(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 March 2003 (06.03.2003)

PCT

(10) International Publication Number
WO 03/018814 A2

(51) International Patent Classification[7]: C12N 15/52, 9/10, 9/16, 9/00, 9/02, 15/11, C07K 16/40

(21) International Application Number: PCT/IN02/00176

(22) International Filing Date: 29 August 2002 (29.08.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/315,757 30 August 2001 (30.08.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for all designations

Published:
without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO / 8814 A2

(54) Title: A NOVEL TRIACYLGLYCEROL BIOSYNTHESIS IN THE! CYTOSOL OF EUKARYOTES

(57) Abstract: This invention describes novel catalytically active cytosolic enzymes for triacylglycerol biosynthesis from eukaryotic systems. The complex from oleaginous yeast was enzymatically characterized, and was found to contain lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl-acyl carrier protein synthetase, superoxide dismutase and acyl carrier protein. The triacylglycerol biosynthetic machinery rapidly incorporates free fatty acids as well as fatty acyl-coenzyme A into triacylglycerol and its biosynthetic intermediates. Lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase and diacylglycerol acyltransferase from the complex were microsequenced. Acyl carrier protein, superoxide dismutase and diacylglycerol acyltransferase genes were cloned and expressed in bacterial system. The soluble triacylglycerol biosynthetic enzymes (lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase) in yeast, rat adipocytes and human hepatocyte cell-line (HepG2) exist in the cytosol either as free enzymes or as a multienzyme complex.

A NOVEL TRIACYLGLYCEROL BIOSYNTHESIS IN THE CYTOSOL OF EUKARYOTES

TECHNICAL FIELD

This invention relates to novel catalytically active cytosolic enzymes for triacylglycerol biosynthesis from eukaryotic systems. The complex from oleaginous yeast was enzymatically characterized and was found to contain lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl-acyl carrier protein synthetase, superoxide dismutase and acyl carrier protein. The triacylglycerol, biosynthetic machinery rapidly incorporates free fatty acids as well as fatty acyl-coenzyme A into triacylglycerol and its biosynthetic intermediates. Lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase and diacylglycerol acyltransferase from the complex were microsequenced. Acyl carrier protein, superoxide dismutase and diacylglycerol acyltransferase genes were cloned and expressed in bacterial system. The triacylglycerol biosynthetic enzymes such as lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase and diacylglycerol acyltransferase in baker's yeast, rat adipocytes and human hepatocyte cell-line can exist in the cytosol as free enzymes.

BACKGROUND ART

The de novo biosynthesis of triacylglycerol has been shown to occur 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 that are catalyzed by dihydroxyacetone phosphate acyltransferase (5) and acyl-dihydroxyacetone phosphate reductase *(6,* 7), respectively. The acylation of LPA is catalyzed by LPA acyltransferase to form phosphatidic acid (PA), which is a branch point for the synthesis of diacylglycerol (DAG) and phospholipids. PA phosphatase catalyzes the dephosphorylation of PA to DAG that is an immediate precursor for triacylglycerol (TAG), phosphatidylcholine and phosphatidylethanolaine. DAG can also be derived from phospholipids by the action of phospholipase C (8), which is an important signal molecule that activates protein kinase C **(9). DAG** acyltransferase catalyzes the acylation of DAG, which is the committed step in TAG biosynthesis. Recently, it has been shown in plants and yeast cells that an acyl-CoA

independent enzyme for TAG synthesis that uses phospholipid as acyl donor and DAG as acyl acceptor. This reaction is catalyzed by phospholipid:DAG acyltransferase (10). The same reaction is also catalyzed by lecithin:cholesterol acyltransferase (11). All the enzymes in these pathways are shown to be membrane-bound in eukaryotic systems (1-4, 12, 13). Both mitochondria1 membranes and endoplasmic reticulum (ER) have been identified as the major sites for phospholipid and TAG synthesis in S. cerevisiae (3, 6, 14).

A number of fungi are known to have high levels of TAG. Understanding the lipid biosynthesis would enable to genetically engineer fungi and plants with desired fatty acid composition and the altered oil content (15).

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.

TAG enzymes in yeast comprise of lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl-acyl carrier protein synthetase and acyl carrier protein. These TAG biosynthetic enzymes may exist as either free or rnultienzyme complex. Among these enzymes, lysophosphatidic acid acyltransferase can be identified in any yeast strain by immunological cross reactivity to the peptide sequence ALELQADDFNK (peptide SEQ ID **2),** diacylglycerol acyltransferase identified by immunological cross reactivity to the peptide sequence XLWAVVGAQPFGGARGS (peptide SEQ ID **7)** and phosphatidic acid phosphatase identified by immunological cross reactivity to peptides NALTGLHMGGGK (peptide SEQ ID **4)** and YVEGARP (peptide SEQ JD ***6).*** TAG biosynthetic enzymes thus isolated from oleaginous yeast can utilize free fatty acids or fatty acyl-CoA or acyl-ACP as substrates.

In oleaginous yeasts and other lipid rich fungi, biochemical and genetic study of these enzyme systems would enable to genetically engineer fungi and.plants with desired fatty acid composition and altered oil content. Using modem methods of genetics, these enzymes may be produced by recombinant gene expression; the recombinant proteins may be reconstituted to active TAG biosynthetic complex; the complex may be suitably assayed to identify

specific inhibitors of TAG biosynthesis, which may have a potential value as lipid lowering drugs in humans.

Keeping with and to further studies conducted, Applicant investigated cytosolic TAG biosynthetic pathway using Rhodotorula glutinis and other Saccharomyces cerevisiae, rat adipocytes and human hepatocytes cell-line.

OBJECTS OF THE INVENTION

The main object of the present invention is to determine the source of the biosynthetic pathway in yeasts.

Another object is to isolate TAG biosynthetic enzymes using yeast species.

Yet another object is to identify and isolate novel TAG biosynthetic enzymes in yeast.

Still another object is to identify the gene sequences encoding TAG biosynthetic enzymes.

Another object is to develop an assay for the isolation of cytosolic TAG biosynthetic enzymes from yeast using free fatty acids or fatty acyl CoA or Acyl ACP as substrates.

Another object is to demonstrate the cytosolic TAG biosynthetic enzymes from *Saccharomyces cerevisiae*, rat adipocytes and human hepatocytes cell-line.

DETAILED DESCRIPTION

In accordance with the above objects, the invention provides a novel source for isolation of TAG enzymes. The invention also provides novel TAG biosynthetic enzymes in yeast and gene sequences and gene sequences encoding these enzymes. The invention is described in detail herebelow with reference to the examples, which should not be construed as limitations on the inventive concept herein.

This invention provides the identification of soluble triacylglycerol biosynthetic machinery in yeasts, rat adipocytes (white and brown), human hepatocytes (HepG2 cell line), which are involved in triacylglycerol biosynthesis.

Thus, the invention provides novel soluble triacylglycerol (TAG) biosynthetic enzymes in eukaryotic cells. These soluble TAG enzymes are soluble in water and detergent solutions.

In the invention, the soluble **TAG** enzymes in yeast comprise lysophosphatidic acid acyltransferase with peptide SEQ ID 1 XALELQADDFNK and peptide SEQ ID 3 XXVNNVXPGXIEQ, phosphatidic acid phosphatase with peptide SEQ ID **4** NALTGLHMGGGK and peptide SEQ ID *5* YVEGARPXK, diacylglycerol acyltransferase with peptide SEQ ID **7** XLWAVVGAQPFGGARGS, acyl-acyl carrier protein synthetase with peptide SEQ ID 8 VHLAVALYGLAAVRVSRIVR, superoxide dismutase encoded by the gene sequence as in SEQ ID 9, and acyl carrier protein encoded by the gene sequence as in SEQ ID 10. The gene sequence encoding superoxide dismutase and diacylglycerol acyltransferase acyl carrier protein have sequence homology to DNA sequences as in SEQ ID 9, SEQ ID 11 and SEQ ID 10, respectively. The lysophatidic acid acyltransferase is identified by immunological cross reactivity to the peptide sequence ALELQADDFNK (as in peptide SEQ ID 2), diacylglycerol acyltransferase is identified by immunological cross reactivity to the pepetide sequence XLWAVVGAQPFGGARGS (as in peptide SEQ ID **7)** and phosphatidic acid phosphatase is identified by immunological cross reactivity to peptides NALTGLHMGGGK **(as** in peptide SEQ ID **4)** and YVEGARP (as in peptide SEQ ID 6).

The enzymes responsible for TAG synthesis **and** accumulation are either in free or multi enzyme complex form.

The novel soluble TAG biosynthetic enzymes can be used to identify compounds that are capable of altering for TAG synthesis and accumulation.

This invention provides method for isolation of soluble triacylglycerol biosynthetic machinery from eukaryotes, which are responsible for triacylglycerol biosynthesis. The eukaryotic cells are selected from yeast, rat adipocytes and human cell-lines (HepG2).

This invention provides the soluble triacylglycerol biosynthetic machinery from oleaginous yeast, which are responsible for tnacylglycerol accumulation.

This invention provides a method of obtaining a polypeptide(s) in purified form which comprises: (a) introducing the vector comprising the isolated nucleic acid which encodes a

superoxide dismutase (SOD)(SEQ ID 9) and acyl carrier protein (ACP) (SEQ ID 10) and diacylglycerol acyltransferase (SEQ ID 11) into a suitable host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered.

This invention provides purified polypeptides, which are lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein, acyl-ACP synthetase and superoxide dismutase.

This invention provides an oligopeptide of at least 5 to 20 amino acids (peptide SEQ ID 1 to 8) capable of specifically identifying with a unique sequence of proteins present within a triacylglycerol biosynthetic complex (TBC).

This invention provides antibodies to an at least 5 to 20 amino acids (peptide SEQ ID 1 to 8) from the isolated polypeptides from TBC, which are capable of specifically identifying the proteins present within a triacylglycerol biosynthetic complex.

This invention provides an oligonucleotide of at least 15 nucleotides from SEQ ID 9 capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid, which encodes a superoxide dismutase (SOD).

This invention provides an oligonucleotide of at least 15 nucleotides from SEQ ID 10 capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid, which encodes an acyl carrier protein (ACP).

This invention provides a nucleic acid having a sequence complementary to the sequences (SEQ ID 9 to 11) of the isolated nucleic acid, which encode superoxide dismutase and acyl carrier protein and diacylglycerol acyltransferase.

This invention provides an in vitro method of detecting the soluble tnacylglycerol biosynthetic enzymes, which exist either as free or as complex in the cytosol.

This invention provides a method for determining whether a subject known to have an imbalance in triacylglycerol has the imbalance due to a defect in the synthesis of triacylglycerol.

This invention provides a method for treating a subject who has an imbalance in triglyceride (triacylglycerol) levels due to a defect in the synthesis of soluble triglyceride, which comprises introducing the isolated nucleic acid which encodes lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycero1 acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase into the subject under conditions such that the nucleic acid expresses the soluble triacylglycerol biosynthetic enzymes individually or in combination, so as to thereby treat the subject.

This invention provides a method for inhibiting the soluble triacylglycerol biosynthetic enzymes in a subject which comprises transforming appropriate cells from the subject with a vector which expresses the nucleic acid which encodes the components in TBC, and introducing the transformed cells into the subject so as to thereby inhibit the soluble enzyme and/or complex.

This invention provides a method for inhibiting 'the soluble triacylglycerol biosynthetic enzymes in a subject which comprises introducing the any of the above-described oligonucleotides of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encode lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase into the subject so as to thereby inhibit the triglyceride formation and accumulation.

This invention provides a method for identifying a chemical compound which is capable of inhibiting soluble triacylglycerol biosynthetic enzyme(s) in a subject which comprises: (a) contacting soluble triacylglycerol biosynthetic enzyme(s) with chemical compound under conditions permitting binding between the soluble triacylglycerol biosynthetic enzyme(s) and the chemical compound; (b) detecting specific binding of the chemical compound to the soluble triacylglycerol biosynthetic enzyme(s); and c) determining whether the chemical

compound inhibits the activity of the soluble triacylglycerol biosynthetic enzyme(s) so as to identify a chemical compound which is capable of inhibiting soluble triacylglycerol biosynthetic enzyme(s) in a subject.

This invention provides a method for identifying a chemical compound which is capable of enhancing soluble triacylglycerol biosynthetic enzyme(s) in a subject which comprises: (a) contacting the soluble triacylglycerol biosynthetic enzyme(s) with the chemical compound under conditions permitting binding between the soluble triacylglycerol biosynthetic enzyme(s) and the chemical compound; (b) detecting specific binding of the chemical compound to the soluble triacylglycerol biosynthetic enzyme(s); and c) determining whether the chemical compound enhances the activity of the soluble triacylglycerol biosynthetic enzyme(s) so as to identify a chemical compound which is capable of enhancing soluble triacylglycerol biosynthetic enzyme(s) in a subject.

This invention provides a pharmaceutical composition comprising the chemical compound(s), which is capable of inhibiting soluble triacylglycerol biosynthetic enzyme(s) identified by the above-described method in an amount effective to inhibit soluble triacylglycerol biosynthetic enzyme(s) in a subject and a pharmaceutically effective carrier.

This invention provides a pharmaceutical composition comprising the chemical compound, which is capable of enhancing soluble triacylglycerol biosynthetic enzyme(s) activity identified by the above-described method in an amount effective to enhance soluble triacylglycerol biosynthetic enzyme(s) activity in the subject and a pharmaceutically effective carrier.

This invention provides a method of treating a subject who has atherosclerosis comprising the pharmaceutical composition comprising the chemical compound which is capable of inhibiting soluble triacylglycerol biosynthetic enzyme(s) identified by the above-described method in an amount effective to inhibit soluble triacylglycerol biosynthetic enzyme(s) in a subject and a pharmaceutically effective carrier.

This invention provides a method of treating a subject who has hyperlipidemia comprising the pharmaceutical composition comprising the chemical compound which is capable of

inhibiting soluble triacylglycerol biosynthetic enzyme(s) identified by the above-described method in an amount effective to inhibit soluble triacylglycerol biosynthetic enzyme(s) in a subject and a pharmaceutically effective carrier.

This invention provides a method of reducing the deposition of fat cells in a subject by decreasing the amount of triglycerides produced in adipose cells of the subject comprising administering the pharmaceutical composition comprising the chemical compound which is capable of inhibiting soluble triacylglycerol biosynthetic enzyme(s) identified by the above-described method in an amount effective to inhibit diacylglycerol acyltransferase in a subject and a pharmaceutically effective carrier.

This invention provides a transgenic, nonhuman mammal comprising the isolated nucleic acid, which encode lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase.

This invention provides a method of obtaining soluble triacylglycerol (TAG) biosynthetic enzymes using polyacrylmide gel electgrophoresis, chromatographic procedures or density gradient centrifugation, said method comprising subjecting blue sepharose, DEAE matrix octail sepharose, antibodies raised from the peptide SEQ ID 1 to 8 and identifying and isolating the complex as a whole or individual components thereof.

This invention provides antibodies directed to an epitope of a purified lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein, acyl-ACP synthetase and superoxide dismutase. This invention provide antibodies capable of specifically binding to a purified lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein, acyl-ACP synthetase and superoxide dismutase.

This invention provides a vector comprising the isolated nucleic acid which encode SOD and ACP related gene products.

This invention provides a purified lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein, acyl-ACP synthetase and superoxide dismutase related gene products.

This invention provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encode lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase related gene products lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase.

This invention provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid which encode lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase related gene products.

This invention provides antibodies directed to epitopes of a purified components of TBC lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein (ACP), acyl-ACP synthetase and superoxide dismutase related gene products. This invention provides antibodies capable of specifically binding to'a purified TBC and its components related gene products.

The invention is illustrated by the following figures wherein:

Figure 1. Growth, cells viability, TAG synthesis and accumulation of R. glutinis. **(A)** Exponentially growing cultures in malt-yeast extract medium were diluted to $OD_{600\ nm}$ = 0.12, and incubated at 30 "C. At regular time intervals, $OD_{600\ nm}$ was measured, and aliquots were diluted, and incubated at 30 °C overnight. (B) Viable cells were counted and expressed as colony-forming units/ml. Open circle indicates $OD_{600\ nm}$; closed circle indicates colony-forming unit/ml. (C) Nile blue A' staining and phase contrast fluorescence microscopy of yeast cells grown at various time intervals. (D) Incorporation of [^{14}C]acetate into TAG of

yeast cells grown at various time intervals. Incorporation was carried out as described in Materials and Methods.

Figure **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 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 co-localized in both the gel filtration and density gradient studies.

Figure **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 2^{nd} 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 focussing.

Figure **4.** LPA acyltransferase is a part of the 10S TAG biosynthetic complex (A) Purified multienzyme complex was treated with 0.1% SDS and 50 mM DTT 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 5^{th} cm of the gel exhibited LPA acyltransferase activity. The molecular size of LPA acyltransferase (LPAAT) corresponded to **32** kDa. (B) A synthetic peptide corresponding to the sequence of the major peptide of LPA acyltransferase was conjugated to BSA for generation of polyclonal antibodies. The antiserum was used to probe the TBC, cytosol and CHAPS solubilized membranes after electrophoresis on native/SDS- polyacrylamide gels and subsequent transfer to nitrocellulose membrane. **TBC** and a **32** kDa protein were visualized in immunoblots under native and

denaturing conditions, respectively. This antibody also reacted with a 28 kDa protein in the solubilized membrane fraction (lane **4).** (C) [^{35}S]Labeled R. glutinis lysate was immunoprecipitated with anti-LPA acyltransferase' antibody. The immunoprecipitate was analyzed on **7%** native gel **as** well as 12% SDS-polyacrylamide gel and proteins visualized by flurography. Native and SDS-gel flurographs showed the presence of TBC and five polypeptides corresponding to the proteins of the TBC, respectively. Normal rabbit serum was used as the negative control in immunoprecipitations.

Figure 5. PA phosphatase is a part of the 10S TAG biosynthetic complex

(A) Purified multienzyme complex was treated with 0.1% SDS and 50 mM DTT 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 PAPase antibodies. Both 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) [^{35}S]Labeled R. glutinis lysate 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 visualized by flurography. Native and SDS-gel flurographs 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.

Figure 6. Nucleotide and deduced amino acid sequence of superoxide dismutase, a component in TBC. The nucleotide sequence of the isolated cDNA (upper line) is presented with its deduced amino acid sequence (lower line). Nucleotides are numbered in the 5'-3' direction whereas amino acids are numbered from amino terminus starting from the first methionine residue (bold face). Polyadenylated tail of the transcript is indicated in Italics and

putative polyadenylation signal is underlined. Asterisk indicates the stop codon of the ORF. The first 20 amino acid residues (shown in bold) were matched with the N-terminal sequence obtained from the 21 kDa protein of TBC.

Figure 7. Multiple sequence alignment of superoxude dismutase. The deduced amino acid sequence of the isolated clone was used to search the public databse (Swissprot) using **BLAST** algorithm. The first few significant hits were superoxide dismutases from many different organisms (*E. coli, S. cerevisiae,* microsporum, maize, pea, capsicum, human, bovine and rabbit). Multiple sequence alignment was performed with these polypetide sequences with our isolated clone using the ClustalW algorithm. Hyphens represent gaps introduced to obtain the best alignment. On the line underneath each section of alignment, asterisk indicates the conserved residues in all sequences in the alignment, colon indicates conserved substitutions and the dot indicates semi-conserve substitutions. Three most conserved domains present among all the SOD's are marked as I, II and III; among them motifs I and II are present in isolated clone but motif III is absent.

Figure 8. Nucleotide and deduced amino acid sequence of ACP. The nucleotide, sequence of isolated ACP clone (upper line) is presented with its deduced amino acid sequence (lower line). Nucleotides are numbered in the 5'-3' direction whereas amino acids are numbered from amino terminus starting from the first methionine residue-(bold face). Asterisk indicates the stop codon of the ORF.

Figure 9. Multiple sequence alignment isolated clone. A, The conceptual translation product of the cDNA identified from TBC was used to search the swissprot database. Six polypeptides [60S ribosomal protein II from cladosporium (P42038) S. pombe (P08094); major allergen of alternaria (P42037); rat (P02401); human (P05387) and maize (024415) ribosomal proteins], which appeared as significant hits (scoring over 60) were multiply aligned with the cloned cDNA using ClustalW. B, Polypeptide sequences of different Acyl Carrier Proteins (ACP) [E, coli, S. cerevisiae, H. pylori, plasmodium, pseudomonas, brassica] were obtained from public database (Swissprot) and were used to do a multiple sequence alignment with the isolated ACP clone using ClustalW algorithm. On the consensus line underneath each

section of the alignment, in both panel A and B, asterisk indicates identical or conserved residues in all sequences in the alignment, colon indicates conserved substitutions and dots denote semi-conserved substitutions. C, Local alignment of the probe used to screen the cDNA for the ACP gene with the isolated cDNA sequence. The asterisk denotes the absolute match between both the sequence fragments.

Figure 10. Partial nucleotide and amino acid sequences of soluble diacylglycerol acyltransferase. Nucleotide and deduced amino acid sequence of diacylglycerol acyltransferase. The nucleotide sequence of isolated diacylglycerol acyltransferase clone (upper line) is presented with its deduced amino acid sequence (lower line). Nucleotides are numbered in the 5'-3' direction whereas amino acids are numbered from amino terminus.

The invention is described in details hereafter and the examples provides are for illustration purposes only without any intention to limit the scope of the invention.

MATERIALS AND METHODS

Materials - *Rhodotorula glutinis* (MTCC 1[2-^{3}H]G3P (12 Ci/mmol), [1-^{14}C]fatty acid (55 mCi/mmol) and [^{35}S]protein labeling mix were obtained from New England 151) was obtained from Institute of Microbial Technology, Chandigarh, India. [1-^{14}C]Palmitoyl-CoA (51 mCi/mmol), [1-oleoyl-9,10-^{3}H]LPA ((50 Ci/mmol), [glycerol-U-^{14}C]PA (100 mCi/mmol), Nuclear. [1-^{14}C]sodium acetate (56.4 mCi/mmol) was from Board of Radiation and Isotope Technology, Mumbai, India. Superose 12 (10/30) FPLC column and gel filtration molecular mass standards were from Pharmacia Biotech. Protein assay reagents were obtained from Pierce. Thin layer chromatography plates were from Merck. All other reagents were obtained from Sigma.

Growth conditions – R. glutinis cells were grown to the logarithmic phase in malt-yeast extract medium (pH 7.0) containing 0.3% yeast extract, 0.5% peptone, 0.3% malt extract supplemented with 1% glucose for 21 h with aeration at 30 °C. Cell density was determined by colony-forming unit of each culture (one $OD_{600\,nm} = 3 \times 10^{7}$ cells).

Saccharomyces cerevisiae cells were grown in yeast extract-peptone and dextrose medium (pH 7.0) containing 0.3 % yeast extract, 0.5 % peptone supplemented with 1 % dextrose with aeration at 30 "C.

Human hepatocellular carcinoma cells (HepG2) were obtained from American Type Culture Collection (ATCC) and maintained in DMEM, supplemented with 39 mM sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 U/ml), gentamycin (50 U/ml), nystatin (5 U/ml) and 10% (v/v) FBS. The cultures were grown at 37 °C in a humidified incubator containing 5% CO_2. HepG2 cells were stored frozen (in liquid nitrogen) to be able to revive them when required. The cells in logarithmic phase of growth were centrifuged at 300 x g and the cell pellet was resuspended in ice cold freezing mixture (50% DMEM, 40% FBS and 10% DMSO) by dropwise addition and transferred to –70 °C and subsequently to liquid nitrogen. For reviving the cells, the vials were removed from liquid nitrogen and warmed rapidly to 37 "C. Freezing mixture was removed by centrifugation at 300 x g and the cells transferred to culture flasks containing 5 ml DMEM.

Animals: Brown adipose tissue, White adipose tissue and liver tissue – Six male Wistar rats, reared at 25-28 "C, weighing about 200 g were obtained from Central Animal Facility, Indian Institute of Science, Bangalore. On the day of arrival (day 0), the rats were divided into two groups. One group (3 rats) was placed in separate cages and transferred to a cold room (4-6 "C; 12 h light/12 h dark) for 5 weeks, ad libitum. The rats in the other group were kept under normal animal house conditions at an ambient temperature of 25-28 "C. After 5 weeks of cold treatment, the rats were decapitated, the intrascapular and cervical brown fat, liver and white fat surrounding the digestive tract was quickly dissected out and placed in preweighed vials, wet weight determined and frozen in liquid nitrogen. The tissues were stored at –80 "C. At appropriate time, 1 g of the tissues were thawed, suspended in lysis buffer containing 1 mM EDTA and homogenized with four to five strokes in a Potter-Elvehjem glass-Teflon homogenizer followed by sonication (5 min at 50% duty cycle, power setting of 8, Vibra cell, Sonics materials). The homogenates were precleared by centrifugation at 300 x g for 15 min. The supernatant was subjected to centrifugation at 10,000 x g for 15 min. The supernatant (10,000 x g) thus obtained was centrifuged at 240,000 x g for 60 min to obtain the soluble fraction (cytosol). The pellet was washed with the lysis buffer and centrifuged again at 240,000 x g for 60 min to obtain membranes. All the operations were carried out at 4 "C.

Incorporation of [1-^{14}C]acetate into TAG – R. glutinis cells (8 x 10^7 cells/ml of 10 mM Tris-HCl, pH 8.0) were labeled with 2.5 μCi of [1-^{14}C]acetate for 2 h. Cells were harvested by centrifugation and the cell pellet washed with ice-cold lysis buffer. To the pellet, 0.5 ml of 10% acetic acid in isopropanol was added and boiled for 5 min. To the mixture, 1 ml of hexane was added and vortexed thoroughly. The hexane layer was removed, concentrated and the lipids were separated on silica gel G thin-layer plates developed with neutral lipid solvent system - petroleum ether: diethyl ether: acetic acid (70:30:1, v/v). Lipids were identified by their migration with standards (TAG, FFA, DAG, PC) and then scraped from the plate and counted in liquid scintillation counter.

Preparation of subcellular fractions - Logarithmic phase cells (R. glutinis – 21 h and S. cerevisiae – 15 h grown) (10 g wet weight) were suspended in minimum volume of 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin and 5% sucrose (–1 g wet weight cells/ml buffer). The cells were lysed using glass beads (0.45-0.6 pm) in the absence of detergent.*Differential centrifugation was used to fractionate intracellular components. The supernatant (10,000 x g) thus obtained was centrifuged at 240,000 x g for 60 min to obtain the soluble fraction (cytosol). The pellet was washed and resuspended in lysis buffer and centrifuged again at 240,000 X g for 60 min to obtain the pellet (membranes). All the operations were carried out at 4 "C.

Enzyme assays - The assay mixtures consisted of all the components of lysis buffer except protease inhibitiors; with labeled acyl donor, 20 μM [1-^{14}C]palmitoyl-CoA (100,000 dpm), 5 to 25 μg 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) and phospholipid solvent system - chloroform:methanol:acetic acid:water (170:25:25:4, v/v) as the solvent systems for separating neutral lipids and phospholipids, respectively. The lipids were visualized with iodine vapor and spots corresponding to LPA, PA, DAG and TAG scraped off for measurement of radioactivity in liquid scintillation counter (Wallac 1409 liquid scintillation counter) using toluene based scintillation cocktail containing 0.5 % POP and 0.05 % POPOP. In addition, acyltransferases

were also assayed using labeled acyl acceptors [2-^{3}H]G3P (50 μM, 100,000 dpm) for G3P acyltransferase or [1-oleoyl-9,10-^{3}H]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-^{14}C(U)]PA (dipalmitoyl) (1.1 x 10^{5} dpm).

ACP and acyl-ACP synthetase assays were carried out as described (22). The reaction mixture consisted of 0.1 M Tris-HCl (pH 8.0), 0.4 M LiCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 0.2% Triton X-100, [1-^{14}C]palmitate (0.5 μCi) and 25-50 μg enzyme source in a final volume of 100 μl. The reaction mixture was incubated at 30 "C for 30 min. The amount of labeled acyl-ACP formed was determined by spotting the reaction mixture on Whatman no. 3MM and washed the filter paper thrice (10 min each wash) with chloroform: methanol: acetic acid (3:6:1, v/v) followed by liquid scintillation counting using toluene based scintillationcocktail containing 0.5 % PPO and 0.05 % POPOP.

The in gel superoxide dismutase assay was performed as described below. The purified TBC from R. glutinis or E. coli lysates, transformed as well as untransformed, were run on a 7 % native PAGE at 4 "C. The gel was soaked in a solution containing 0.025 % nitro blue tetrazolium (NBT) and 0.01 % riboflavin for 20 rnin in dark. The gel was then soaked in a solution containing 0.01 % (w/v) TEMED for 5 min and exposed to intense light. The band position of SOD was white in the dark blue background of the gel.

Sucrose density gradient – The soluble fraction of (75-100 mg/0.5 ml) or the purified complex (–50 pg) of R. glutinis was layered onto a 10 to 30% linear sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 0.1 M NaCl.. The tubes were centrifuged for 18 h at 200,000 x g (Beckman SW 41 rotor). After the' centrifugation, fractions (1 ml) were collected and assayed for TAG biosynthetic enzyme activities as described previously.

Purification of TAG biosynthetic enzyme complex.- All operations were conducted at 4°C except FPLC purification step, which was conducted at ambient temperature. The soluble fraction from the exponentially growing R. glutinis cells was used for the purification. Cytosol was loaded onto 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 mM Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl, 5 mM $MgCl_2$ and 5 % sucrose, and incubating overnight at 4 "C. The gel eluted protein was used for further studies.

Size exclusion chromatography – The soluble fraction from R. glutinis was concentrated (2 mg protein/ml) and applied onto a preparative Superose 12 FPLC column fitted with Bio-Rad BioLogic low-pressure chromatography system. The column was pre-equilibrated with 10 mM Tris-HCl, pH 7.5 containing 0.1 M NaCl and the elution was with the same buffer at a flow rate of 0.3 ml/min. Fractions (1 ml) were collected and assayed for TAG biosynthetic enzyme activities (LPA acyltransferase, PA phosphatase and DAG acyltransferase). The column was calibrated with standard M_r markers – apofemtin (440,000 Da), amylase (200,000 Da), yeast alcohol dehydrogenase (150,000 Da), bovine serum albumin (66,000 Da) and carbonic anhydrase (29,000 Da).

Antisera production - Rabbits were immunized by subcutaneous injection of 250 μg of R. glutinis purified ACP emulsified in Freund's complete adjuvant (FCA). Two booster doses of 125 μg protein emulsified in Freund's incomplete adjuvant (FIA) were administrated at 3 weekly intervals. Ten days after the last injection, blood was collected, serum separated and stored at –20 °C.

The major peptide, CY-ALELQADDFNK peptide SEQ ID 2 corresponding to LPA acyltransferase and phosphatidic acid phosphatase peptides (major peptide CY-NALTGLHMGGGK peptide SEQ ID 4, and minor peptide C-YVEGARP peptide SEQ ID 6) and peptide SEQ ID 7 of diacylglycerol acyltransferase from R. glutinis were conjugated to bovine serum albumin using m-maleimidobenzoyl-N-hydroxysuccinimide ester (18). The conjugated peptides (300 pg) were emulsified and injected into rabbits. The antibody production, specificity and titer were analyzed by ELISA (19). Proteins were separated by gel electrophoresis and transferred onto a nitrocellulose membrane for immunoblotting as described (20).

Cross-linking of proteins – Cross-linking was carried out as described (reference). Preparations of the R. glutinis purified complex (5 pg) 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 M

Tris-HCl, pH 7.5. The cross-linked products were resolved on 6 % SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was probed with anti-ACP and anti-LPA acyltransferase antibodies for the detection of cross-linked products.

Protein labeling and immunoprecipitation - Logarithmic phase R. glutinis or S. cerevisiae cells (10^7cells/ml) were labeled with 30 μCi/ml of [^{35}S]protein labeling mix for 3 h at 32 °C. The labeled cells were lysed and centrifuged at 10,000 x g for 15 min to obtain the lysate. Primary antibodies were incubated with lysate for 1 h and the immunocomplex was precipitated with Protein A beads. The bound proteins were analyzed by gel electrophoresis followed by autoradiography.

Nile blue A staining - A smear of cells was prepared on a glass slide and heat fixed. The slides were immersed in 1% aqueous solution of Nile blue A stain for 10 min at 30 "C. The slides were washed with water and air-dried followed by visualizing under fluorescence microscope.

Indirect immunofluorescence - Logarithmic phase (21 h) *R. glutinis* cells were fixed with 4% para-formaldehyde for 20 min followed by 4 % formaldehyde for 60 min. The fixed cells were washed thrice with 0.1 M phosphate buffer (pH 5.9) and resuspended in 50 mg lytic enzyme with 1.2 M 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 coated with R. glutinis spheroplasts were treated with ice-cold methanol and acetone. The primary antibodies were added with a dilution of 1:10 except for anti-ACP antibody, which was used at a dilution of 1:200. Secondary antibody FITC/TRITC conjugates were used for detection. The slides were viewed in a confocal laser-scanning microscope (Leica TCS SP, Heidelberg, Germany) to locate TBC.

Example I

Growth, TAG synthesis in oleaginous yeast – The growth of oleaginous yeast cells were monitored by both $OD_{600\ nm}$ and colony-forming units at 30 °C, both $OD_{600\ nm}$ and viable cell count increased proportionally with time (Fig. 1A). Yeast cells grown at various time intervals were stained with fluorescent dye (Nile blue A) followed by phase contrast

fluorescence microscopy of cells revealed that 24 h grown cells accumulate TAG to the lesser extent. However, stationary phase cells showed an intense Nile blue A staining (Fig. 1B) suggesting the large accumulation of TAG at the stationary phase. Cells were isolated from different growth time intervals and cell number was adjusted to give 2.0 $OD_{600\ nm}$. Lipids were extracted and separated by silica-TLC using neutral lipid solvent system and the results indicated that TAG accumulation was found even at the early logarithmic phase (Fig. 1C). To metabolically label TAG formation, [^{14}C]acetate incorporation was carried out at various growth time intervals using 8 x 10^7 cells/time point revealed that the active lipid synthesis occur at 21 h (Fig. 1D). 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. Table 1 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 to 69% total activity) of LPA and DAG acyltransferases, and PA phosphatase activities as compared to the corresponding enzymes in the particulate fraction (Table I). On the contrary, a negligible amount of glycerol-3-phosphate acyltransferase activity could be detected in the soluble fraction. The pattern of distribution of activities remained the same under different lysis procedures such as French press and sonication. 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).

Example II

Identification of soluble TAG biosynthetic activity - The TAG synthase activity in the cytosol could be due to the presence of non-sedimentable cellular membrane fragments and lipid particles generated during isolation procedures. To demonstrate that the soluble fraction had TAG biosynthetic activity, which was loaded onto a gel exclusion column (Superose 12). TAG synthase activity (12-15%) was also found in the wide-volume fraction and this activity could be due the to the lipid particles. Most of the TAG synthase activity was eluted between 158 to 200 kDa gel filtration molecular weight standards (Fig. 2A). This experiment demonstrated that the TAG biosynthetic activity is soluble.

Example III

Purification of TAG synthase -TAG biosynthetic activity was found high in the soluble fraction and this fraction was loaded onto a 7% native-polyacrylamide gel electrophoresis (PAGE) and the run was carried out at 4 °C. To determine which region of the gel corresponds to TAG synthase, the eluted proteins were assayed for activity. LPA acyltransferase, PA phosphatase and DAG acyltransferase were detected in the same region of the gel (Fig. 3A). To identify the number of bands present in the active gel eluted fraction, the same was reloaded onto a native gel and a single band was visualized upon silver staining (Fig. 3B). Overall summary of the purification procedure is shown in Table II. Native-PAGE step was effective and resulted in 469, 426-, and 409-fold purification for LPA acyltransferase, PA phosphatase and **DAG** acyltransferase, respectively, with the recovery of 39 to 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 eluted as a single peak with the native molecular size of 180 kDa. The active fraction from gel filtration column contained LPA acyltransferase, PA phosphatase and DAG acyltransferase activities. These results suggested the possibility of an enzyme complex for TAG biosynthesis in ***R. glutinis.***

Example IV

Identification of a 10S multienzyme complex - To examine if the enzymes were present as a multifunctional protein or multienzyme complex. Gel eluted active fraction containing TAG synthase were 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 focussing (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). The sedimentation value of the purified complex **was** estimated by loading the native polyacrylamide gel eluted active fraction onto a 10-30% linear sucrose gradient and the various fractions were analyzed for TAG synthase activity. The analysis revealed that LPA acyltransferase, PA phosphatase and DAG acyltransferase activities were associated with one fraction. The sedimentation value of the active fraction was calculated to be 10S (Fig. **2B).** These data, in conjunction with the native-PAGE of the purified complex suggest the presence of a 10S-multienzyme complex for TAG biosynthesis in the cytosol of R. glutinis.

Example V

10S complex and its polypeptide composition - To identify the nature of polypeptides in the complex, the purified 10S multienzyme complex was loaded onto a 12% SDS-PAGE 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, protein eluted from the gel and assayed for TAG synthase activity. As shown in Figure 4A and D, LPA acyltransferase and PA phosphatase activities were predominantly found at the 4^{th} and 5^{th} cm, respectively and the yield was 7-10 %. The gel-eluted LPA acyltransferase and PA phosphatase migrated separately with the molecular masses of **32** kDa and 48 kDa, respectively. DAG acyltransferase activity could not be localized in the gel. These results indicated that two of the polypeptides in the 10S TBC were LPA acyltransferase and PA phosphatase. The electrophoresed proteins were blotted onto PVDF membrane and the polypeptides corresponding to molecular sizes 32 and 48 kDa were excised, digested with trypsin and the tryptic peptide sequences determined. The internal sequences (Major peptide XALELQADDFNK, peptide SEQ ID 1 and Minor peptide XXVNNVXPGXIEQ, peptide SEQ ID **3)** of LPA acyltransferase **(32** kDa) did not match with any known sequences in the database. Tryptic peptide sequences (Major peptide NALTGLHMGGGK, peptide SEQ ID 4 and Minor peptide YVEGARPXK, peptide SEQ ID 5) of PA phosphatase (48 kDa) showed 40-100% identity with homosapien **PA** phosphatase 2a and 2b isoforms and with Musculus domesticus kidney PA phosphatase.

To confirm that 10S 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 of molecular size of **28** kDa. 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).* 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** kDa and **45** kDa protein in the microsomal membranes.

To determine whether the five different proteins identified in the TBC were held together by physical interactions, immunoprecipitations were carried out with antibodies raised to three peptides. All three peptide specific antisera, one to LPA acyltransferase and two to PA phosphatase specifically immunoprecipitated the TBC while normal rabbit serum or protein Sepharose A could not immunoprecipitate the complex. When the immunoprecipitate was resolved by SDS-PAGE followed by autoradiography, which exhibited five distinct bands corresponding to TBC (Fig. 4C and 5D). Importantly, the autoradiograms of both native and 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 111).

DAG acyltransferase (56 kDa) polypeptide was microsequenced and the internal sequence (XLWAVVGAQPFGGARGS, peptide SEQ ID 7) showed 40-80% identity to the known DAG acyltransferase sequences available in the database. DAG acyltransferase was found to be the most labile enzyme of the 10S multienzyme TBC. The presence of DAG acyltransferase was confirmed by assaying the activity in the immunoprecipitate (Table 111).

To study the formation of TAG, the purified complex was incubated with either [^{14}C]palmitic acid in the presence of ATP, $MgCl_2$ and LPA or [^{14}C]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 linoleic>oleic>stearic>palmitic >myristic acids. During fatty acid synthesis, activation of fatty acids was shown to be via the formation of acyl-ACP in the cytosol (21). Fatty acyl-CoAs 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 (22) and polyclonal antibodies were raised to the purified ACP. To ensure that the 10S 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 in Western blots and the complex under native conditions. The membrane fraction was devoid of ACP. The gene encoding for 21-kDa polypeptide was isolated and found that this polypeptide was superoxide dismutase.

Example VI

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. 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 confirmed the cytosolic nature of the TBC. We have proposed a model for the TAG biosynthesis in R. glutinis.

Example VII

Cytosolic **TAG** enzymes in other yeasts - In oleaginous yeast, the TAG biosynthetic enzymes were predominantly localized in the cytosol. In order to determine if the TAG biosynthetic enzymes are present in the soluble fraction in non-oleaginous yeast, subcellular distribution was performed on S. cerevisiae lysate. About 20 % of the total TAG biosynthetic enzyme activities were found to be present in the soluble fraction (Table IV). LPAAT, PA phosphatase and DAGAT activities were detected in the cytosol. **As** compared to oleaginous yeast, the TAG biosynthetic enzymes in the non-oleaginous yeast are primarily localized in the membrane fraction however a small fraction of the enzyme activities is associated with the soluble fraction.

In an attempt to analyze the soluble TAG biosynthetic enzymes from S. cerevisiae, the cytosol and membrane fractions were resolved on SDS-polyacrylamide gels and the proteins transferred onto nitrocellulose membranes. The immunoblots were probed with the LPA acyltransferase, PA phosphatase and acyl-ACP synthetase antibodies (that were generated to R. glutinis proteins). Lysophosphatidic acid acyltransferase antibodies specifically recognized a **38** kDa polypeptide whereas acyl-ACP synthetase and PA phosphatase antibodies recognized a 30 and a **48** kDa polypeptide, respectively. The cytosolic isoforms were different from the membrane-bound TAG biosynthetic enzymks that have been reported previously. This also ruled out the possibility of any membrane contamination in the soluble fraction during cell lysis and differential centrifugation. To establish the nature of interactions between the **TAG** biosynthetic enzymes from S. cerevisiae, logarithmic phase grown cells

were metabolically labeled with [^{35}S]methionine followed by obtaining the immunecomplexes with antibodies to LPA acyltransferase, PA phosphatase and acyl-ACP synthetase. All the three antibodies pulled down only their respective proteins. Protein A Sepharose or normal rabbit serum could not pull down any of these proteins. Resolution of the immunecomplexes by SDS-PAGE followed by fluorography revealed the presence of a 33, 48 and 30 kDa polypeptides being immunoprecipitated by the LPAAT, PA phosphatase and acyl-ACP synthetase antibodies. This exhibited that the soluble TAG biosynthetic enzymes of S. cerevisiae are not organized in the form of a complex. This was confirmed by assaying for the various enzyme TAG biosynthetic enzyme activities in the immunecomplexes. Hence, in non-oleaginous yeast, the soluble TAG biosynthetic enzymes exist as free enzymes.

Example VIII

Cytosolic TAG biosynthetic enzymes in mammalian systems

TAG biosynthetic enzyme in HepG2 cells – TAG formation in the sub-confluent HepG2 cells showed that the particulate fraction contributed maximally to the biosynthesis of TAG. About 16-26% of the TAG synthesizing capacity was associated with the soluble (240,000 x g supernatant) fraction. Table IV summarizes the TAG biosynthetic enzyme activity profile from the various fractions of HepG2 cells. Glycerol-3-phosphate acyltransferase activity could not be detected in either the soluble or the membrane fraction. The total activity of lysophosphatidic acid acyltransferase and phosphatidic acid phosphatase in the cytosolic fraction of HepG2 was slightly greater than diacylglycerol acyltransferase. The membrane fraction had considerable amount (74-84%) of TAG biosynthetic enzyme activity.

TAG biosynthetic enzyme activities in rat adipose tissue and liver – Adipose tissues are the classical oleaginous tissues. The profile of the TAG biosynthetic enzymes in the various fractions of the rat brown and white adipose tissues and the cold exposed rat liver were analyzed. Upon subcellular fractionation, cytosol, membrane and lipid body fractions were obtained. The cytosolic fraction of brown and white adipose tissues had ~20-35% of TAG biosynthetic enzyme activities as compared to the membrane fraction. As in HepG2 cells, total activities of LPA acyltransferase and PA phosphatase were higher in the soluble fraction as compared to DAG acyltransferase. A small amount of glycerol-3-phosphate acyltransferase activity could be detected in the membrane fraction of HepG2 cells. The cold

treated rat liver TAG biosynthetic enzymes exhibited a modest increase in their activities over the untreated rat liver (25-26 °C maintained rats).

Example IX

Generation and screening of TAG-deficient R. glutinis – Wild type cells were subjected to ethyl methane sulfonate mutagenesis to generate TAG biosynthesis mutants. Subjecting the mutagenized cells through a simple screen enhanced the TAG-deficient cell population. Due to accumulation of lipids, the wild-type (WT) cells float on 50% sucrose cushion whereas the mutagenized cells due to defective TAG biosynthesis have 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, the total lipid was extracted from all these mutants and the lipid profile was analyzed 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 neutral lipid formation. We chose two mutants TAG1 (high FFA, low DAG and TAG) and TAG2 (high FFA, DAG and low TAG) for further studies.

Example X

Characterization of TAG biosynthetic mutants - TAGl and TAG2 were characterized to determine the nature of defect in TAG biosynthesis. The growth rate of the WT oleaginous yeast cells and mutants at 30 °C was monitored by measuring the $OD_{600\ nm}$. The TAGl and TAG2 mutants had almost similar growth pattern. The number of cells in the mutants were significantly low as compared to WT. Accumulation of TAG in 21 h grown cells indicated that the TAG amount was negligible in TAGl and TAG2 as compared to the WT cells when observed by Nile blue A staining.

Example XI

Labeling studies on TAG mutants – Logarithmic phase (21 h) yeast cells (8×10^7 cells/ml) were labeled with 2.5 μCi [^{14}C]acetate or 2.5 μCi [^{32}P]orthophosphate 2 μCi [^{14}C]glycerol-3-phosphate or 2 μCi [^{14}C]oleic acid for 3 h at 30 °C. Before labeling, [^{14}C]oleic acid was converted to its potassium salt by boiling the dried fatty acid in 10 μl of 4.5 % KOH at 70 °C

for 10 min. and then added to the cells. Prior to addition of [^{14}C]glycerol-3-phosphate, the cells were sonicated for 3 min at 50 % duty cycle, power setting of 8, Vibra cell, Sonics materials. Cells were harvested by centrifugation and the cell pellet washed with ice-cold phosphate-buffered saline. To the pellet, 0.5 ml of 10% acetic acid in isopropanol was added and boiled for 5 min. To the mixture, 1 ml of hexane was added and vortexed thoroughly (19). The hexane layer was removed, concentrated and lipids were separated on silica gel G thin-layer plates developed with petroleum ether: diethyl ether: acetic acid (70:30:1, v/v) to separate the neutral lipids or chloroform: methanol: acetone: acetic acid: water (10:2:4:3:1, v/v) 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 measured in liquid scintillation counter.

Studies carried out on the incorporation of [^{14}C]acetate into lipids indicated that the incorporation of acetate into TAG and PC reached maximum at 3 h of labeling. At this time point, 22 % of the radiolabel was associated with TAG, 18% with PC and 48 % with FFA. Analysis of the neutral lipid profile of the cells indicated that both the mutants were defective in TAG production, as there was no visible TAG spot on the TLC autoradiograph. The pattern of incorporation of [^{14}C]acetate into lipids of WT, TAG1 and TAG2 was compared by silica-TLC. TAG1 accumulated only 6 % and TAG2, 10 % TAG as compared to the WT cells. In addition to negligible TAG, TAG1 also had significantly low DAG indicating a defective DAG synthesis. When the [^{14}C]acetate labeled lipids were resolved on TLC using phospholipid solvent system, TAG1 showed reduced PA as compared to the WT whereas the phospholipid profile of TAG2 was similar to that of the WT.

In an attempt to understand the biosynthesis of phospholipids, the mutants and the WT cells were labeled with [^{32}P]orthophosphate and the total lipids extracted and resolved on TLC using a phospholipid solvent system. TAG2 showed a higher amount of PC than the WT R. glutinis. On the other hand, TAG1 phospholipid profile indicated 60% decrease in the PA content of the cell, which corroborated with the [^{14}C]acetate labeling studies.

Incorporation of [^{14}C]oleate into TAG biosynthetic intermediates was determined for the WT and mutant yeast cells. The total lipids were resolved on TLC using neutral and phospholipid solvent systems. The neutral lipid profile of both the mutants indicated a significant decrease in the TAG content as compared to WT. TAG1 showed a 90 % decrease whereas TAG2 an

86 % decrease in the TAG content as compared to the WT. Incorporation of oleate into PL indicated that there was no obvious change in the PL pattern of TAG2 as compared to WT whereas TAG1 had compromised PA formation. Metabolic labeling of the WT and mutant yeast cells with [^{14}C]glycerol-3-phosphate yielded similar labeling pattern as obtained with [^{14}C]acetate.

Example XII

Determination of the mutation in TAG1 and TAG2 – In order 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. TAG1 had a defective **LPA** acyltransferase and thus, there was a reduction in PA, DAG and TAG formation. The DAG acyltransferase and PA phosphatase activities were comparable to that of the WT enzyme activities. On the other hand, TAG2 had a defective DAG acyltransferase and thus, there was no TAG formation. These results indicated that the TAG1 had a defective LPA acyltransferase and TAG2 a defective DAG acyltransferase.

Example XIII

Activities of the isoforms of TAG biosynthetic enzymes – To get an insight into the isoforms of enzymes of the TAG biosynthetic pathway, the cytosolic and the membrane-bound TAG biosynthetic enzymes were isolated from the mutants and WT and their activities measured. It was surprising to observe that the membrane-bound TAG biosynthetic enzyme activities were unaltered. WT and TAG mutants had comparable LPA and DAG acyltransferases, and PA phosphatase activities. The purified cytosolic LPA acyltransferase activity in TAG1 and DAG acyltransferase activity in TAG2 were significantly lower in the mutants than the corresponding WT enzyme activities. The loss of enzyme activities was comparable to the decrease in TAG accumulation in the mutants. This experiment led us to conclude that the cytosolic isoform of LPA and DAG acyltransferases were defective in the mutants, thereby resulting in reduced TAG formation.

Example XIV

Cytosolic TAG biosynthetic enzyme profile of TAG1 and TAG2 - The TBC from the TAG biosynthetic mutants was isolated and analyzed by SDS-PAGE. The gel profile of TAG1 was

identical to that of the WT indicating that defective enzyme was present as a part of the TBC. However, TAG2 had a reduced DAG acyltransferase as compared to WT cells. To establish the apparent difference observed in the LPA and DAG acyltransferase on SDS-polyacrylamide gels, immunoblotting was performed on the purified TBC from the WT, TAGl and TAG2 under native and denaturing conditions. There was no evident change in the LPA acyltransferase from the TBC and cytosol of TAGl and TAG2 when compared to the WT. PA phosphatase enzyme profile monitored by the PA phosphatase major and minor peptide antibodies indicated that there was no change in the amount of this enzyme in the cytosol and the TBC of TAGl and TAG2 when compared to the WT enzyme. DAG acyltransferase was comparable in WT and TAGl cells whereas, there was a considerable decrease **(39-48** %) in the amount of cytosolic DAG acyltransferase in the TAG2 mutant.

Example XV

Identification of acyl-ACP synthetase - To understand the mechanism by which fatty acids were utilized by the TBC to generate TAG, we examined the formation of acyl-ACP **and** acyl-CoA. The formation of acyl-ACP was monitored by performing the acyl-ACP synthetase assay with [1-^{14}C]linoleic acid or [1-^{14}C]palmitic acid and resolving the reaction mixture on SDS-polyacrylamide gel followed by fluorography. It was evident that acyl-ACP was formed. Incubation of the purified TBC with [1-^{14}C]linoleic acid or [1-^{14}C]oleic acid or [1-^{14}C]stearic acid or [1-^{14}C]palmitic acid in the presence of 5 mM ATP further confirmed the formation of acyl-ACP by the TBC. Among the fatty acids tested, linoleic acid exhibited higher activity as compared to other fatty acids. These results demonstrated that the complex was capable of acylating ACP and this could be due to the presence of acyl-ACP synthetase. However, the formation of acyl-CoA was not detected under the standard assay conditions. In order to identify acyl-ACP synthetase in the complex, photoaffinity labeling with [^{32}P]azido-ATP was carried out and it was observed that the azido-ATP was covalently bound to a 35 kDa polypeptide under denaturing and reducing conditions (Fig. 1C). These results indicate the presence of acyl-ACP synthetase in the triacylglycerol biosynthetic complex of R. glutinis.

Example XVI

Purification of acyl-ACP synthetase - Acyl-ACP synthetase activity was found only in the soluble fraction of R. glutinis and this fraction was electrophoresed on a 7% native-polyacrylamide gel. Acyl-ACP synthetase, LPA acyltransferase, PA phosphatase, DAG acyltransferase and ACP were detected in the same region of the gel. The native polyacrylamide gel electrophoresis step was very effective and resulted in a 358-fold purification of acyl-ACP synthetase, with a recovery of 42%. The specific activity was determined to be 494 pmol min^{-1} mg^{-1} for palmitic acid while the total activity was 5.43 pmol min^{-1}. The purity of the complex was determined by 2-dimensional electrophoresis. Acyl-ACP synthetase was further purified to homogeneity from the TBC by subjecting the native polyacrylamide gel eluted active fraction to SDS-PAGE. The 35-kDa band, which corresponded to acyl-ACP synthetase, was excised out of the SDS-polyacrylamide gel, protein eluted and reloaded onto a 12% SDS-polyacrylamide gel. A single band of M_r 35-kDa was observed upon silver staining. Although the enzyme preparation was pure, the specific activity dropped to 60 pmol min^{-1} mg^{-1} and the yield was 1.1 %. The pI of acyl-ACP synthetase was estimated to be ~8.7 by isoelectric focussing and 2D gel electrophoresis. The purified protein was subjected to N-terminal sequencing and the sequence VHLAVALYGLAAVRVSRIVR, (**peptide SEQ ID**) showed 46% similarity towards the 276-295 amino acid region of the putative acyl-ACP synthetase from Campylobacter jejuni. The N-terminal sequence of R. glutinis acyl-ACP synthetase did not show any similarity to the E. coli 2-ac yl-glycerophosphatidylethanolamine acyltransferase/acyl-ACP synthetase.

Example XVII

Properties of acyl-ACP synthetase - Maximum enzyme activity was observed in the presence of both fatty acids and LPA in the reaction mixture. Labeling of acyl-ACP synthetase with [γ-^{32}P]ATP was maximum when both the substrates were supplied in the reaction mixture. Enzyme-AMP complex was not detected in the absence of fatty acid. This suggested that the acyl-ACP synthetase from R. glutinis required both FFA and LPA in the presence of ATP to be maximally active. The acylation of ACP followed by synthesis of **TAG** by the TBC in the presence of exogenous **ACP** from either E. coli or R. glutinis and acyl-ACP synthetase were determined. The results indicated that exogenous supply of either ACP or acyl-ACP synthetase to the TBC had no significant effect in the TAG formation.

To establish that the 10S TBC contained acyl-ACP synthetase, the purified complex, the cytosol and the membranes were probed with polyclonal antibodies raised against the purified acyl-ACP synthetase by immunoblotting. The antibody recognized a single band of 35-kDa protein in the SDS polyacrylamide gel immunoblot from the complex as well as the cytosol. The same antibody was used for probing the 10 mM **CHAPS** solubilized microsomal membranes and found that there was no immunoreactive protein in this fraction suggesting the absence of this enzyme in the membranes. The antibodies reacted with E. coli acyl-ACP synthetase but did not show any reactivity towards acyl-CoA synthetase. When the R. glutinis acyl-ACP synthetase was probed with anti-ACP antibodies and found that the antibodies did not recognize the 35-kDa protein.

To validate whether acyl-ACP synthetase was a part of the TBC, logarithmic phase grown R. glutinis cells were metabolically labeled with [^{35}S]methionine followed by immunoprecipitation with acyl-ACP synthetase antibodies. The antibodies specifically immunoprecipitated the TBC while neither normal rabbit serum nor protein Sepharose A did not immunoprecipitate the complex. Resolution of the immunorecipitate by SDS-PAGE followed by fluorography, exhibited five distinct polypeptides corresponding to TBC that contained 35 kDa acyl-ACP synthetase. This result demonstrated that acyl-ACP synthetase is indeed a part of the triacylglycerol biosynthetic complex,

The acyl-ACP synthetase antibodies were capable of inhibiting only 40% of the enzyme activity. The inhibition of the acyl-ACP synthetase activity with the antibodies subsequently reduced the TAG formation by the TBC. There was almost complete inhibition of acyl-ACP and TAG formation when the complex was pre-incubated with anti-ACP antibodies. This corroborated that one of the components of the 10S complex is indeed acyl-ACP synthetase.

The TBC was subjected to cross-linking using a homobifunctional cross-linker, disuccimidyl suberate, in order to rule out the possibility of any lipid-protein interactions. As we were unsuccessful in detecting the complex with anti-acyl-ACP synthetase antibodies, we probed the cross-linked product with ACP antibodies. It was observed that the cross-linked product migrated at ~200 kDa indicating that the complex was held together by protein-protein interactions.

Example XVIII

Molecular cloning and sequence analysis of the cDNA's encoding **SOD** – One liter yeast cell culture was grown for 21 h and total RNA was isolated by following GITC method. mRNA was purified from total RNA using oligo (dT) cellulose column. The cDNA library was constructed from the purified mRNA using the cDNA library construction kit by following the manufacturer's protocol. The vector used for constructing the library was pBK-CMV ***ZAP*** expression vector and the cDNA was directionally cloned in EcoRI and XhoI sites. The cloned vector was packaged in Gigapack packaging extract provided by the manufacturer and propagated in host XL-1 blue MRF' E. coli cells. The titer of the amplified library was 10^{11} pfu/ml. N-terminal sequence of the 21 kDa protein (superoxide dismutase) was obtained by micro-sequencing of the protein transferred onto PVDF membrane following Edman degradation. Protein sequencing was performed using an Applied Biosystems gas phase sequenator at the protein sequencing facility of Rockefeller University. The nucleotide probes generated from protein sequence (AYNKIPAVALPKLPFATNAL, peptide SEQ ID 12) were radiolabeled at the 3' end using TdT and [α-^{32}P]dATP. Filters carrying 1 x 10^5 clones from R. glutinis cDNA library were hybridized with the radiolabeled probe. The positive λZAPII phage plaques, identified by autoradiography were purified through another two rounds of screening using the same probe. After the tertiary screening, three positive clones were obtained and in vivo excision was performed using a helper phage (ExAssist^{TM}). The clones obtained were sequenced by the dideoxy chain termination method using a Dye terminator cycle sequencing kit (PerkinElmer Life Sciences) with a DNA sequencer (Applied Biosystems Model 377). All the cDNAs were sequenced in both directions using T7 and T3.

Cloning of SOD – To obtain the fill-length cDNA clone that encodes R. glutinis 21 kDa (ACP antibody reactive protein), R. glutinis cDNA library was screened with the oligonucleotide degenerate primer, 5'-TACAACAAGATCCCIGTICTCCCTAAGCTCCCITTCGCITACAAC-3' (SEQ ID 13), designed based on the N-terminal amino acid sequence of the protein, AYNKIPAVALPKLPFATNAL (peptide **SEQ** ID 12). The nucleotide probe was radiolabeled at the 3' end using TdT and [α-^{32}P]dATP. A large number of positive clones were isolated in the screen, and the plasmids from three clones that contained the longest inserts (–0.7 kb) were subjected to nucleotide sequencing. The sequences obtained were identical except a few

base pairs changes from the 3'-untranslated regions. The complete primary structure of a cDNA (732 base pairs) is shown in Figure *6.* Analysis of the nucleotide sequence revealed an open reading frame of 191amino acids, with translation initiating at the ATG codon at nucleotides 25-27 and consensus Kozak translation initiation sequence (GCCATGG). The 3'-untranslated region contained a noncanonical polyadenylation signal (AATAC) near the poly(A) tail (Fig. *6)* indicating that the isolated clone represents full length cDNA. The calculated molecular mass of the R. glutinis protein is 20.9 kDa, close to that of estimated by SDS-polyacrylamide electrophoresis. When the sequence of N-terminal amino acid was aligned with the deduced amino acid sequence, there was a complete match, confirming that the cloned cDNA codes for 21 kDa protein (Fig. 6). Hydropathy plot of the predicted protein suggested there was no transmembrane domain, which is in agreement with the observation that TBC is located at the cytosol. When the deduced amino acid sequence of 21 kDa was examined for a number of structural motifs, we identified a single potential Asn-linked glycosylation site at ^{80}NHTL^{83} and a casein kinase II phosphorylation site at ^{187}SLSE^{190}. In addition, there were six protein kinase C dependent phosphorylation sites and seven N-myristoylation sites. BLAST search for the deduced amino acid sequence in all the available databases for the isolated cDNA clone revealed that the protein is homologous to mitochondria1 manganese-SOD. The deduced amino acid sequence shared more than 30% identity with Ganoderma microsporum (38%), human (33%) maize (31%), S. cerevisiae (29%) and E. coli (24%). In addition, we used the ClustalW program to obtain an optimized multiple sequence alignment of known manganese-SOD (Fig. **7).** All protein posses three conserved domains I, II and III, the isolated clone showed homology to the domains I and II but not to domain III. This sequence has been submitted to GenBank™ with the identification as Mn^{2+} superoxide dismutase. Phylogenetic analysis of SOD revealed that the protein is more closely related to Ganoderma microsporum.

Expression of Recombinant SOD - The SOD gene was transformed to E. coli JM109 cells independently and induced with 0.5 mM IPTG for 4 h. The crude cell lysate was sonicated and run in a 12 % SDS-PAGE. Untransformed cell lysate was used as a control. The full-length R. glutinis cDNA in pBK-CMV expression vector containing T7lac promoter, which was then used to transform E. coli cells (JM109). Transformed bacteria were induced by treatment with IPTG to produce the recombinant protein. Protein extracts were prepared from

the induced bacteria and assayed for SOD activity and found that there was no increase in SOD activity in JM109 cells transformed with the recombinant plasmid as compared to untransformed cells. SDS-polyacrylamide gel electrophoresis demonstrated that bacteria transformed with the recombinant plasmid contained a polypeptide of the expected size, whereas crude extracts of bacteria lacking R. glutinis SOD-coding sequence did not show the 25-kDa protein. Western blot analysis of the transformed JM109 cells with anti-ACP antibodies showed an intense signal at around 25 kDa and no signal was detected in the untransformed cells.

Example XIX

Cloning of Acyl Carrier Protein – For ACP, screening was performed using polyclonal antisera raised against the purified R. glutinis ACP. To screen the expression library with antibody, plaques were grown for **4** h at 42 °C after plating and overlaid with IPTG soaked nitrocellulose membrane and incubated at **37** "C for 6-8 h. Membranes were developed by following the western blot procedure with primary antibody dilution 1:1000 in 0.5 % gelatin in phosphate buffered saline containing 0.05 % Tween 20. Three rounds of screening were performed and a cross-reactive 21 kDa SOD clones were eliminated by checking the tertiary positive clones with anti-SOD antibody. Three positive clones were sequenced as described above.

Cloning of Acyl Carrier Protein – To obtain the full-length cDNA clone that encodes R. glutinis ACP, R. glutinis cDNA expression library was screened with the antibodies raised against the purified ACP. A large number of positive clones were isolated in the screen, and the plasmids from three clones that contained the longest inserts (–0.7 kb) were subjected to nucleotide sequencing. The sequences obtained were identical except a few base pairs changes from the 3'-untranslated regions. The complete primary structure of a cDNA is shown in Figure 8. BLAST search for the deduced amino acid sequence in all the available databases for the isolated cDNA clone revealed that the protein is homologous to ribosomal protkin (Fig. 9).

Expression of ACP in E. coli - To prepare a vector suitable for expression of recombinant ACP in E. coli, we first generated an 351-bp DNA fragment containing the coding sequence

for ACP by PCR of the aforementioned cDNA clone using forward primer (5'-CGAAGCTAGCATGAAGCACGTCGCCGCCTACCTC-3', SEQ ID 14) and reverse primer (5'-GCGAATTCTTAGTCGAAGAGACCGAAGCCCAT -3', SEQ ID 15). The forward primer contains a NheI site followed by the beginning of the open reading frame; the reverse primer contains the end of the open reading frame followed by a EcoRI cleavage site. PCR (1 min of denaturation at 95 °C, 1 rnin of annealing at 55 "C, and 1 min of elongation at 72 "C) was performed using pfu polymerase for 30 cycles with 25 pmol of each primer in a final volume of 50 pl. After electrophoresis on a 1% agarose gel, the amplified DNA, visible by ethidium bromide staining, **was** digested with NheI and EcoRI and cloned into NheI and EcoRI -digested pRSET plasmid vector to obtain the construct pRSET-A. The insert was sequenced to ascertain that no mutations had been introduced during amplification. The construct was used to transform E. coli JM109 (Invitrogen) for plasmid preparation and E. coli BL21(pLysS) for protein expression. The full-length R. glutinis cDNA in pBK-CMV expression vector containing T7lac promoter, which was then used to transform E. coli cells (JM109). Transformed bacteria were induced by treatment with IPTG to produce the recombinant protein. Protein extracts were prepared from the induced bacteria and assayed for ACP activity and found that there was an increase in ACP activity in JM109 cells transformed with the recombinant plasmid **as** compared to untransformed cells. SDS-polyacrylamide gel electrophoresis demonstrated that bacteria transformed with the recombinant plasmid contained a polypeptide of the expected size. Western blot analysis of the transformed JM109 cells with anti-ACP antibodies showed an intense signal at around 10 to 12 kDa and no signal **was** detected in the untransformed cells.

Expression of Recombinant R. glutinis ACP-- Transformed BL21 cells were grown at 37 "C to an A_{600} of 0.5 in LB medium (50 ml) containing 100 µg/ml ampicillin. **A** 10-ml portion was used to inoculate 1 liter fresh LB medium containing 100 µg/ml ampicillin, and the mixture was incubated overnight at 120 rpm at 37 "C. Protein production was then induced by 0.5 mM IPTG. After a 3-h induction at 37 "C, cells were harvested by centrifugation (10,000 × g for 10 min at 4 "C. The cells were harvested by centrifugation and then lysed by treating with lysozyme (0.1 mg/ml) at pH 8.0 (50 mM Tris-HCl) in the presence of 0.1% Triton X-100 for 20 min at 30 "C. The mixture, cooled in ice, was sonicated two times for 10 s each using a probe sonicator (Branson). The sonicated mixture was centrifuged at 12,000 × g for

10 min, and the supernatant was collected. This supernatant was used both to measure the ACP activity and for the affinity purification of the recombinant His-tagged enzyme.

Purification of Recombinant R. glutinis ACP - All steps were performed at 4 °C. For the affinity purification of the recombinant enzyme, the bacterial extract (5 ml) in 20 mM Tris buffer (pH 8.0), 0.5 M NaCl and 5 mM imidazole was loaded onto a 2.5-ml nickel-nitrilotriacetic acid-agarose (Qiagen) column. The column was washed first with 10 volumes of the above binding buffer and further washed with six volumes of the same buffer but containing a higher concentration of imidazole (60 mM). The enzyme was eluted with six volumes of the buffer containing 0.3 M imidazole, followed by elution with four volumes of 1.0 M imidazole.

Example XX

Cloning of diacylglycerol acyltransferase (DAG-AT) – cDNA library screening was performed using polyclonal antisera raised against peptide ID 5. To screen the expression library with antibody, plaques were grown for 4 h at 42 "C after plating and overlaid with IPTG soaked nitrocellulose membrane and incubated at 37 "C for 6-8 h. Membranes were developed by following the western blot procedure with primary antibody dilution 1:1000 in 0.5 % gelatin in phosphate buffered saline containing 0.05 % Tween 20. Three positive clones were sequenced as described above.

Expression of DAG-AT - The DAG-AT gene was transformed to E. coli JM109 cells independently and induced with 0.5 mM IPTG for 4 h. The crude cell lysate was sonicated and run in a 12 % SDS-PAGE. Untransformed cell lysate was used as a control. The full-length R. glutinis cDNA in pBK-CMV expression vector containing T7lac promoter, which was then used to transform E. coli cells (JM109). Transformed bacteria were induced by treatment with IPTG to produce the recombinant protein. Protein extracts were prepared from the induced bacteria and assayed for DAG-At activity and found that there was a slight increase in DAG-AT activity in JM109 cells transformed with the recombinant plasmid as compared to untransformed cells.

Table I.

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

Fraction	LPA acyltransferase		PA phosphatase		DAG acyltransferase	
	Specific activity pmol/min/mg	Total activity pmol/min	Specific activity pmol/min/mg	Total activity pmol/min	Specific activity pmol/min/mg	Total activity pmol/min
10,000 x g Sup	1.59	32.42 ± 4.54	1.14	27.35 ± 0.75	0.98	23.62 ± 1.65
240,000 x g Sup	1.60	27.00 ± 3.78	1.16	19.5 ± 0.58	0.83	13.95 ± 1.12
240,000 x Pellet	1.88	12.29 ± 0.31	1.49	9.75 ± 0.20	1.96	14.75 ± 0.74

The logarithmic phase oleaginous yeast cells from 1 litre culture were lysed with glass Bead and fractions were obtained by differential centrifugation. Enzyme activities were measured as described in Materials and Methods. Values are the mean ± SEM of nine separate experiments, each performed in duplicate. Sup, supernatant.

Table II

Purification of triacylglycerol biosynthetic enzymes from oleaginous yeast

LPA acyltransferase

Step	Protein	Specific activity	Total activity	Yield	Purification
	µg	pmol/min/mg	pmol/min	%	-fold
Cytosol	9500	1.57	14.92	100	1
Native-PAGE	11	736	8.10	56	469

PA phosphatase

Step	Protein	Specific activity	Total activity	Yield	Purification
	µg	pmol/min/mg	pmol/min	%	-fold
Cytosol	9500	1.29	12.26	100	1
Native-PAGE	11	550	6.25	51	426

DAG acyltransferase

Step	Protein	Specific activity	Total activity	Yield	Purification
	µg	pmol/min/mg	pmol/min	%	-fold
Cytosol	9500	0.96	9.12	100	1
Native-PAGE	11	393	3.56	39	409

Table III

TAG biosynthesizing capacity of the immunoprecipitate

Antiserum used	LPA acyltransferase	PA phosphatase	DAG acyltransferase
		pmol/min	
LPA acyltransferase	1.52 ± 0.06	0.88 ± 0.03	0.48 f 0.02
PA phosphatase (major)	1.38 f 0.06	0.86 ± 0.04	0.42 ± 0.01
PA phosphatase (minor)	1.44± 0.04	0.84 f 0.05	0.51 ± 0.06

Table IV

Distribution of TAG biosynthetic enzyme activities in the soluble and the particulate fractions of Hepg2, cold adapted liver and adipose tissues

	LPA acyltransferase		PA phosphatase		DAG acyltransferase	
	(% Total activity, pmol/min/g tissue)					
	Cytosol	Membranes	Cytosol	Membranes	Cytosol	Membranes
HepG2	25±2.1	75f6.2	17f1.5	83f6.2	15f0.9	85f5.3
Liver	23f1.3	77k5.7	31±2.6	69±4.5	21f1.1	79±6.7
BAT	38±1.8	62f4.6	33±1.4	67k5.2	32f2.3	68±5.1 ,
WAT	33f2.3	67k5.1	3552.7	65f4.9	29f1.8	71±6.4

Sub-confluent HepG2 cells (1 g wet weight), 1 g wet weight of brown adipose tissue (BAT) and white adipose tissue (WAT) cell lysates were subjected to differential centrifugation. Values are the mean ± SEM.

SEQUENCE LISTING

Lysophosphatidic Acid Acyltransferase

Peptide SEQ ID **1** XALELQADDFNK

Peptide SEQ ID 2 ALELQADDFNK

Peptide SEQ ID 3 XXVNNVXPGXIEQ

Phosphatidic Acid Phosphatase

Peptide SEQ ID **4** NALTGLHMGGGK

Peptide SEQ ID ***5*** YVEGARPXK

Peptide SEQ ID 6 YVEGARP

Diacylglycerol Acyltransferase

Peptide SEQ ID 7 XLWAWGAQPFGGARGS

Acyl-Acyl Carrier Protein Synthetase

Peptide SEQ ID 8 VHLAVALYGLAAVRVSRIVR

Superoxide dismutase

SEQ ID 9

Peptide SEQ ID **12** AYNKIPAVALPKLPFATNAL

SEQ ID 13 **TACAACAAGATCCCIGTICTCCCTAAGCTCCCITTCGCITACAAC-3'**

Acyl carrier protein

SEQ ID **10**

SEQ ID **14** **CGAAGCTAGCATGAAGCACGTCGCCGCCTACCTC**

SEQ ID **15** **GCGAATTCTTAGTCGAAGAGACCGAAGCCCAT**

Diacylglycerol acyltransferase

SEQ ID **11**

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The abbreviations used are: **ACP,** acyl carrier protein; DAG, diacylglycerol; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; TAG, triacylglycerol; **TBC,** triacylglycerol biosynthetic complex, SOD, superoxide dismutase.

SEQID 9

Superoxide Dismutase Gene Sequence

GGCACCAGGGGCGACGACTCGAGCCATGGCCGCCTATAACAAGATCCCTGCT
GTTCTCCCGAAGCTTCCCTTCGCGTACAACGCTCTGGAGCCGGCCATTTCCTC
CCAGATCATGGAGCTCCACCACTCGAAGCACCACGCCACCTACGTCGCCAAC
TTCAACAAGGCCCACGAGGACATCCAGGCTGCTTCGCAGGCCCAGGACATCA
AGAAGCAGATTGCCCTCCAGGCCACCGTCAAGTTCAACGGCGGTGGCCACAT
CAACCACACCCTCTTCTGGGAGAACCTCGCCCCCCAGTCGCAGGGCGGCGGC
CAGTTCCCCTCGTCGGGCAAGCTCCACGACCAAGTCCAGCAGGACTTCGGCG
GTCTCGACGGCTTTGAAGAAGGCCGTCAACGCCGCTGCCCTCGGTATCCAGG
GATCTGGATGGGCCTGGCTCGGGGTACAACCCGACGACCACCTTGAGG
CTGTCTCGACCGCAACCAGGGACCCGCTTCTCGGCTACGTTCCCCCCGTCGGC
ATGGACATGTGGGAGCACGCTTACTACATCGACTACAAG~GTC~GGCCTC
GTACCTCGAAGTCTCTCGGAGGCCTAAGTCTCGCCCTCCCTTCTCGAGCCCGC
TTGGAAGGAAGGAAGGAAAGGAATGCGCTTGCGCTTGAACCCATGTAGTACGCGAAA
AGTCGAAATACGAAATCCCCTCAGTCGTTGCAAAAAAAAAAAAAAAAAAAA
AAA

SEQ ID 10

Acyl Carrier Protein Gene Sequence

GGCACGAGGCTCTCTCACATCGTTTGCCGCTACATTTCAGCTT~GGGTCTA
TTCAGCTCACGATGAAGCACGTCGCCGCCTACCTCCTCCTCGTCTCTGCCGGC
AACACCTCGCCCTCGGCCGAGGACGTCAAGAAGGTCCTCGCCGCCGCCGACA
TCCAGGCCGACGAGGAGCGCCTCTCGGTCCTCATCAAGGAGCTCGAGGGC~
GGACGTCAACGAGGTCATTGCCGAGGGATCCAAGAAGCTCGCTTCCGTCCCC
TCGGGCGGCGCCGCCCCCGCCGCTGCCGCTGGCGGCGCTGCCGCTGGCGGTG
CCGCCGAGGAGAAGGCTGAGGACAAGCCCGCTGAGAAGGATGAGGAGAGCG
ACGACGACATGGGCTTCGGTCTCTTCGACTAAGCTCTCGTCTCGCCTCTCCCC
CTCTGCGACGACGCACAACTTTCCCGACCTTCCTCGACTTGCCGAAGCGTTTC

ATCTCTGTAGTTTGGGATCGATGGATTGCGCTAGGGAAGCCCTGACGGW
GGGGGGGTGGTTTGGCTTCTCAAAAAAAAAAAAAAAAAAAAA

SEQ ID 11

Diacylglycerol Acyltransferase

ACACTAGTGGATCCAAAGAATTCGGCACGAGGCTCGGCTCTCTCGCGTCTT
TACGTCCCGAAGGGTCTGTGGGAGGGCGAGGGCAAGTTCAAGGAGATCCT
TCTCTCCGAGGTTGCCAAGATCACTCTCGGCCCCGTCACCGAGTTCGAGCA
CTTCATGGGTCCCGTCATCTCGCAGGCTTCGTTCGACAAGTGCCTCAGCTA
CGTTGAGAAGGCCAAGCAGGCAGGTGGCGAGGTCCTCGCCGGCGGCMG
GGCGACGCGTCGAGCGGTTACTACGTCGAGCCGACCATCATCCTGACCAA
GGACCCTCGCTCGCCTACCATGGTCGACGAGATCTTCGGCCCGGTCCTCA
CTGTTTACATCTTCGAGGACGACCAGTACGAGGAGACGTGCAAGTTGATCG
ACCAGACGACGACGTACGCCCTCACTGGCTGCATCTTCTCGGACGACCGT
GCCGCGACTGTCAAGGCCGGTGCTCTCCTCCGCCACGCTGCGGGTAACTA
CTACATCAACGACAAGTCGACCGGTGCTGTTGTCGGTGCCCAGCCTTTCGG
TGGCGCACGCGGATCGGGCACGAACGACAAGGCGGGCTCGATGACG
TTCTTCACCCGCTGGTGCCAGCCGCGGAGTGTGAAGGAATCCTTCTGCCCGCC
CGAATCTTTCCCTTACCCGTCGAACCAGCGCGATTAAATGGAGGAGTTGGGG
AGGAGGAGGACGTCGAGGGAGCTGGGGAGGCGGAGGACGTCGAGGAGGAG
TTGGGGAGGAGGAGGACGTCGAGGGAGCTGGGGAGGCGGAGGACGTCGAGG
AGGAGTTGGGGAGGAGTTTGTCGAGGAGGAGGAGAAGGGGTTTCTCCTCGCCT
GTAGTTGTACAAAATCAGCACGCCTTTGCTTCCACCGCCA
AAAAAAAACTCGAGAGTACTTCTAGAGCGGCCGCGGGCCCATCGATTTTCCA
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TTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAAT
AGCGAAGAGGCCCGGACCGATCGCCCTTCCAACAGTTGCGCAGCCTGATGGC
GAATGGAGATCCAATTTTTAAGTGTATAAGGGGTAAACTACTGATCTAATTGT
GGGGTTTTTAAAT

Claims:

1. Novel soluble triacylglycerol (TAG) biosynthetic enzymes in eukaryotic cells comprising of lysophosphatidic acid acyltransferase with peptide SEQ ID 1 XALELQADDFNK and peptide SEQ ID 3 XXVNNVXPGXIEQ, phosphatidic acid phosphatase with peptide SEQ ID **4** NALTGLHMGGGK and peptide SEQ ID 5 sequence YVEGARPXK, diacylglycerol acyltransferase with peptide SEQ ID **7** XLWAVVGAQPFGGARGS, acyl-acyl carrier protein synthetase with peptide SEQ ID 8 sequence VHLAVALYGLAAVRVSRIVR, superoxide dismutase encoded by the gene sequence as in SEQ ID 9, and acyl carrier protein encoded by the gene sequence as in SEQ ID 10 and diacylglycerol acyltransferase encoded by the gene sequence as in SEQ ID 11.

2. Novel soluble TAG enzymes as in claim 1, being soluble in aqueous solutions.

3. Novel soluble TAG enzymes as in claim 1, where in the said eukaryotic cells are oleaginous yeast, backer's yeast, rat adipocytes and human cell-lines (HepG2).

4. Novel soluble TAG enzymes in yeast as in claim 1, comprising of lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl-acyl carrier protein synthetase, superoxide dismutase and acyl carrier protein.

5. Novel soluble TAG enzymes in oleaginous yeast as in claim 1, comprising of lysophosphatidic acid acyltransferase with peptide SEQ ID 1 XALELQADDFNK and peptide SEQ ID 3 XXVNNVXPGXIEQ, phosphatidic acid phosphatase with peptide SEQ ID **4** NALTGLHMGGGK and peptide SEQ ID 5 YVEGARPXK, diacylglycerol acyltransferase with peptide SEQ ID 7 XLWAVVGAQPFGGARGS, acyl-acyl carrier protein synthetase with peptide SEQ ID 8 VHLAVALYGLAAVRVSRIVR, superoxide dismutase encoded by the gene sequence as in SEQ ID 9, and acyl carrier protein encoded by the gene sequence as in SEQ ID 10 and diacylglycerol acyltransferase encoded by the gene sequence as in SEQ ID 11.

6. Novel soluble TAG enzymes in oleaginous yeast as in claim 1, comprising of lysophosphatidic acid acyl transferase, phosphatidic acid phosphatase, diacylglycerol acyl transferase, acyl-acyl carrier protein synthetase, superoxide dismutase and acyl carrier protein wherein the gene sequence encoding superoxide dismutase and acyl carrier protein and diacylglycerol acyltransferase have sequence homology to DNA sequences as in SEQ ID 9, SEQ ID 10 and SEQ ID 11, respectively.

7. Novel soluble TAG biosynthetic enzymes in yeast as in claim 1, where in lysophosphatidic acid acyltransferase is identified by immunological cross reactivity to the peptide sequence ALELQADDFNK (as in peptide SEQ ID **2),** diacylglycerol acyltransferase is identified by immunological cross reactivity to the peptide sequence XLWAVVGAQPFGGARGS (as in peptide SEQ ID **7)** and phosphatidic acid phosphatase is identified by immunological cross reactivity to peptides NALTGLHMGGGK (as in peptide SEQ ID **4)** and YVEGARP (as in peptide SEQ ID 6).

8. Novel soluble TAG biosynthetic enzymes as in claim 1 where in the enzymes are responsible for TAG synthesis and accumulation either in free or multi enzyme complex form.

9. The uses of novel soluble TAG biosynthetic enzymes to identify compounds that are capable of altering for TAG synthesis and accumulation.

10. Polypeptide of superoxide dismutase **(SOD)as** encoded by SEQ ID 9..

11. Polypeptide as 'claimed in claim 10, wherein the said polypeptide is prepared by expressing the gene defined by SEQ ID 9 in *E.coli.*

12. Polypeptide of acyl carrier protein (ACP), **as** encoded by SEQ ID 10.

13. Polypeptide as claimed in claim 12, wherein the said polypeptide is prepared by expressing the gene defined by SEQ ID 10 in *E.coli.*

14. Polypeptide of diacylglycerol acyltransferase, as encoded by SEQ ID 11.

15. Polypeptide as claimed in claim 14, wherein the said polypeptide is prepared by expressing the gene defined by SEQ ID 11 in *E.coli*.

16. The newly identified novel TAG biosynthetic enzyme sequence can be used as a target site for controlling fat.

17. The identified individual enzymes can also be used as a target for controlling fat.

18. An oligopeptide of at least 5 to 20 amino acids (peptide SEQ ID 1 to 8) capable of specifically identifying with a unique sequence of proteins present within a triacylglycerol biosynthetic complex (TBC).

19. Antibodies to at least 5 to 20 amino acids (peptide SEQ ID 1 to 8) from the isolated polypeptides from TBC, which are capable of specifically identifying the proteins present within a triacylglycerol biosynthetic complex.

20. An oligonucleotide of at least 15 nucleotides selected from SEQ ID 9 capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid, which encodes a superoxide dismutase.

21. An oligonucleotide of at least 15 nucleotides selected from SEQ ID 10 capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid, which encodes an acyl carrier protein.

22. A nucleic acid having a sequence complementary to the sequences (SEQ ID 9 to 11) of the isolated nucleic acid, which encode superoxide dismutase and acyl carrier protein and diacylglycerol acyltransferase.

23. An in vitro method of detecting the soluble triacylglycerol biosynthetic enzymes, which exist either as free or as complex in the cytosol.

24. A method for determining whether a subject known to have an imbalance in triacylglycerol has the imbalance due to a defect in the synthesis of triacylglycerol.

25. A method for treating a subject who has **an** imbalance in triglyceride (triacylglycerol) levels due to a defect in the synthesis of soluble triglyceride, which comprises introducing the isolated nucleic acid which encodes lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl camer protein (ACP), and acyl-ACP synthetase into the subject under conditions such that the nucleic acid expresses the soluble triacylglycerol biosynthetic enzymes individually or in combination, so as to thereby treat the subject.

26. A method for inhibiting the soluble triacylglycerol biosynthetic enzymes in a subject which comprises transforming appropriate cells from the subject 'with a vector which! expresses the nucleic acid which encodes the components in TBC, and introducing the transformed cells into the subject so as to thereby inhibit the soluble enzyme and/or complex.

27. A method for inhibiting the soluble triacylglycerol biosynthetic enzymes in a subject which comprises introducing the any of the above-described oligonucleotides of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encode lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl camer protein (ACP), acyl-ACP synthetase and superoxide dismutase into the subject so as to thereby inhibit the triglyceride formation and accumulation.

28. A method of obtaining soluble triacylglycerol (TAG) biosynthetic enzymes using polyacrylmide gel electgrophoresis, chromatographic procedures or density gradient centrifugation, said method comprising subjecting blue sepharose, DEAE matrix octail sepharose, antibodies raised from the peptide SEQ ID 1 to 8 and identifying and isolating the complex as a whole or individual components thereof.

29. Novel Lysophosphatidic Acid Acyltransferase

Peptide SEQ ID 1 XALELQADDFNK

Peptide SEQ ID **3** XXVNNVXPGXIEQ

30. Novel Phosphatidic Acid Phosphatase

Peptide SEQ ID **4** NALTGLHMGGGK

Peptide SEQ ID **5** YVEGARPXK

31. Novel Diacylglycerol Acyltransferase

Peptide SEQ ID **7** XLWAVVGAQPFGGARGS

32. Novel Acyl-Acyl Carrier Protein Synthetase

Peptide SEQ ID 8 VHLAVALYGLAAVRVSRIVR

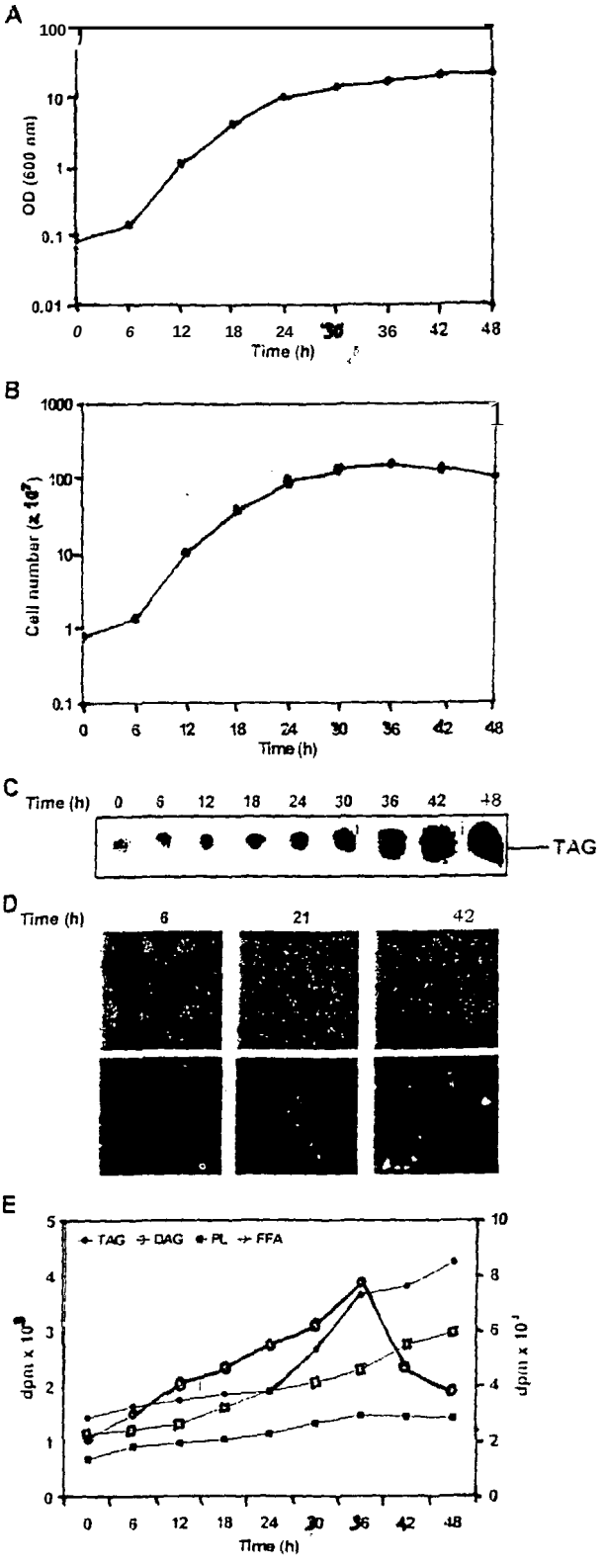

A
OD (600 nm)
Time (h)
B
Cell number
Time (h)
C
Time (h)
TAG
D
Time (h)
6
21
42
E
TAG
DAG
PL
FFA
Time (h)

FIGURE 1

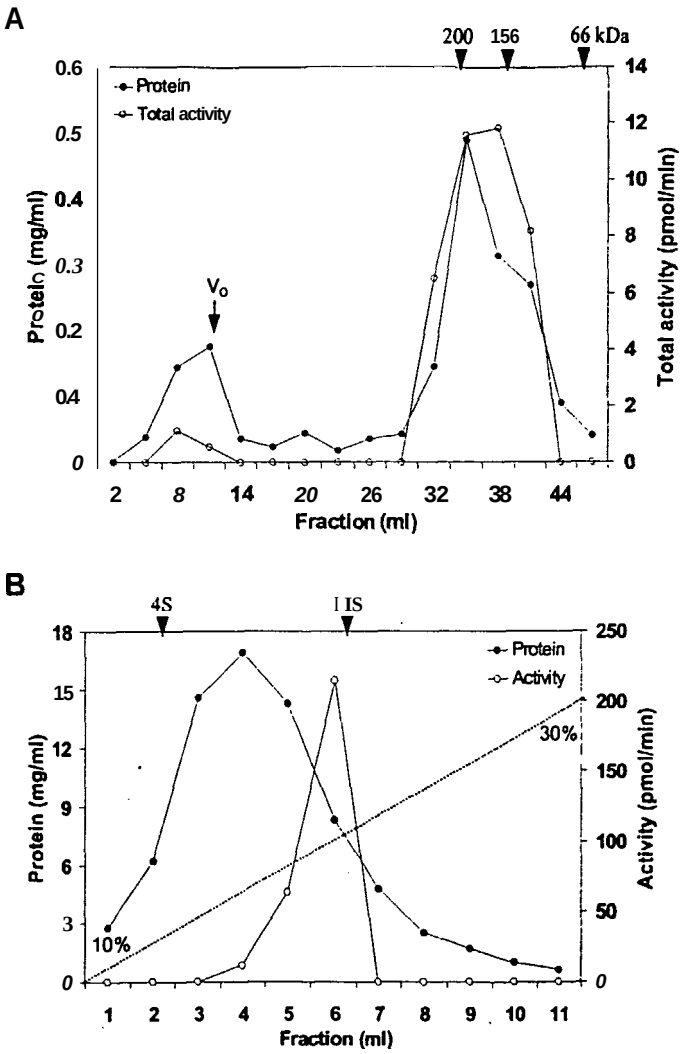

A
200 156 66 kDa
Protein
Total activity
Protein (mg/ml)
Total activity (pmol/min)
Vo
Fraction (ml)
B
4S
IIS
Protein
Activity
30%
10%
Protein (mg/ml)
Activity (pmol/min)
Fraction (ml)

FIGURE 2

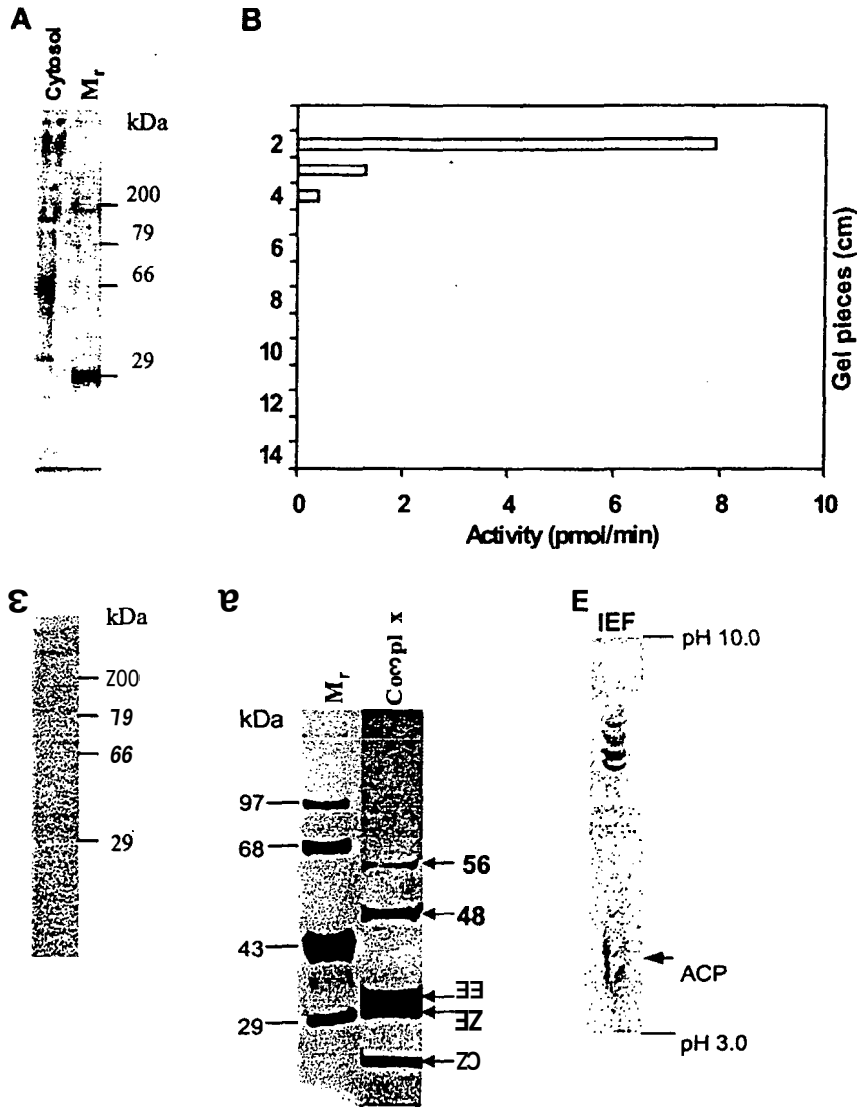
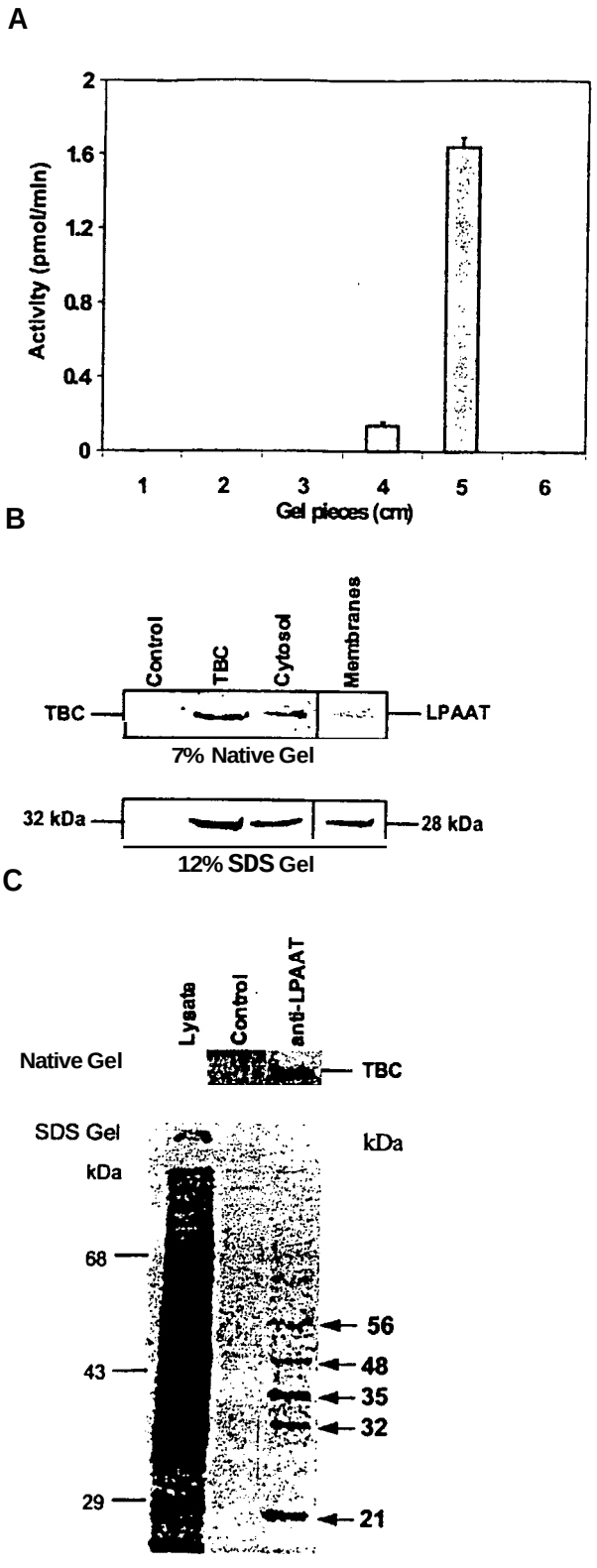

A
Cytosol
M_r
kDa
200
79
66
29
B
Gel pieces (cm)
Activity (pmol/min)
kDa
97
68
43
29
56
48
ACP
IEF
pH 10.0
pH 3.0

FIGURE 3


A
Activity (pmol/min)
2
1.6
1.2
0.8
0.4
0
1
2
3
4
5
6
Gel pieces (cm)
B
Control
TBC
Cytosol
Membranes
TBC
LPAAT
7% Native Gel
32 kDa
28 kDa
12% SDS Gel
C
Lysate
Control
anti-LPAAT
Native Gel
TBC
SDS Gel
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kDa
68
43
29
56
48
35
32
21

FIGURE 4

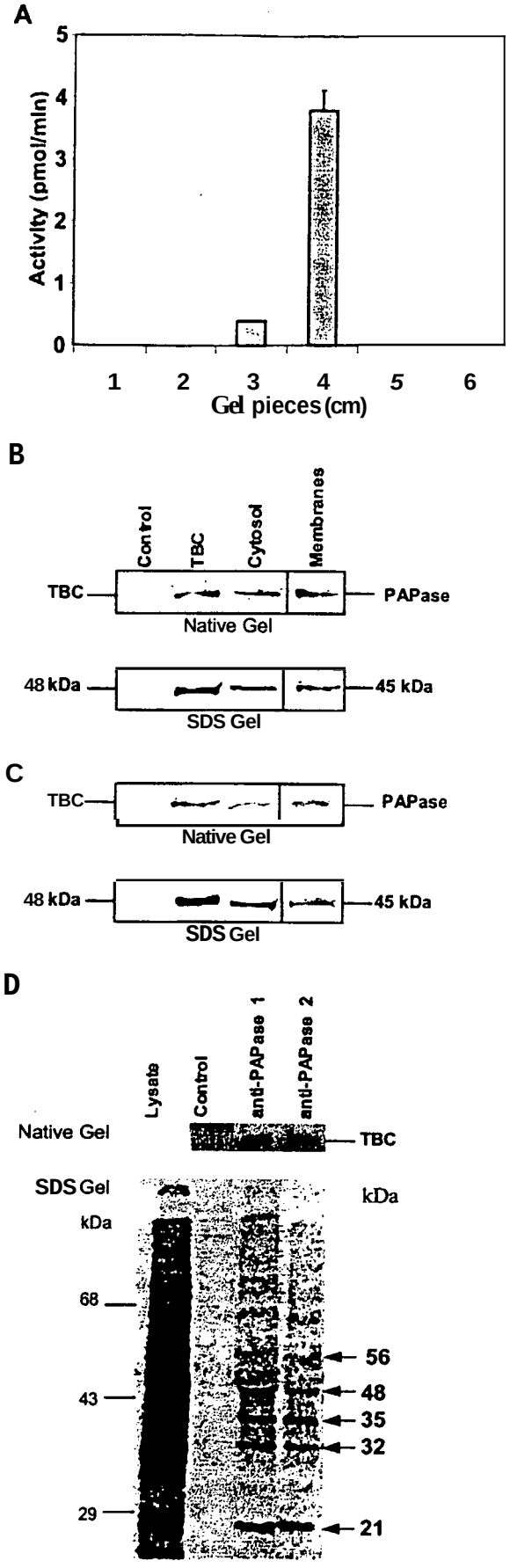
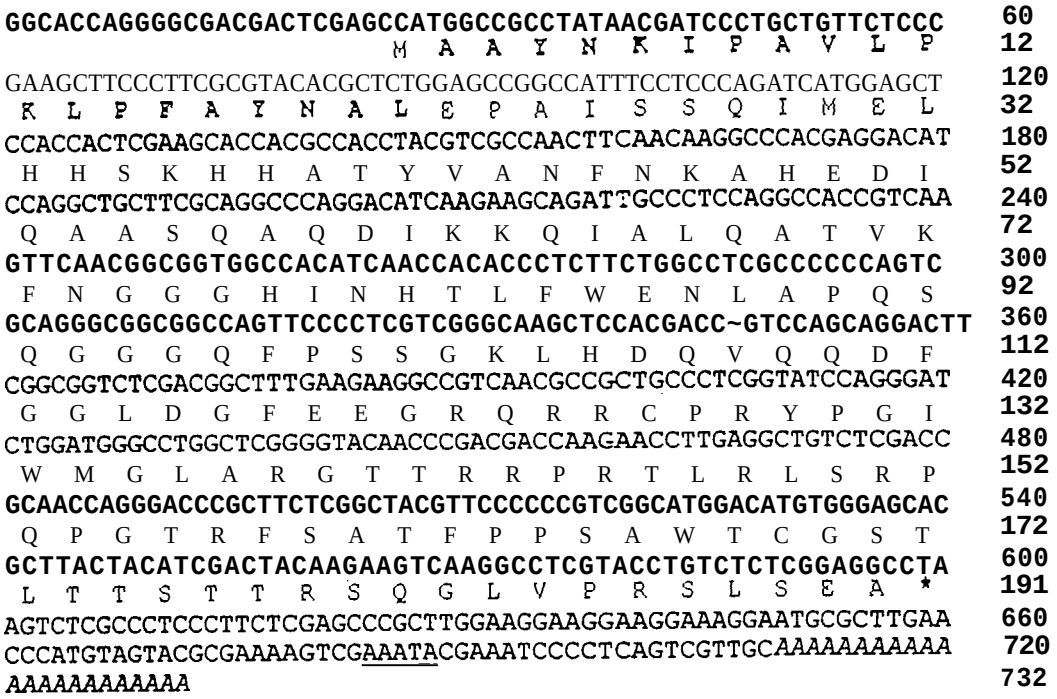

A
Activity (pmol/min)
0
1
2
3
4
5
1
2
3
4
5
6
Gel pieces (cm)
B
Control
TBC
Cytosol
Membranes
TBC
PAPase
Native Gel
48 kDa
45 kDa
SDS Gel
C
TBC
PAPase
Native Gel
48 kDa
45 kDa
SDS Gel
D
Lysate
Control
anti-PAPase 1
anti-PAPase 2
Native Gel
TBC
SDS Gel
kDa
kDa
68
43
29
56
48
35
32
21

FIGURE 5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M A A Y N K I P A V L P 12
GAAGCTTCCCTTCGCGTACACGCTCTGGAGCCGGCCATTTCCTCCCAGATCATGGAGCT 120
K L P F A Y N A L E P A I S S Q I M E L 32
CCACCACTCGAAGCACCACGCCACCTACGTCGCCAACTTCAACAAGGCCCACGAGGACAT 180
H H S K H H A T Y V A N F N K A H E D I 52
CCAGGCTGCTTCGCAGGCCCAGGACATCAAGAAGCAGATTGCCCTCCAGGCCACCGTCAA 240
Q A A S Q A Q D I K K Q I A L Q A T V K 72
GTTCAACGGCGGTGGCCACATCAACCACACCCTCTTCTGGCCTCGCCCCCCAGTC 300
F N G G G H I N H T L F W E N L A P Q S 92
GCAGGGCGGCGGCCAGTTCCCCTCGTCGGGCAAGCTCCACGACC~GTCCAGCAGGACTT 360
Q G G G Q F P S S G K L H D Q V Q Q D F 112
CGGCGGTCTCGACGGCTTTGAAGAAGGCCGTCAACGCCGCTGCCCTCGGTATCCAGGGAT 420
G G L D G F E E G R Q R R C P R Y P G I 132
CTGGATGGGCCTGGCTCGGGGTACAACCCGACGACCAAGAACCTTGAGGCTGTCTCGACC 480
W M G L A R G T T R R P R T L R L S R P 152
GCAACCAGGGACCCGCTTCTCGGCTACGTTCCCCCCGTCGGCATGGACATGTGGGAGCAC 540
Q P G T R F S A T F P P S A W T C G S T 172
GCTTACTACATCGACTACAAGAAGTCAAGGCCTCGTACCTGTCTCTCGGAGGCCTA 600
L T T S T T R S Q G L V P R S L S E A * 191
AGTCTCGCCCTCCCTTCTCGAGCCCGCTTGGAAGGAAGGAAGGAAAGGAATGCGCTTGAA 660
CCCATGTAGTACGCGAAAAGTCGAAATACGAAATCCCCTCAGTCGTTGCAAAAAAAAAAA 720
AAAAAAAAAAAA 732

FIGURE *6*

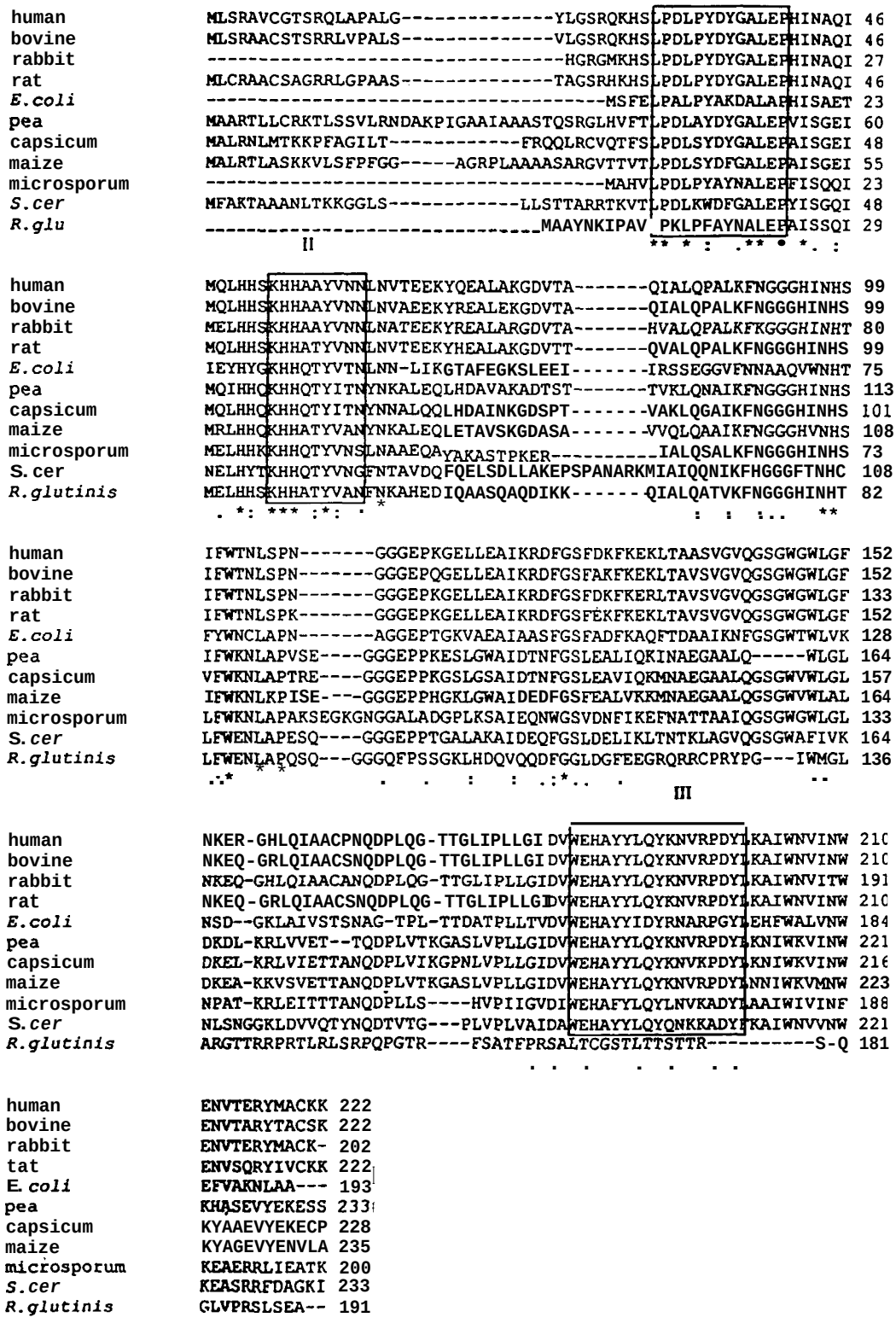
human MLSRAVCGTSRQLAPALG----------YLGSRQKHSLPDLPYDYGALEPHINAQI 46
bovine MLSRAACSTSRRLVPALS----------VLGSRQKHSLPDLPYDYGALEPHINAQI 46
rabbit ----------HGRGMKHSLPDLPYDYGALEPHINAQI 27
rat MLCRAACSAGRRLGPAAS----------TAGSRHKHSLPDLPYDYGALEPHINAQI 46
E.coli ----------MSFELPALPYAKDALAPHISAET 23
pea MAARTLLCRKTLSSVLRNDAKPIGAAIAAASTQSRGLHVFTLPDLAYDYGALEPVISGEI 60
capsicum MALRNLMTKKPFAGILT----------FRQQLRCVQTFSLPDLSYDYGALEPAISGEI 48
maize MALRTLASKKVLSFPFGG-----AGRPLAAAASARGVTTVTLPDLSYDFGALEPAISGEI 55
microsporum ----------MAHVLPDLPYAYNALEPFISQQI 23
S.cer MFAKTAAANLTKKGGLS----------LLSTTARRTKVTLPDLKWDFGALEPYISGQI 48
R.glu ----------MAAYNKIPAV PKLPFAYNALEPAISSQI 29
II
human MQLHHSKHHAAYVNNLNVTEEKYQEALAKGDVTA-------QIALQPALKFNGGGHINHS 99
bovine MQLHHSKHHAAYVNNLNVAEEKYREALEKGDVTA-------QIALQPALKFNGGGHINHS 99
rabbit MELHHSKHHAAYVNNLNATEEKYREALARGDVTA-------HVALQPALKFKGGGHINHT 80
rat MQLHHSKHHATYVNNLNVTEEKYHEALAKGDVTT-------QVALQPALKFNGGGHINHS 99
E.coli IEYHYGKHHQTYVTNLNN-LIKGTAFEGKSLEEI-------IRSSEGGVFNNAAQVWNHT 75
pea MQIHHQKHHQTYITNYNKALEQLHDAVAKADTST-------TVKLQNAIKFNGGGHINHS 113
capsicum MQLHHQKHHQTYITNYNNALQQLHDAINKGDSPT-------VAKLQGAIKFNGGGHINHS 101
maize MRLHHQKHHATYVANYNKALEQLETAVSKGDASA-------VVQLQAAIKFNGGGHVNHS 108
microsporum MELHHKKHHQTYVNSLNAAEQAYAKASTPKER----------IALQSALKFNGGGHINHS 73
S.cer NELHYTKHHQTYVNGFNTAVDQFQELSDLLAKEPSPANARKMIAIQQNIKFHGGGFTNHC 108
R.glutinis MELHHSKHHATYVANFNKAHEDIQAASQAQDIKK-------QIALQATVKFNGGGHINHT 82
human IFWTNLSPN-------GGGEPKGELLEAIKRDFGSFDKFKEKLTAASVGVQGSGWGWLGF 152
bovine IFWTNLSPN-------GGGEPQGELLEAIKRDFGSFAKFKEKLTAVSVGVQGSGWGWLGF 152
rabbit IFWTNLSPN-------GGGEPKGELLEAIKRDFGSFDKFKERLTAVSVGVQGSGWGWLGF 133
rat IFWTNLSPK-------GGGEPKGELLEAIKRDFGSFEKFKEKLTAVSVGVQGSGWGWLGF 152
E.coli FYWNCLAPN-------AGGEPTGKVAEAIAASFGSFADFKAQFTDAAIKNFGSGWTWLVK 128
pea IFWKNLAPVSE----GGGEPPKESLGWAIDTNFGSLEALIQKINAEGAALQ-----WLGL 164
capsicum VFWKNLAPTRE----GGGEPPKGSLGSAIDTNFGSLEAVIQKMNAEGAALQGSGWVWLGL 157
maize IFWKNLKPISE---GGGEPPHGKLGWAIDEDFGSFEALVKKMNAEGAALQGSGWVWLAL 164
microsporum LFWKNLAPAKSEGKGNGGALADGPLKSAIEQNWGSVDNFIKEFNATTAAIQGSGWGWLGL 133
S.cer LFWENLAPESQ----GGGEPPTGALAKAIDEQFGSLDELIKLTNTKLAGVQGSGWAFIVK 164
R.glutinis LFWENLAPQSQ---GGGQFPSSGKLHDQVQQDFGGLDGFEEGRQRRCPRYPG---IWMGL 136
III
human NKER-GHLQIAACPNQDPLQG-TTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINW 210
bovine NKEQ-GRLQIAACSNQDPLQG-TTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINW 210
rabbit NKEQ-GHLQIAACANQDPLQG-TTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVITW 191
rat NKEQ-GRLQIAACSNQDPLQG-TTGLIPLLGIDVWEHAYYLQYKNVRPDYLKAIWNVINW 210
E.coli NSD--GKLAIVSTSNAG-TPL-TTDATPLLTVDVWEHAYYIDYRNARPGYLEHFWALVNW 184
pea DKDL-KRLVVET--TQDPLVTKGASLVPLLGIDVWEHAYYLQYKNVRPDYLKNIWKVINW 221
capsicum DKEL-KRLVIETTANQDPLVIKGPNLVPLLGIDVWEHAYYLQYKNVKPDYLKNIWKVINW 216
maize DKEA-KKVSVETTANQDPLVTKGASLVPLLGIDVWEHAYYLQYKNVRPDYLNNIWKVMNW 223
microsporum NPAT-KRLEITTTANQDPLLS----HVPIIGVDIWEHAFYLQYLNVKADYLAAIWIVINF 188
S.cer NLSNGGKLDVVQTYNQDTVTG---PLVPLVAIDAWEHAYYLQYQNKKADYFKAIWNVVNW 221
R.glutinis ARGTTRRPRTLRLSRPQPGTR----FSATFPRSALTCGSTLTTSTTR----------S-Q 181
human ENVTERYMACKK 222
bovine ENVTARYTACSK 222
rabbit ENVTERYMACK- 202
tat ENVSQRYIVCKK 222
E.coli EFVAKNLAA--- 193
pea KHASEVYEKESS 233
capsicum KYAAEVYEKECP 228
maize KYAGEVYENVLA 235
microsporum KEAERRLIEATK 200
S.cer KEASRRFDAGKI 233
R.glutinis GLVPRSLSEA-- 191

FIGURE 7

```
GGCACGAGGCTCTCTCACATCGTTTGCCGCTACATTTCAGCTT~GGGTCTATTCAGCT     60
CACGATGAAGCACGTCGCCGCCTACCTCCTCCTCGTCTCTGCCGGC~CACCTCGCCCTC   120
     M  K  H  V  A  A  Y  L  L  L  V  S  A  G  N  T  S  P  S     19
GGCCGAGGACGTCAAGAAGGTCCTCGCCGCCGCCGACATCCAGGCCGACGAGGAGCGCC~  180
 A  E  D  V  K  K  V  L  A  A  A  D  I  Q  A  D  E  E  R  L     39
CTCGGTCCTCATCAAGGAGCTCGAGGGCAAGGACGTCAACGAGGTCATTGCCGAGGGATC  240
 S  V  L  I  K  E  L  E  G  K  D  V  N  E  V  I  A  E  G  S     59
CAAGAAGCTCGCTTCCGTCCCCTCGGGCGGCGCCGCCCCCGCCGCTGCCGCTGGCGGCGC  300
 K  K  L  A  S  V  P  S  G  G  A  A  P  A  A  A  A  G  G  A     79
TGCCGCTGGCGGTGCCGCCGAGGAGAAGGCTGAGGACAAGGGATGAGGA             360
 A  A  G  G  A  A  E  E  K  A  E  D  K  P  A  E  K  D  E  E     99
GAGCGACGACGACATGGGCTTCGGTCTCTTCGACTAAGCTCTCGTCTCGCCTCTCCCCCT  420
 S  D  D  D  M  G  F  G  L  F  D  *                           109
CTGCGACGACGCACAACTTTCCCGACCTTCCTCGACTTGCCG~GCGTTTCATCTCTGTA   480
GTTTGGGATCGATGGATTGCGCTAGGGAAGCCCTGACGGAGGGGGGGTGGTTTGGCT    540
TCTCAAAAAAAAAAAAAAAAAAAA
```

FIGURE *8*

A

```
Cladosporium    MKYMAAYLLLGLAGNSSPSAEDIKTVLSSVGIDADEERLSSLLKELEGKDINELISSGSQ 60
Alternata       MKHLAAYLLLGLGGNTSPSAADVKAVLESVGIEADSDRLDKLISELEGKDINELIASGSE 60
R.              MKHVAAYLLLVSAGNTSPSAEDVKKVLAAADIQADEERLSVLIKELEGKDVNEVIAEGSK 60
S.              MRYLAAYLLLTVGGKDSPSASDIESVLSTVGIEAESERIETLINELNGKDIDELIAAGNE 60
maize           MKVIAAYLLAVLGGNTSPTADDVKSILESVGAEADEEKLEFLLTELKDKDITEVIAAGRE 60
rat             MRYVASYLLARLGGNSNPSAKDIKKILDSVGIEADDERLNKVISELNGKNIEDVIAQGVG 60
human           MRYVASYLLAALGGNSSPSAKDIKRILDSVGIEADDDRLNRVISELNGKNIEDVIAQGIG 60
                *: :*:***   .*: .*:* *:: :* :.. :*:.::::. ::.**:.*:: :.:*:. *

Cladosporium    KLASVPSGGSGAAPSAGGAAAAGG--ATEAAPEAAKEEEKEE---SDDDMGFGLFD 111
Alternata       KLASVPSGGAGGAAASGGAAAAGGSAQAEAAPEAAKEEEKEE---SDEDMGFGLFD 113
R.              KLASVPSGGAAPAAAAGG-AAAGG--AAEEKAEDKPAEKDEE---SDDDMGFGLFD 110
S.              KLATVPTGG---AASAAPAAAAGG--AAPAAEEAAKEEAKEEEE-SDEDMGFGLFD 110
maize           RLSSVPSGGGAIDMGAPAAVAGGGAAPAEEAKKEEKVEEKEE---SDEDMGFSLFD 113
rat             KLASVPAGGAVAVSAAPGSAAPAAG-SAPAAAEEKKDEKKEESEESDDDMGFGLFD 115
human           KLASVPAGGAVAVSAAPGSA-SAPAAAEEKKDEKKEESEESDDDMGFGLFD 115
                :*::**:**     ..  .* ..  :   .  • .**  **:****.***
```

B

```
pylori          ----------------------------------------------MALFED-IQAVIAEQ 14
plasmodium      MFVVLSYVYGVSLQILKKKRSNQVNFLNRKNDYNLIKNKNPSSSLKSTFDD-IKKIISKQ 59
pseudomonas     ----------------------------------------MSTIEERVKKIVAEQ 15
e.coli          ----------------------------------------MSTIEERVKKIIGEQ 15
s.cer           MFRSVCRISSRVAPSAYRTIMGRSVMSNTILAQRFYSANLSKDQVSQRVIDVIKAFDKNS 60
brassica        ----------------------------------------NLSFNLGRSIPTR 13
rglutinis       ----------MKHVAAYLLLVSAGNTSPSAEDVKKVLAAADIQADERLSVLIKELEGKD 49
                                                        .  :

pylori          LNVDAAQVTPEAEFVKDLGADSLDVVELIMALEEKFNIEIPDEQAEKIVNVGDVVKYIED 74
plasmodium      LSVEEDKIQMNSNETKDLGADSLDLVELIMALEEKFNVTISDQDALKINTVQDAIDYIEK 119
pseudomonas     LGVKEEEVTNSASFVEDLGADSLDTVELVMALEEEFETEIPDEKAEKITTVQEAIDYIVA 75
e.coli          LGVKQEEVTNNASFVEDLGADSLDTVELVMALEEEFDTEIPDEEAEKITTVQAAIDYING 75
s.cer           PNIANKQISSMQFHKDLGLDSLDTVELLVAIEEEFDIEIPDKVADELRSVGETVDYIAS 120
brassica        LSVS---CAAKPETIEKVS----KIVKKQLSLKDD-QKVVAETKFADLG--ADSLDTV-- 61
rglutinis       VNEVIAEGSKKLASVPSGGAAPAAAAG-GAAAGGAAEEKAEDKPAEKDEESDDDMGFGLF 108
                                  . .

pylori          NKLA- 78
plasmodium      NNKQ- 123
pseudomonas     HQQ-- 78
e.coli          HQA-- 78
s.cer           NPDAN 125
brassica        -----
rglutinis       D---- 109
```

FIGURE 9

Rhodoturula glutinis soluble DAGAT sequence:

ACACTAGTGGATCCAAAGAATTCGGCACGAGGCTCGGCTCTCTCGCGTCTTTACGTCCCG
T L V D P K N ' S A R G S A L S R L Y V P
AAGGGTCTGTGGGAGGGCGAGGGCAAGTTCAAGGAGATCCTTCTCTCCGAGGTTGCCAAG
K G L W E G E G K F K E I L L S E V A K
ATCACTCTCGGCCCCGTCACCGAGTTCGAGCACTTCATGGGTCCCGTCATCTCGCAGGCT
I T L G P V T E F E H F M G P V I S Q A
TCGTTCGACAAGTGCCTCAGCTACGTTGAG~GGCCAAGCAGGCAGGTGGCGAGGTCCTC
S F D K C L S Y V E K A K Q A G G E V L
GCCGGCGGCAAGGGCGACGCGTCGAGCGGTTACTACGTTACTACGTCGAGCCGACCATCATCCTGACC
A G G K G D A S S G Y Y V E P T I I L T
AAGGACCCTCGCTCGCCTACCATGGTCGACGAGATCTTCGGCCCGGTCCTCACTGTTTAC
K D P R S P T M V D E I F G P V L T V Y
ATCTTCGAGGACGACCAGTACGAGGAGACGTGCAAGTTGATCGACCAGACGACGACGTAC
I F E D D Q Y E E T C K L I D Q T T T Y
GCCCTCACTGGCTGCATCTTCTCGGACGACCGTGCCGCGACTGTC~GGCCGGTGCTCTC
A L T G C I F S D D R A A T V K A G A L
CTCCGCCACGCTGCGGGTAACTACTACATCAACGACAAGTCGACCGGTGCTGTTGTCGGT
L R H A A G N Y Y I N D K S T G A V V G
GCCCAGCCTTTCGGTGGCGCACGCGGATCGGGCACGAACGGGCGGGCTCGATGACG
A Q P F G G A R G S G T N D K A G S M T
TTCTTCACCCGCTGGTGCCAGCCGCGGAGTGTG~GG~TCCTTCTGCCCGCCCG~TCT
F F T R W C Q P R S V
TTCCCTTACCCGTCGAACCAGCGCGATTAAATGGAGGAGTTGGGGAGGAGGAGGACGTCG
AGGGAGCTGGGGAGGCGGAGGACGTCGAGGAGGAGTTGGGGAGGAGGAGGACGTCGAGGG
AGCTGGGGAGGCGGAGGACGTCGAGGAGGAGTTGGGGAGGAGTTTGTCGAGGAGGAGGAG
AAGGGTTTCTCCTCGCCTGTAGTTGTACAAAATCAGCACGCCTTTGCTTCCACCGCCAAA
AAAAAAAAAAAAAAAAAACTCGAGAGTACTTCTAGAGCGGCCGCGGGCCCATCGATTTTC
CACCCGGGTGGGGTACCAGGTAAGTGTACCC~TTCGCCCTATAGTGAGTCGTATTAC~
TTCACTGGCCGTCGTTTTACAACGTCGTCGTGACTGGG~CCCTGGCGTTACCC~CTT~
TCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATGAGGCCCGGACCGA
TCGCCCTTCCAACAGTTGCGCAGCCTGATGGCGAATGGAGATCCAATTTTTAAGTGTATA
AGGGGTAAACTACTGATCTATTGTGGGGTTTTTAAAT

FIGURE 10