids were separated on a TLC plate. In a converse experiment, when \(^{14}\text{C}\)DAG was used as an acyl acceptor and oleoyl-CoA as an acyl donor, the enzyme exhibited similar kinetics of TAG biosynthesis (Fig. 3e). These results show that the novel gene identified from R. glutinis utilizes acyl-CoA as a substrate for TAG biosynthesis.

While in yeast, DGAT1 and DGAT2 enzymes exhibit similar fatty acyl-CoA specificities, in plants, DGAT enzymes exhibit varied substrate specificities. For example, in Racinus communis, RcdDGAT2 can increase unusual fatty acid levels in TAG, while RcdDGAT1 cannot. To assess the preference for acyl-CoA by the DGAT3 enzyme, the assays were performed using long-, medium- and short-chain fatty acids, oleoyl-, palmitoyl-, stearoyl-, lauroyl-, myristoyl- and butyryl-CoAs as acyl donors and DAG as an acyl acceptor. The enzyme was found to prefer acyl-CoAs in the following pattern: 18:1-CoA > 16:0-CoA = 18:0-CoA (Fig. 3f). The enzyme did not prefer the medium- and short-chain fatty acids. This also supports the fact that major constituents of microbial fat of R. glutinis are oleic (47%) and palmitic (37%) (Misra et al., 1984). Higher activity was observed with oleoyl-CoA [6.25 nmol TAG formed min\(^{-1}\) (mg protein)\(^{-1}\)] compared with palmitoyl-CoA [5.1 nmol TAG formed min\(^{-1}\) (mg protein)\(^{-1}\)]. When stearoyl-CoA was used as an acyl donor, activity was found to be 4.3 nmol TAG formed min\(^{-1}\) (mg protein)\(^{-1}\) under the standard assay conditions. These data suggest that RgDGAT exhibits broad specificity for acyl-CoAs, with greater preference for oleoyl-CoA.

In yeast, the two membrane DGATs, DGAT1 and DGAT2 which utilize acyl-CoA as an acyl acceptor, differ with respect to their co-factor dependence (Cases et al., 2001; Yen et al., 2008). While DGAT2 is an Mg\(^{2+}\)-dependent enzyme, DGAT1 does not require Mg\(^{2+}\) for its activity. The effect of the metal ions, Mg\(^{2+}\) and Ca\(^{2+}\) on the DGAT
**Fig. 3.** Biochemical characterization of RgDGAT. (a) Immunoblot analysis with anti-DGAT antibodies. Lanes: 1, *R. glutinis* cell lysate; 2, quadruple mutant yeast cells overexpressing RgDGAT; M, protein molecular mass marker. (b) Ni²⁺-NTA purification of the recombinant DGAT enzyme from yeast. F1–F3, represents the purified enzyme fractions. (c) A protein-dependent DGAT
activity assay was performed using different amounts of a purified recombinant RgDGAT protein. NE represents the reaction in which no enzyme was added but that contains DAG and \([^{14}C]\)-labelled oleoyl-CoA. Different amounts of protein (0.2, 0.4, 1, 2 and 4 \(\mu\)g) were taken to perform the DGAT assay. (d) A time-dependent DGAT assay performed with 50 \(\mu\)M diolein and 10 \(\mu\)M \([^{14}C]\)-labelled oleoyl-CoA. The purified recombinant enzyme (5 \(\mu\)g) was used in the assay. Aliquots were withdrawn at different time intervals (0, 15, 30, 45 and 60 min), and the lipids were separated by TLC. NE represents a reaction in which no enzyme was added but that contains DAG and \([^{14}C]\)-labelled oleoyl-CoA, which was incubated for 60 min. '0' indicates the zero time point at which the enzyme was added after stopping the reaction. (e) A time-course DGAT assay was carried out using \([^{14}C]\)DAG and \([^{14}C]\)-labelled oleoyl-CoA to further validate the DGAT activity of the enzyme. Aliquots were taken at different intervals (0, 5, 10, 15, 20, 25 and 30 min), and lipids were extracted with chloroform/methanol (2:1), resolved on TLC and then visualised using a phosphorimager. NE represents a reaction in which no enzyme was added but that contains \([^{14}C]\)DAG and \([^{14}C]\)-labelled oleoyl-CoA incubated for 30 min. A representative TLC is given. (f) Acyl donor-specificity with purified RgDGAT enzyme (1 \(\mu\)g) using palmitoyl-, oleoyl-, stearoyl-, lauroyl-, myristoyl- and butyryl-CoAs. Radiolabelled DAG and non-radiolabelled fatty acyl-CoAs were used for the assay, with an incubation time of 20 min. (g) The effect of monovalent and divalent ions on DGAT activity tested with metal ions \(\text{Mg}^{2+}, \text{K}^+, \text{Ca}^{2+}\) (1 mM). The assay was performed using 1 \(\mu\)g purified enzyme. (h) A DGAT assay was carried out at different pH ranges using 1 \(\mu\)g purified protein. The graphs in (c–h) depict the specific activity of TAG formation obtained from three independent experiments; error bars, SEM.

Overexpression and functional analysis of the recombinant CTD of RgDGAT

The short cDNA clone isolated from the library happened to be the CTD of the RgDGAT; a map of the full-length and truncated versions of RgDGAT is in Fig. 4(a). The protein possesses a neutral lipid-binding motif and an acyltransferase motif containing a putative DGAT active site. The recombinant CTD of RgDGAT was purified by Ni-NTA affinity column chromatography and the enzyme overexpressed cell lysate (50 \(\mu\)g) was analysed by immunoblotting with an anti-His-tag antibody as well as with anti-DGAT polyclonal antibodies (Fig. 4b). Due to the presence of putative acyltransferase motifs and the DGAT active site at the CTD of RgDGAT, we investigated whether the enzyme retains the acyltransferase activity. To test the TAG biosynthetic activity, a time-dependent DGAT assay was carried out. The purified recombinant RgDGAT-CTD showed an increased incorporation of \([^{14}C]\)-labelled oleoyl-CoA into TAG in a time-dependent manner (Fig. 4c). A protein-dependent DGAT assay also confirmed the increase in TAG biosynthesis (Fig. 4d). These data suggest that the CTD of RgDGAT that harbours the putative acyltransferase motif is sufficient for DGAT functionality. A comparison of CTD with the RgDGAT protein revealed that the full-length protein exhibits ~1.3-fold higher activity (Fig. 4e).

Rescue of quadruple mutant cells by RgDGAT from oleic acid toxicity

Yeast can import exogenous fatty acids and convert them to their respective acyl-CoA derivatives. These fatty acyl-CoAs are then channelled to TAG for storage or into membrane phospholipids. Unusual fatty acids are usually utilized for storage to prevent perturbation of the membrane structure and hence fatty acid toxicity to the cell (Connorth et al., 2010; Siloto et al., 2009). Thus, TAG protects cells by acting as a sink for these modified/unusual fatty acids. The channelling of these fatty acids into TAG is carried out by DGAT enzymes.

Wild-type yeast cells grow normally without any growth defect in the presence of unsaturated fatty acids, especially when oleic acid is in the medium. However, quadruple mutant cells exhibit a growth defect in the presence of a fatty acid-containing medium. To test whether soluble acyltransferase is equally efficient in rescuing yeast cells from free fatty acid toxicity, the quadruple mutant cells were transformed with pYES2 (vector alone), pYES2-RgDGAT and pYES2-CTD. In media devoid of fatty acids, both wild-type and quadruple mutant cells exhibited normal growth. As the concentration of oleic acid in the medium was increased to 0.5 mM, growth of the quadruple mutant was arrested. However, upon overexpression of the RgDGAT enzyme, growth of the quadruple mutant was rescued at the critical oleic acid concentration compared with the vector control cells (Fig. 5).

Lipid particles are present at abundant levels in the wild-type and completely absent in the quadruple DGAT-disrupted strain. To assess whether the cells rescued from free fatty acid toxicity form lipid particles, quadruple mutant cells transformed with the pYES2 vector alone or pYES2-RgDGAT were visualized by confocal microscopy after BODIPY staining. The BODIPY493/503 fluorophore specifically binds to lipid bodies containing neutral lipids such as TAG (Gocze & Freeman, 1994). Confocal microscopy analysis revealed cytoplasmic lipid droplet formation in quadruple mutant cells transformed with pYES2-RgDGAT when compared with cells transformed with pYES2 alone (Fig. 6). From these studies, it is evident that soluble DGAT is efficient in mobilizing the free fatty acid pool into storage lipids.
DISCUSSION

*R. glutinis*, an oleaginous yeast isolated from soil accumulates several times more oil than normal yeast. However, the molecular pathways that govern this physiological behaviour are poorly understood. Since TAG is a major component of oil and is the rate-limiting step in the pathway, we postulated that the accumulation of an enormous amount of lipids in oleaginous yeast could be due to the presence of multiple TAG synthases. Owing to the lack of an *R. glutinis* genomic database, we isolated a cDNA clone encoding a full-length DGAT and determined the specificity of the enzyme.

The following lines of evidence indicate that cytosolic TAG biosynthetic machinery is involved in the accumulation of TAG in *R. glutinis*. (i) The bacterially expressed, purified recombinant cDNA was examined for possible acyltransferase activities. It was found to specifically acylate DAG in an acyl-CoA-dependent manner to form TAG. It showed a high activity with DAG and radiolabelled oleoyl-CoA by incubating with various amounts of enzyme (0.2, 0.4, 0.8 and 1 µg) for 20 min. (ii) The graph shows the protein-dependent DGAT activity.

(iii) Comparison of the DGAT-specific activities of *RgDGAT* and its C-terminal domain. Lanes: 1, the reaction in which no enzyme was added but that contains DAG and radiolabelled oleoyl-CoA; 2, full-length DGAT; 3, CTD. Data represent the values from three independent experiments; error bars, SEM.
Lipid acyltransferases have the conserved signature sequence HX4D (Heath & Rock, 1998) in which the conserved His and Asp residues are involved in catalysis. It is proposed that the His residue removes a proton from the hydroxyl group of the acyl acceptor, thereby facilitating its nucleophilic attack on the thioester bond of the acyl donor. The closely spaced Asp residue is hypothesized to stabilize the positive charge generated during the catalysis (Heath & Rock, 1999). It is observed that in some DGAT genes, serine or glutamine substitutes for the active site histidine of the acyltransferase motif (Daniel et al., 2004). We observed that RgDGAT contains the modified acyltransferase motif 435SXXD437. A pairwise alignment of RgDGAT and human acyl-CoA : cholesterol acyltransferase (GenBank accession no. L21934) showed that a conserved RLXXXE motif (Yang et al., 1997) aligns in the vicinity of the active site residues. Interestingly, the multiple sequence analysis indicated that the protein belongs to the dehydrogenase enzyme superfamily. In plants, a dehydrogenase-like gene has been shown to be differentially expressed during TAG accumulation (Francki et al., 2002), indicating that a subfamily of dehydrogenases catalyse acyl group transfer.

These results differ from those obtained with other eukaryotic systems, where TAG biosynthesis is associated solely with the membrane fraction. Our results provide evidence for the presence of a soluble acyl-CoA : DGAT in the eukaryotic system. The overexpression of RgDGAT in yeast enhances the TAG level and, thereby, overall neutral lipid biosynthesis. Our study on the fate of phospholipids indicates a significant reduction in the level of phospholipids on RgDGAT overexpression in yeast (data not shown). Similar studies in human lung SV40-transformed fibroblast cells suggest a decrease in the level of phospholipids on DGAT overexpression (Bagnato & Igal, 2003).

The biosynthesis of TAG is shown to occur in microsomal membranes (Bishop & Bell, 1988; Kent et al., 1991; Raetz & Dowhan, 1990) catalysed by four DGAT enzymes belonging to independent gene families namely DGAT1, DGAT2, bi-functional wax-ester/DGAT and PDAT (Cases et al., 1998; Dahlqvist et al., 2000; Lardizabal et al., 2001; Oelkers et al., 2000; Sorger & Daum, 2002; Yang et al., 1996; Yu et al., 1996). Although they are all involved in TAG biosynthesis, they differ in sequence similarity and substrate specificity (Turchetto-Zolet et al., 2011; Yen et al., 2008). It has also been speculated that these gene families play different roles across species, tissues and organs.
over time during development. The existence of cytosolic enzymes that provide important precursors for TAG biosynthesis were also documented in other systems (Ghosh et al., 2008a, b, 2009; Parthibane et al., 2012; Rani et al., 2010; Reddy et al., 2008, 2010; Saha et al., 2006). In plants, it has been shown that a soluble TAG biosynthetic pathway operates during post-germinative seedling growth when sucrose is supplied externally (Hernández et al., 2012). Recent studies with soluble DGAT from A. thaliana have shown that the enzyme functions in cutin biosynthesis by incorporating hydroxyl fatty acids into TAG. We presume that the isolated DGAT from R. glutinis (GenBank accession no. DQ315417) plays an important cellular role in oleaginous yeast (Gangar et al., 2002). Alternatively, the enzyme might function in a stress response; one such example may be at nutrient deficient conditions. There are reports of TAG accumulation under different stress conditions in both bacteria as well as in eukaryotes. For example, in Mycobacterium tuberculosis, TAG is accumulated under acidic, static or hypoxic growth conditions (Sirakova et al., 2006). Similarly, in Chlamydomonas, TAG is accumulated when cells are exposed to nitrogen starvation conditions (Boyle et al., 2012). The growth of R. glutinis on various growth media indicated that there is a drastic reduction in cell growth and TAG production upon glucose starvation. The decrease in the TAG levels was correlated with lower levels of cellular RgDGAT, indicating that the physiological levels of the enzyme are regulated.

In conclusion, the results presented here demonstrate that cDNA encoding DGAT enzymically catalyses the transfer of an acyl group to DAG in a membrane-independent manner. Although the physiological relevance of this reaction by soluble DGAT is not completely understood, the present study offers a biochemical explanation for the abundant lipid accumulation in oleaginous yeasts and highlights the importance of an alternative pathway in TAG biosynthesis.

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**REFERENCES**


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