Isolation of a Gene Encoding a 1,2-Diacylglycerol-sn-acetyl-CoA Acetyltransferase from Developing Seeds of Euonymus alatus*

Received for publication, September 7, 2004, and in revised form, November 16, 2004
Published, JBC Papers in Press, December 3, 2004, DOI 10.1074/jbc.M410276200

Anne Milcamps, Ajay W. Tumaney, Troy Paddock, David A. Pan, John Ohlrogge, and Mike Pollard‡

From the Department of Plant Biology, Michigan State University, East Lansing, Michigan 48824

1,2-Diacyl-3-acetyl-sn-glyceroles (ac-TAG) are unusual triacylglycerols that constitute the major storage lipid in the seeds of Euonymus alatus (Burning Bush). These ac-TAGs have long-chain acyl groups esterified at both the sn-1 and sn-2 positions of glycerol. Cell-free extracts of developing seeds of E. alatus contain both long-chain acyl-CoA and acetyl-CoA sn-1,2-diacylglycerol acyltransferase (DGAT) activity. We have isolated a gene from developing seeds of Euonymus alatus that shows a very high sequence similarity to the members of the DGAT1 gene family (i.e. related to acyl-CoA:cholesterol acyltransferases). This Euonymus DGAT1 gene, when expressed in wild type yeast, results in a 5-fold enhancement of long-chain triacylglycerol (lc-TAG) accumulation, as well as the appearance of low levels of ac-TAG. Hydrogenated ac-TAG molecular species were identified by gas chromatography-mass spectrometry. Microsomes isolated from this transformed yeast show diacylglycerolacetyl-CoA acetyltransferase activity, which is about 40-fold higher than that measured in microsomes prepared from yeast transformed with the empty vector or with the Arabidopsis thaliana DGAT1 gene. The specific activity of this microsomal acetyltransferase activity is of the same order of magnitude as the microsomal long-chain DGAT activities measured for yeast lines transformed with the empty vector or either the Arabidopsis or Euonymus DGAT1 genes. Despite this, ac-TAG accumulation in yeast transformed with the Euonymus DGAT1 gene was very low (0.26% of lc-TAG), whereas lc-TAG accumulation was enhanced. Possible reasons for this anomaly are discussed. Expression of the Euonymus DGAT1-like gene in yeast lines where endogenous TAG synthesis has been deleted confirmed that the gene product has both long-chain acyl- and acetylacyltransferase activity.

The occurrence and the structural characterization of the 3-acetyl-1,2-long-chain diacyl-sn-glyceroles (1,2-diacyl-3-acetins, or ac-TAG)3 in seed oils were reported by Kleiman et al. (1). Unlike most long-chain triacylglycerols (lc-TAG), these ac-TAGs exhibit a strong optical activity because of asymmetry at the central carbon atom of the glycerol moiety introduced by the acetyl group. They have been found mainly in plants of the family Celastraceae but also in the families Lardizabalaceae, Ranunculaceae, Rosaceae, and Balsaminaceae. These unusual triacylglycerols are found in varying amounts, but in the genus Euonymus they can represent up to 98% of the total triacylglycerols in the seed oil. In Euonymus the sn-1 and sn-2 positions of the acyl glyceroles are esterified with common long-chain fatty acids, predominantly palmitate, oleate, and linoleate. Short-chain fatty acids are also found esterified in the triacylglycerols of milk from ruminants, particularly at the sn-3 position (2), and ac-TAGs have been identified in bovine udder lipids (3). Thus it is likely that ac-TAGs are often a minor component in the human diet. In addition, SALATRIM (short and long acylglyceride molecule) triacylglycerols have been developed as a commercial reduced calorie fat (4, 5). These are interesterified TAGs containing saturated fatty acids (largely stearate) and short-chain fatty acids (acetal, propionate, and/or butyrate). Lipase-catalyzed interesterification has also been used to produce acetylatedglycerols (6).

Triacylglycerols can be synthesized by sn-1,2-diacylglycerol:acyl-CoA acyltransferases (EC 2.3.1.20) (DGAT) or various transacylases. The first DGAT gene to be cloned was from mouse (7) and is a member of the DGAT1 gene family, which is closely related to the cholesterol:acyl-CoA acyltransferase gene family. Homologous DGAT1 genes have been cloned from Arabidopsis and other plants (8–11). In Arabidopsis there is only one DGAT1 gene, and studies with mutant lines generated by chemical mutagenesis or TDNA tagging indicate that this gene contributes significantly to seed oil content (10, 11). A second family of DGAT genes (DGAT2) was first identified in the oelignous fungus Mortierella ramanniana, based upon purification of a DGAT activity, peptide sequencing, and subsequent isolation of the corresponding genes (12). DGAT2 genes do not share sequence similarity to DGAT1 genes. Several DGAT2 homologues are reported in fungi, plants, and mammals (13, 14). Most recently, a gene encoding a bifunctional wax ester synthase/acyl-CoA diacylglycerol acyltransferase was characterized in Acinetobacter calcoaceticus (15). Several related open reading frames have been identified in Arabidopsis. This novel long-chain acyl-CoA acyltransferase is not related to the DGAT1 or DGAT2 gene families described above, and any role in triacylglycerol synthesis in higher plants still has to be established. Transacylases can produce triacylglycerols via acyl-CoA-independent reactions. A phospholipid:1,2-diacylglycerol acyltransferase has been reported to be involved in triacylglycerol synthesis in yeast and higher plants (16).

Triacylglycerol synthesis has been described in developing seed extracts from various plant species using medium-chain acyl-CoAs and/or medium-chain diacylglycerols in DGAT as-
Diacylglycerol:acetyl-CoA Acetyltransferase

says (17–20). Frequently the substrate specificity and selectivity of these DGATs are fairly broad. Our in vivo labeling of developing Euonymus seeds with exogenous [14C]acetate produced [acetetyl-14C]1,2-diacyl-3-acetin with negligible kinetic lag phase, suggesting that there is no intermediate acetyl lipid involved in biosynthesis, i.e. that a transacylase mechanism of synthesis was unlikely. In this paper we present evidence for a 1,2-diacylglycerol:acetyl-CoA acetyltransferase activity in extracts of developing Euonymus seeds, and we describe the isolation and characterization of a Euonymus DGAT1 gene that encodes this DGAT with both long-chain acyl- and acetyltransferase activity.

EXPERIMENTAL PROCEDURES

Materials—Seeds of Euonymus alatus were harvested during the full fall of 1999 in Michigan State University campus, courtesy of the Grounds Department of Campus Parks, and the endosperm plus embryo tissue were dissected and stored at −80 °C. Arabidopsis thaliana (ecotype Colombia-2) developing seeds were collected from the silicles of 6–8-week-old plants, grown in the growth chambers (22 °C, 16-h light period at 80–100 microeinstins of light intensity). Yeast (Saccharomyces cerevisiae) cultures (“wild type” is Invitrogen MATα ura3::::TRP1 ADE2, 100 μg/ml ampicillin (pYES2CT) or 50 μg/ml kanamycin (pE1776). Isolation of Euonymus and Arabidopsis cDNAs—Encoding DGAT1—Total RNA was extracted from developing seeds of Euonymus according to the protocol of Chung et al. (22) and from developing seeds of Arabidopsis by the method of Ruuska and Ohlrogge (23). For Euonymus gene isolation, alignment of the deduced amino acid sequence of several plant and mammalian DGAT1 genes showed very well conserved sequences. On the basis of the most conserved sequences between these DGAT1 genes, a set of degenerated primers were designed and tested in a PCR for accuracy with the Arabidopsis DGAT1 EST AA042298 as template. Two sets of primers (MP1 and MP6) were found to be successful and were used for an RT-PCR with Euonymus total RNA of developing seeds. The cDNA of the total RNA was made with an oligo(dT) primer (Invitrogen). Because the resolution of the products on gel was not clear, the primers were labeled with 32P, and the products were purified with a Beckman Biozym spin column and was recovered, cloned into the TopoPCR2.1 vector, and sequenced. With fidelity polymerase DagrFayYes (containing a BamHI site) and DagrRYes (containing an XhoI site). The 1.6-kb PCR fragment was purified with the kit from Qiagen and cloned in the vector pYES2CT. Whole cell PCR of obtained colonies with DGAT1-specific primers confirmed the presence of the insert, and the DNA sequence was verified, yielding the clone pYES2EaPCR5.1. The PCR primers used are as follows: MP1, TANTTNTATGNTG-CNCNAC; MP6, NCNTGNAANACNGCGN; MP10, TACCCTATGTTGCCAGAG; MP11, ATGCCATTGAGAGATTTTT; MP16, TTGTTCCGATGTTCTACTG; MP30, CAGTCTCTGGAACACATCGA; MP31, CTTCTCTAGGTGACATAAAAAAAM; MP5, GAGTAGAACATGCAGAACCC; DagrFayYes, ATATCGTACATATGCTCTACAATTTGCAAG; DagrRYes, ATATCGTACGACA-AAAACTGCGCTCTACTCCA; DagrFayYes, ATATCTCGAGCAC-AACATGCAGAACC; DagrFayYes, ATATGGATCCAATAATGTCTA-GTGCA; MP31, CTCTCTCAATGGCATACAAAAAG; MP15, GCAGTAGACGATCAGAACCC; DagrFayYes, ATATCGTACATATGCTCTACAATTTGCAAG; DagrRYes, ATATCGTACGACA-

Expression of Euonymus Ea DGAT1 and Arabidopsis (At) DGAT1 in Yeast Strains—The Ea cDNA and the At cDNA, cloned into the yeast vector pYES2CT, as well as the empty vector pYES2CT, were introduced into the S. cerevisiae strains INVY51 and H1266 via transformation. Three yeast colonies for each construct were grown in liquid medium and analyzed for lipid content. For the semi-quantitative lipid analysis by TLC, a small 5-ml culture of each colony was started in SC medium with 2% glucose and grown overnight. This culture was diluted 1:100 in a volume of 200 ml and grown overnight in the OD between 1 and 2. This culture was subsequently centrifuged and washed with sterile water and resuspended in 400 ml of SC medium supplemented with 2% galactose and 1% raffinose, with a starting OD of 0.4. Growth was followed over time, and 40-ml samples were taken at early and mid-exponential phase and beginning, mid-, and late stationary phase. These samples were washed, pelleted, and stored at −20 °C and analyzed for TAG content. For this work, the semiquantitative analysis of lipids, 800 ml of yeast cultures of S. cerevisiae and H1266 carrying either pYES2CT, pYES2EaPCR5.1, or pYES2At2.2 were grown until the start of the stationary phase and were treated as described above.

Lipid Analysis—Yeast pellets were quenched by heating in 5 volumes of hot isopropyl alcohol for 10 min to inactivate lipases. The lipids were then washed with chloroform and added to hot isopropyl alcohol. The pellet was extracted with hexane/isopropyl alcohol (25). The initial hot isopropyl alcohol extracts were combined with 1.5 volumes of hexane and added to the lipid extraction. The lipids recovered from the extractions were evaporated to dryness under nitrogen for semi-quantitative lipid analysis, the total lipid extract was separated by TLC (0.25-mm thickness K6 Silica Gel 60 A plates, Whatman). Lipids were then visualized with a 10% H2SO4 in methanol solution and were compared with mass standards. Visualization of bands was carried out with iodine staining, and bands were compared with mass standards.

For quantitative lipid analysis and identification, GC standards were prepared. 3-Acetyl-1,2-dipalmitin was prepared by the acetylation of 1,2-dipalmitoylglycerol with acetic anhydride in pyridine and purified by preparative TLC. A mixture of palmitoylstearoyl-3-acetin and distearoyl-3-acetin was prepared by hydrogenation of the purified 1,2-diacyl-3-acetin fraction isolated from E. alatus seeds. Dipentadecanoyl-α-acetin was prepared by the interesterification of triacetin (1 mmol) and tripentaenadecanoic acid (2 mmol). These triacylglycerols were heated in a capped tube at 250 °C in the presence of sodium methoxide (0.2 mmol) catalyst for 1 h. The resulting dipentaenadecanoyl-α-acetin was separated from the scrambled products by preparative TLC. The lipids recovered from the large scale yeast cultures were first weighed, and then tripeptdadenoin (l-TAG) and dipentaenadecanoyl-α-acetin (ac-TAG) internal standards were added. An aliquot of the sample was hydrogenated. Hydrogenations were carried out by continuous stirring of lipids (5–20 mg) in hexane (2–3 ml) with 2–3 mg of platinum(IV) oxide catalyst and hydrogen at slightly above atmospheric pressure at room temperature for 2 h. The hydrogenated lipids were then treated with ethereal diazomethane to convert free fatty acids to fatty acid methyl esters, because free fatty acids will co-chromatograph with ac-TAG on silica TLC. An aliquot was then transmethylated by using the method of Ichihara et al. (26) and analyzed by GC (with flame ionization detector (FID)) to determine total fatty acid content (allison 17:0 from added tripeptdadenoin l-TAG and ac-TAG fractions were isolated by preparative TLC using 1-mm thickness K6 Silica Gel 60 A plates (Whatman) and were developed multiple times with hexane/diethyl ether/acidic acid 80:20:1 (v/v/v)). Plates were viewed under UV light after spraying with ethanolic dichlorofluorescein (0.2% w/v) to locate the bands. Lipids were eluted from the silica with hexane/isopropyl alcohol 3:2 (v/v). The hydrogenated triacylglycerols were analyzed by high temperature GC using a DB-5ht capillary column (30 m × 0.25 mm inner diameter, 25 M. Pollard, unpublished data.
0.1-μm film thickness) with helium carrier gas at 1.5 ml/min. The column was temperature programmed at 5 °C/min to 360 °C, with the injector set at 380 °C and a 40:1 split ratio, and the FID detector temperature was also 380 °C. For GC-MS to identify the deuterated ac-TAG fractions, a Hewlett-Packard 5890 gas chromatograph-coupled MSD 5972 mass analyzer was used, with the mass analyzer set in electron impact mode scanning from 40 to 70 atomic mass units.

Microsomal Assays for DGAT Activity— Cultures of S. cerevisiae strains INVSc1 and H1266 carrying pYES2CT, pYRS2EaPCR5.1, or pYRS2At2.2 were grown in SC medium lacking uracil and supplemented with 1% raffinose and 2% galactose. At the beginning stationary phase, 100-ml cultures were centrifuged (−0.5 g of yeast pellet), and microsomes were isolated as described by Dahlqvist et al. (16) with some minor modifications. The yeast pellet was resuspended in 4 ml of ice-cold buffer (20 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 1 mM EDTA, 5% (v/v) glycerol, 1 mM dithiothreitol, and 0.3 M ammonium sulfate) and vortexed vigorously with 2-ml glass beads for 5 min. The resulting (v/v) glycerol, 1 mM dithiothreitol, and 0.3 M ammonium sulfate) and vortexed vigorously with 2-ml glass beads for 5 min. The resulting suspension was centrifuged at 15,000 × g for 15 min at 6 °C. The supernatant was subsequently centrifuged at 100,000 × g for 1.5 h at 6 °C. The pellet was resuspended in cold 100 mM potassium phosphate, pH 7.2, to 1.5–3.5 mg of protein/ml, and aliquots were stored at −80 °C. Protein concentrations of the yeast microsomes were determined according to the dye-binding method of Bradford (27) using bovine serum albumin as a standard. DGAT assays contained 2–5 μl of microsomes (5–15 mg/mg protein). 150–200 nCi of [1-14C]acetyl-CoA or [1-14C]oleoyl-CoA as substrates (20–50 μM), and 50 mM potassium phosphate buffer in a total volume of 100 μl. The reaction was carried out at 30 °C for 15 min. The reaction mixture was immediately quenched in hot isopropanol alcohol, and lipids were extracted with hexane/isopropanol alcohol (25:75) as described by Bligh and Dyer (19). Total lipids were analyzed on K6 Silica TLC plates developed with hexane/diethyl ether/acetic acid, 80:20:1 (v/v/v). Labeled bands were located and quantified using an Instant Imager. To identify the putative DGAT activity responsible for this sn-3 acetylation reaction, a unique exogenous DGAT, 1,2-dihexanoyl-sn-glycerol, was added to the assays, resulting in a novel band that co-chromatographed with the synthetic 1,2-dihexanoyl-sn-glycerol standard was small (5 to 10%, or −5 to +15%, respectively), no response factor correction was included.

DGAT Assays with Eucalyptus Seed Extracts—Frozen embryo and endosperm tissue was homogenized in 2 volumes of chilled buffer containing 0.3 M sucrose, 10 mM NaF, 5 mM MgCl2, 2 mM dithiothreitol, 1 mM EDTA, and 40 mM HEPES-NaOH, pH 7.4, and filtered through two layers of Miracloth. The residue was re-homogenized in 2 more volumes of buffer and filtered. The filtrates were combined and centrifuged at 10,000 × g for 10 min at 4 °C. The postnuclear supernatant was mixed with 0.5 g of yeast pellet, and DGAT activity was determined in a total volume of 4 ml of buffer and filtered. The filtrates were combined and constitute the enzyme activity responsible for this sn-3 acetylation reaction. Labeled lipids from incubation of cell-free homogenates prepared from developing E. alatus seed tissues with [1-14C]acetyl-CoA were analyzed by TLC as shown in Fig. 1. In the absence of exogenous diacylglycerol (DAG), normal phase TLC showed a major labeled band (Fig. 1, lane 2) that co-eluted with endogenous ac-TAG. When this labeled band was recovered and analyzed by C18 reversed phase TLC, the radioactivity migrated with the mass bands corresponding to the major ac-TAG molecular species (Fig. 1, lane 4). To confirm further the reaction as a 1,2-diacyl-sn-glycerol:acyl-CoA acyltransferase reaction, a unique exogenous DAG, 1,2-dihexanoyl-sn-glycerol, was added to the assays, resulting in a novel band that co-chromatographed with the synthetic 3-acyl-1,2-dihexanoin standard in both normal (Fig. 1, lanes 1 and 3) and reverse phase TLC systems (lanes 5 and 6). The acyltransferase assay gave a linear initial rate of incorporation into ac-TAG up to 15 min, and activity was proportional to protein up to 20 μl of extract (0.3 mg of protein) (data not shown). Extract boiled for 5 min was inactive. There was no measurable lag phase before the label appears in the ac-TAG product, indicating that no detectable [1-14C]acyl-lipid intermediate was formed. Thus the activity was defined as a DGAT by the incorporation of the labeled acetyl group from acetyl-CoA, by the acetyl incorporation kinetics, and by the use of a distinctive acyl acceptor. When the homogenate was assayed for long-chain TAG synthesis at 5–60 μM palmitoyl-CoA concentrations, the le-DGAT activity was about half the acyltransferase-specific activity measured at the same acetyl-CoA concentration.

Identification of a DGAT1 Gene in Eucalyptus—Total RNA was isolated from developing E. alata seeds at early to mid-maturation, a period during which the accumulation of acetyl glycerides reaches a maximum, and thus mRNA levels for the responsible biosynthetic enzyme(s) would be high. An RT-PCR gene-cloning strategy was used for the isolation of putative TAG synthesis genes. Several classes of TAG-synthesizing genes/proteins have been described with the DGAT1 family being the first to have been identified. Because nulls for the single DGAT1 gene present in A. thaliana result in the reduction of seed oil content by 35–45% (10, 11), the DGAT1 gene product contributes substantially to seed oil content. As other TAG-synthesizing enzymes might compensate for the loss of the DGAT1 activity, it is likely that in Arabidopsis the DGAT1 gene could contribute much more than half of the total oil accumulation in the wild type. Thus our strategy was to isolate a putative DGAT1 gene from E. alatus and investigate whether it would be involved in 1,2-diacyl-3-acetin production.

![Fig. 1. TLC identification of labeled lipid products from assays of E. alatus developing seed extracts with [1-14C]acetyl-CoA. Assays were run with exogenous diacylglycerol (lanes 2 and 4) or with added 1,2-dihexanoin at concentrations to partially (0.25 mM, lanes 3 and 6) or fully (2.5 mM, lanes 1 and 5) compete with the endogenous diacylglycerol. Normal phase TLC of the total lipid product is shown in the left-hand panel (lanes 1–3). The ac-TAG bands, C18/C18, C18/C16, and C16/C18 from the endogenous diacylglycerols or C6/C6 from dihexanoin were isolated and re-analyzed by C18 reversed phase TLC, as shown on the right-hand panel (lanes 4–6). In lanes 4 and 6, the labeled ac-TAG species will include 16/0:18:1 and 18:1/18:1 in the lowest band, 16:0/18:2 and 18:1/18:2 in the middle band, and 18:2/18:2 in the upper band.](http://www.jbc.org/)
We searched for a DGAT1-like gene in Euonymus using degenerate primers that were made on the basis of well conserved regions of several DGAT1 proteins. These primers were used for RT-PCR of total RNA from Euonymus developing seeds. A small fragment of 330 bp was obtained, showing a high degree of similarity with the DGAT1 genes. 3' and 5' RACE, using gene-specific primers, yielded a 1.5-kb fragment that very likely encompassed the full open reading frame. The deduced amino acid sequence is highly similar to all DGAT1 proteins described so far in plants (Fig. 2), with 50.7% identity and 91% similarity to the Arabidopsis DGAT1 protein. The only region of the putative Euonymus DGAT1 protein that is greatly different from the other DGAT1 proteins is the N-terminal end (93 amino acids). DGAT1 enzymes are membrane-associated proteins. The predicted transmembrane regions and putative substrate-binding site and active site residues described by others are present in the Euonymus DGAT1 gene product (8–11, 28).

**Fig. 2.** Alignment of the deduced amino acid sequences of plant DGAT1 genes. *E. alatus* (E.a.) (GenBank™ accession number AY751297), *A. thaliana* (A.t.) (GenBank™ accession number AF051849), *Nicotiana tabacum* (N.t.) (GenBank™ accession number AF129003), and *Perilla frutescens* (P.f.) (GenBank™ accession number AF298815) genes are shown. The putative acyl-CoA binding site motif (1) and active site motif (2), after Jako et al. (28), are shown underlined.
function of the DGAT1 gene of Euonymus, the cDNA was cloned into the yeast vector pYES2CT and expressed in wild type S. cerevisiae cells (strain INVSc1) under inducing conditions. As controls, the yeast was also transformed with the empty pYES2CT vector and the pYES2CT vector containing the A. thaliana DGAT1 gene. Oil extracted from mature Arabidopsis seeds contains only 0.01 ± 0.003% ac-TAG (data not shown), and thus any triacylglycerol-synthesizing gene from Arabidopsis would not be expected to have acetyltransferase activity. Total lipids extracted from yeast cells were analyzed semi-quantitatively by TLC with iodine staining. Triacylglycerols, including 1,2-diacyl-3-acetins, were analyzed quantitatively by the addition of odd-chain internal standards, namely acetyl-1,2-dipalmitadecanoin (for ac-TAG) and triheptadecanoin (for lc-TAG), followed by hydrogenation, TLC separation, and GC. Cultures of all the transformed yeast lines were sampled from early logarithmic to late stationary phase, and TLC analysis showed optimum accumulation of TAG at early stationary phase. This is in agreement with the observations on wild type yeast (13, 29). There was no significant difference in the growth rates of the yeast lines. Thus the full TAG analyses were conducted at 48 h after culture inoculation. We also tested if the supplementation of acetate would increase the lc-TAG or ac-TAG production. Acetate (5 mM) was found to marginally increase TAG synthesis and was included in the medium because it might cause higher acetyl-CoA pools in the yeast cells.

Total lipids were extracted and hydrogenated, and lc-TAG and ac-TAG fractions were isolated by preparative TLC. Chromatograms of the acetyl glyceride fraction from the empty vector and Euonymus DGAT1-transformed yeast are shown in Fig. 3. The major peak is the α-acetyl-α,β-dipalmitadecanoin internal standard (C32, not counting the glycerol carbon atoms). Three additional peaks are observed only for the Euonymus DGAT1-transformed yeast and correspond to C34, C36, and C38 ac-TAG, having retention times identical to synthetic standards. The sample was then analyzed by GC-MS. Fig. 4 shows the mass spectra for the C32 (internal standard) and C36 peaks from Fig. 3. The mass spectrum of the C32 internal standard shows diagnostic ions at m/z = 225 and 341 atomic mass units, corresponding to CH$_3$(CH$_2$)$_{13}$CO$^+$ and (M-CH$_3$(CH$_2$)$_{13}$COO)$^+$ ions. The mass spectrum of the C36 peak shows diagnostic ions at m/z = 239, 267, 355, and 383 atomic mass units, which are assigned as CH$_3$(CH$_2$)$_{13}$CO$^+$, CH$_3$(CH$_2$)$_{15}$CO$^+$, (M-CH$_3$(CH$_2$)$_{15}$COO)$^+$, and (M-CH$_3$(CH$_2$)$_{15}$COO)$^+$ ions, respectively. The molecular weight is given by (RCO) + (M-RCOO) + 16 atomic mass units. Thus the molecular weight is given by (239 + 383 + 16) or (267 + 355 + 16) = 638. In addition there is a small peak at m/z = 578 corresponding to (M-HOAc)$^+$, and the “fingerprint” regions below m/z 200 atomic mass units match very closely for the standard and the C36 peak. Thus the structure of this particular peak is unambiguously acetyl-palmitoyl-stearoyl-glycerol. Likewise, the C34 and C38 peaks shown in Fig. 3, panel 2, are acetyldi-, triacylglycerids. The acyl distribution of the hydrogenated ac-TAG shown in Fig. 3 is a reflection of the fatty acid composition of yeast, which is predominantly a mixture of 16:0, 16:1, 18:0, and 18:1 and which will therefore be expected to produce C34, C36, and C38 acetylglycerides. The GC peaks gave 49, 43, and 8%, respectively, of ac-TAG species.

The results of the expression of the Arabidopsis and Euonymus DGAT1 genes on lipid accumulation are shown in Table I. The Arabidopsis and Euonymus DGAT1 genes provided 2.7- and 6.4-fold increases in total fatty acids, respectively, relative to the empty vector control, and increases in lc-TAG of 5.1- and 19.8-fold, respectively. Thus the expression of DGAT1 genes is not simply diverting acyl groups into TAG but is stimulating net fatty acid accumulation. Most or all of the increase in fatty acid content is 87% in the Arabidopsis DGAT1-transformed yeast line. This is greater than the TAG content reported for wild type S. cerevisiae (10–50%) (30), and for yeast transformed with the LROI gene (66%) (16). The expression of...
TABLE I
Lipid quantification in transformed yeast

| Lipid mass                        | Empty vector | Euonymus DGAT1 | Arabidopsis DGAT1 |
|-----------------------------------|--------------|----------------|-------------------|
| Total lipids (mg/liter culture)   | 17.5         | 36.5           | 58                |
| Total fatty acids (mg/liter culture) | 7            | 18.5           | 43                |
| Total TAG (mg/liter culture)      | 2            | 9.5            | 37.5              |
| Total ac-TAG (μg/liter culture)   | <0.7         | 26             | 35                |

For DGAT activity using [14C]acetyl-CoA or [14C]oleoyl-CoA.

FIG. 6. Additional TLC analysis of the putative labeled acetyl glyceride product from the incubation of [1-14C]acetyl-CoA with microsomes isolated from yeast transformed with the Euonymus DGAT1 gene. The labeled lipid (ac-TAG, Fig. 5) from silica TLC was eluted and analyzed by either C18 reverse phase TLC (A) or silver nitrate TLC (B). The possible combinations of individual molecular species of acetyldiacylglycerols from yeast are shown.

FIG. 7. Specific activities for DGAT measured in transformed yeast lines. DGAT activity (average of four determinations) was measured with either 0.05 mM [1-14C]acetyl-CoA (black bars) or [1-14C]oleoyl-CoA (gray bars) substrates for microsomes isolated from wild type yeast (strain INVSc1) transformed with the Euonymus DGAT1 gene, the Arabidopsis DGAT1 gene, or the empty pYES2CT vector.

The DGAT-specific activities from yeast microsomes in the above experiments are compared in Fig. 7. The results for the acetyltransferase activity are clear; only the Euonymus DGAT1 gene produced significant activity and with an order of magnitude comparable with long-chain DGAT activities. The yeast transformed with the Euonymus DGAT1 gene showed about a 30-fold increase over the empty pYES2CT vector.

The Arabidopsis DGAT1 gene in yeast also resulted in the appearance of very low levels of acetyl glycerides. However, the acetyl glyceride content to total triacylglycerols was 3-fold higher with the Euonymus gene, being 0.26%, whereas with the Arabidopsis gene it was 0.09%. The addition of acetate into the yeast culture had only a small increasing effect on the synthesis of the ac-TAG (data not shown).

DGAT Activity in Wild Type Yeast Expressing the Euonymus or Arabidopsis DGAT1 Genes—Microsomal fractions prepared from wild type yeasts expressing the Euonymus DGAT1 gene, the Arabidopsis DGAT1 gene, or the empty vector were assayed for DGAT activity using [14C]acetyl-CoA or [14C]oleoyl-CoA. Product analysis by TLC is shown in Figs. 5 and 6, and quantification of the relative specific activities is shown in Fig. 7.

When microsomes were incubated with [14C]acetyl-CoA, only the yeast line transformed with the Euonymus DGAT1 gene showed significant accumulation of a labeled product that comigrates with the authentic 1,2-diacyl-3-acetin standard on silica TLC (Fig. 5). When this band was recovered and reanalyzed on two totally different TLC systems, its chromatographic behavior was completely consistent with its identification as [14C]ac-TAG. C18 reverse-phase TLC (Fig. 6A) shows three labeled bands and the assignment of the probable molecular species. The lower band co-migrates with 16:0/18:1 and 18:1/18:1 acetyl glyceride standards, and the spacing of the bands is consistent with two carbon or one double bond differences in acyl composition. On silver nitrate TLC, the putative [14C]ac-TAG sample eluted with monoenoic and dienoic AcTAG standards, as expected (Fig. 6B).
40-fold increase in acetyltransferase activity versus the yeast transformed with the Arabidopsis DGAT1 gene. The long-chain DGAT activity we report in the control yeast (Fig. 7; 180 pmol/min/mg microsomal protein) is similar to that reported for wild type yeast (340 ± 40 pmol/min/mg microsomal protein) by Oelkers et al. (31). However, the relative specific activities for long-chain DGAT (Fig. 7) for the three yeast lines (Euonymus DGAT1 > control > Arabidopsis DGAT1) bear no relationship to the TAG accumulation observed (Table I; Arabidopsis DGAT1 ≫ Euonymus DGAT1 ≫ control). The control DGAT activity comes largely from the DGA1 gene in yeast, which is a member of the DGAT2 gene family and is the dominant TAG synthesizing gene at the onset of stationary phase (31).

TAG Content and DGAT Activity Analyses of TAG-null Yeasts Transformed with Euonymus or Arabidopsis DGAT1 Genes—A triple mutant yeast line, H1266, deleted in the DGA1, LRO1, and ARE2 genes (13), was also transformed with the plant genes or empty vector control. The DGA1 gene encodes a DGAT2 protein that is largely responsible for TAG synthesis during stationary phase, whereas the LRO1 gene encodes a phospholipid:diacylglycerol acyltransferase that contributes significantly to TAG synthesis during logarithmic phase growth. The acyl-CoA:sterol acyltransferase encoded by ARE2 has residual DGAT activity. When all three genes were inactivated, the yeast cell cannot synthesize TAG nor do microsomes exhibit any DGAT activity. TAG accumulation and microsomal DGAT activities with acetyl-CoA and palmitoyl-CoA substrates were determined (Table II) in transformed H1266 lines. With the much reduced endogenous background in H1266, the long-chain DGAT activity of both plant genes becomes clearly apparent. In this background the acetyltransferase activity of the Euonymus DGAT1 gene is still much higher than the Arabidopsis gene, but the long-chain DGAT activities are severalfold higher, primarily because the substrate in these assays was palmitoyl-CoA. Again, the Euonymus DGAT1 gene gives a (2-fold) greater microsomal long-chain DGAT activity, but lc-TAG accumulation is much higher (12-fold) in the yeast line transformed with the Arabidopsis gene.

**DISCUSSION**

Our enzymology experiments with cell-free extracts of developing E. alatus seeds demonstrate an acetyl-CoA-utilizing DGAT activity (Fig. 1). The DGAT1 gene we identified from developing E. alatus seeds clearly has substantial acetyl-CoA transferase activity, as demonstrated by the microsomal assay in transformed yeast and in contrast to yeast transformed with the Arabidopsis DGAT1 gene as a control, where the activity is almost undetectable. The variant acyltransferase we have identified is striking because it involves the complete removal of the hydrophobic domain of the substrate molecule. To our knowledge, this is the first report of a gene from any source encoding an acyltransferase that retains its long-chain acyl-CoA acyltransferase activity and yet can also function with an acyl substrate with no hydrophobic domain.

Euonymus seed oil contains about 95% 1,2-diacyl-3-acytins, whereas Arabidopsis seed oil contains essentially none. The DGAT1 gene with acetyl-CoA transferase activity we describe is likely involved in acetyl glyceride accumulation in developing Euonymus seeds. However, the fact that Euonymus is a genetically uncharacterized plant, even to its ploidy level, would allow for other DGAT1 isoforms, and thus the DGAT1 gene may not be the only gene required for expression of the ac-TAG phenotype. By measuring bulk tissue concentrations of acetyl-CoA in various seeds, we have ruled out that the primary cause of the ac-TAG phenotype may be a very high acetyl-CoA pool concentration (32). However, a channeled supply of acetyl-CoA, perhaps from an association of ATP-citrate lyase with the DGAT1 protein, might not result in a detectable change in the bulk acetyl-CoA concentration.

The DGAT1 gene identified from developing E. alatus seeds has substantial acetyltransferase activity in the transformed yeast (Fig. 7) and also results in accumulation of low but measurable amounts of the expected acetyl glyceride product (Table I). However, the expression of this gene does not result in as strong an acetyl glyceride phenotype as might be expected from consideration of enzyme activity alone. This result is similar to a number of other studies where expression of genes involved in the synthesis of unusual fatty acids has yielded only low levels of the expected product (33–35). In this study the limitation is clearly not the result of low expression of the transgene. By comparing the expression of Euonymus and Arabidopsis DGAT1 genes, which have 51% identity and 91% similarity, it is clear that the yeast long-chain microsomal DGAT activity does not predict the level of lc-TAG accumulation. This is the case whether the genes are expressed in wild type (Table I and Fig. 7) or in a TAG-synthesizing null line of yeast (Table II). In both cell lines, expression of the Euonymus DGAT resulted in higher microsomal DGAT activity, whereas expression of the Arabidopsis DGAT gave 4- and 12-fold higher TAG accumulations for wild type and H1266 backgrounds, respectively. Euonymus seed extracts also contain similar acetyl-CoA and long-chain acyl-CoA DGAT activity, but in the seed the latter activity is barely represented in terms of end-product accumulation (1). Thus, TAG accumulation in both yeast and Euonymus reflects a higher degree of complexity than indicated by *in vitro* enzyme activity assays. Although catalyzing a simple acyl transfer reaction, DGAT1 is a very large integral membrane protein, having 60-kDa subunits with 5–7 transmembrane domains and likely existing as a tetramer (36, 37), and by inference it may have some sophisticated regulatory mechanisms associated with its structure.

With the demonstrated lack of correlation between long-chain DGAT activity and lc-TAG accumulation in yeast, we should hardly be surprised if the acetyltransferase activity of the Euonymus DGAT1 gene product is not reflected in a large accumulation of ac-TAG product. One simple interpretation is that the gene we have cloned produces an enzyme with both acetyl and long-chain acyltransferase activity and can switch between either specificity depending on conditions. Such conditions might include metabolite concentrations or association
with other proteins. This interpretation would fit the observation that expression of the Euonymus DGAT1 protein in yeast causes large increases in lc-TAG. It would be assumed that the conditions in vivo favor the long-chain DGAT activity. In developing Euonymus seeds the broad specificity DGAT1 may function as an acyltransferase because of localized conditions in the membrane or perhaps because of an as yet unidentified additional gene product. It needs to be stressed that, as is usually the case with assay of microsomal acyl-CoA-dependent enzymes of lipid synthesis, even at fairly low acyl-CoA assay concentrations, most of the acyl-CoA will sequester into the membranes, giving a higher molar ratio of acyl-CoA to membrane lipid than is expected in vivo. In vivo, most of the endogenous acyl-CoA pool will be bound to acyl-CoA-binding protein (38). In our long-chain DGAT1 assay at an oleoyl-CoA concentration of 50 \mu M and containing on average 10 \mu M of microsomal protein, there is an \sim 1:2 acyl-CoA to membrane lipid ratio in the assay. This compares with a bulk cellular ratio estimated at about 1:30, using a value for the long-chain acyl-CoA concentration in yeast cells of 1.5–2 nmol/10^9 cells (39), and our estimate of total phospholipids at 40–60 nmol/10^9 cells. With such microsomal assays, the cooperativity of substrate utilization might be hidden, acyl-CoA substrate inhibition might occur, or factors that regulate activity in vivo might be lost during extract preparation.

DGAT enzyme activities with distinct specificities and/or selectivities have been described in assays with microsomes isolated from various developing seeds where the seed oils have unique compositions (18–20), but none of the genes corresponded to isolated from various developing seeds where the seed oils have unique compositions (18–20), but none of the genes corresponded-

Acknowledgment—We thank Prof. Sten Stymne (Department of Crop Science, Swedish University of Agricultural Sciences, S-330 53 Alnarp, Sweden) for the donation of the yeast triple KO line H1266.

REFERENCES

1. Kleiman, R., Miller, R. W., Earle, F. R., and Wolff, I. A. (1967) Lipids 2, 473–478
2. Padley, F. B. (1986) in The Lipid Handbook (Gunstone, F. D., Harwood, J. L., and Padley, F. B., eds) pp. 113–129, Chapman and Hall Ltd., London.
3. Limb, J. K., Kim, Y. H., Han, S.-Y., and Jhon, G.-J. (1999) J. Lipid Res. 40, 2169–2176
4. Smith, R. E., Finley, J. W., and Leveille, G. A. (1994) J. Agric. Food Chem. 42, 432–434
5. Auerbach, M. H., Chang, P. W., Kosmark, R., O’Neill, J. J., Philips, J. C., and Klemann, L. P. (1998) in Structural Modified Food Fats: Synthesis, Biochemistry, and Use (Christophe, A., ed) pp. 89–116, American Oil Chemists’ Society Press, Champaign, IL.
6. Kuo, S.-J., and Parkin, K. L. (1995) J. Agric. Food Chem. 43, 1775–1783
7. Cases, S., Smith, S. J., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C., Welch, C. B., Luiz, A. J., Erickson, S. K., and Farese, R. V. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13018–13023
8. Bovier-Nave, P., Benveniste, P., Oelkers, P., Sturley, S. L., and Schaller, H. (2000) Eur. J. Biochem. 267, 85–96
9. Halsall, D. H., Liu, C., and Hills, M. J. (1999) FEBS Lett. 452, 145–149
10. Routaboul, J., Benning, C., Bechtold, N., Caboche, M., and Leopinie, L. (1999) Plant Physiol. Biochem. 37, 831–840
11. Zou, J., Wei, Y., Jako, C., Kumur, A., Selvaraj, G., and Taylor, D. C. (2001) Plant J. 23, 645–653
12. Lardizabal, K. D., Mai, J. T., Wagner, N. W., Wyrick, A., Voeler, T., and Hawkins, D. J. (2001) J. Biol. Chem. 276, 38682–38687
13. Sandagner, L., Gustavsson, M. H., Stahl, U., Dahleqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., and Stymne, S. (2002) J. Biol. Chem. 277, 6478–6482
14. Cases, S., Stone, S. J., Zhou, P., Yen, E., Tew, B., Lardizabal, K. D., Voeler, T., and Farese, R. V. (2001) J. Biol. Chem. 276, 38870–38876
15. Kalscheuer, R., and Steinbuchel, A. (2003) J. Biol. Chem. 278, 8075–8082
16. Dahleqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandagner, L., Ronne, H., and Stymne, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6487–6492
17. Ichihara, K., and Noda, M. (1982) Phytochemistry 21, 1995–1991
18. Cao, Y.-Z., and Huang, A. H. C. (1987) J. Biol. Chem. 262, 989–992
19. Vogel, G., and Browse, J. (1999) Plant Physiol. 119, 923–931
20. Silhavy, T. J., Berman, M. L., and Enquist, L. W. (1984) Experiments with Gene Fusions, pp. 217–222, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Chung, C. H., Kwon, O. C., Lee, Y. M., and Lee, S. Y. (2000) Mol. Cell. Biol. 20, 108–111
22. Raukas, A. A., and Ohlrogge, J. B. (2001) BioTechniques 31, 752–758
23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
24. Araya, H., and Radin, N. S. (1978) Anal. Biochem. 90, 420–426
25. Ichihara, K., Shibahara, A., Yamamoto, Y., and Nakayama, T. (1996) Lipids 31, 535–539
26. Bradley, M. F. (1976) Anal. Biochem. 71, 248–254
27. Jako, C., Kumur, A., Wei, Y., Zou, J., Barton, D. L., Giblin, E. M., Covelio, P. S., and Taylor, D. C. (2001) Plant Physiol. 126, 861–874
28. Taylor, P. R., and Parks, L. W. (1978) Biochem. Biophys. Acta 575, 204–214
29. Rattray, J. B. M. (1988) in Ratledge, C., and Wilkinson, S. G., eds) Vol. 1, pp. 555–697, Academic Press, London
30. Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J. T., and Sturley, S. L. (2002) J. Biol. Chem. 277, 8877–8881
31. Tumaney, A. W., Ohirgrobe, J. B., and Pollard, M. (2004) J. Plant Physiol. 161, 485–489
32. Reddy, A. S., and Thomas, T. L. (1996) Nat. Biotechnol. 14, 639–642
33. Cahoon, E. B., Shanklin, J., and Ohlrogge, J. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11184–11188
34. Bao, X., Kata, S., Pollard, M., and Ohlrogge, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7172–7177
35. Yu, C., Chen, J., Lin, S., Liu, J., Chang, C. C. Y., and Chang, Y. T. (1999) J. Biol. Chem. 274, 36139–36145
36. Cheng, D., Meegalla, R. L., He, B., Cromley, D. A., Billheimer, J. T., and Young, P. R. (2001) Biochem. J. 359, 707–714
37. Faegeman, N. J., and Krudsen, J. (1997) Biochem. J. 323, 1–12
38. Rosendal, J., and Krudsen, J. (1992) Anal. Biochem. 207, 63–67
Isolation of a Gene Encoding a 1,2-Diacylglycerol-sn-acetyl-CoA Acetyltransferase from Developing Seeds of Euonymus alatus

Anne Milcamps, Ajay W. Tumaney, Troy Paddock, David A. Pan, John Ohlrogge and Mike Pollard

J. Biol. Chem. 2005, 280:5370-5377.
doi: 10.1074/jbc.M410276200 originally published online December 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410276200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 14 of which can be accessed free at http://www.jbc.org/content/280/7/5370.full.html#ref-list-1