Identification of Two Novel Human Acyl-CoA Wax Alcohol Acyltransferases

MEMBERS OF THE DIACYLGlycerol ACYLTRANSFERASE 2 (DGAT2) GENE SUPERFAMILY* [S]

Received for publication, January 3, 2005
Published, JBC Papers in Press, January 25, 2005, DOI 10.1074/jbc.M500025200

Aaron R. Turkish§§, Annette L. Henneberry¶¶, Debra Cromley**, Mahajabeen Padamsee¶¶, Peter Oelkers§§, Hisham Bazzi§§, Angela M. Christiano§§, Jeffrey T. Billheimer**, and Stephen L. Sturley§§*

From the Departments of ¶¶Pediatrics, §§Dermatology, Genetics, and Development and the ^**Center for Experimental Therapeutics, Columbia University Medical Center, New York, New York 10032 and the *Center of Human Nutrition, Columbia University Medical Center, New York, New York 10032 and the Institute of Human Nutrition, Columbia University Medical Center, 650 W168th St., New York, New York 10032.

This paper is available on line at http://www.jbc.org

The esterification of alcohols such as sterols, diacylglycerols, and monoacylglycerols with fatty acids represents the formation of both storage and cytoprotective molecules. Conversely, the overproduction of these molecules is associated with several disease pathologies, including atherosclerosis and obesity. The human acyl-CoA:diacylglycerol acyltransferase (DGAT) 2 gene superfamily comprises seven members, four of which have been previously implicated in the synthesis of di- or triacylglycerol. The remaining 3 members comprise an X-linked locus and have not been characterized. We describe here the expression of DGAT2 and the three X-linked genes in Saccharomyces cerevisiae strains virtually devoid of neutral lipids. All four gene products mediate the synthesis of triacylglycerol; however, two of the X-linked genes act as acyl-CoA wax alcohol acyltransferases (AWAT 1 and 2) that predominantly esterify long chain (wax) alcohols with acyl-CoA-derived fatty acids to produce wax esters. AWAT1 and AWAT2 have very distinct substrate preferences in terms of alcohol chain length and fatty acyl saturation. The enzymes are expressed in many human tissues but predominate in skin. In situ hybridizations demonstrate a differentiation-specific expression pattern within the human sebaceous gland for the two AWAT genes, consistent with a significant role in the composition of sebum.

In eukaryotes, the cytoplasmic storage of fatty acids in the form of triacylglycerol (TAG) serves to provide reservoirs for membrane formation and maintenance, lipoprotein trafficking, detoxification of fatty acid and alcohol substrates, epidermal integrity, and fuel in times of stress or nutrient deprivation (reviewed in Refs. 1–3). By contrast, the subcellular and extracellular accumulation of TAG and other neutral lipids has been linked to several human disease states such as diabetes mellitus (4), obesity (4), atherosclerosis (5, 6), and non-alcoholic fatty liver disease (7). Understanding the metabolic pathways of triglyceride (TG) synthesis and the roles of the enzymes involved in these reactions may hasten the development of therapeutical interventions by clarifying the pathophysiological processes of these diseases.

Diacylglycerol is esterified to triglyceride by an acyl-CoA:diacylglycerol acyltransferase (DGAT) reaction. There are at least two independent mammalian enzymes known to catalyze this reaction, DGAT1 (8) and DGAT2 (9, 10). DGAT1 is a member of the acyl-CoA:cholesterol acyltransferase (ACAT) gene family with high levels of expression in human small intestine, colon, testis, and skeletal muscle. Mice lacking DGAT1 are surprisingly healthy with normal serum TAG but are resistant to diet-induced obesity, and have impaired sebaceous gland secretion (11–14). Subsequent to the discovery of DGAT1, DGAT2, the original member of a second human DGAT family, was identified by sequence similarity to lipid droplet proteins purified from Mortierella ramanniana, an oleaginous fungus (10). When expressed in insect cells, DGAT2 produces robust DGAT activity. DGAT2 shares no sequence similarity with DGAT1 and exhibits widespread expression in humans, with particularly high levels in liver and adipose tissue. Recently it has been shown that DGAT2 mice are severely depleted of triglycerides in their tissues and plasma, and possess poor skin barrier function, leading to early death (15). DGAT1 was unable to fully compensate for the loss of DGAT2, suggesting different roles for the two enzymes, and that DGAT2 is the enzyme responsible for the majority of TAG synthesis in mice.

In Saccharomyces cerevisiae, DGA1 is the sole member of the DGAT2 gene family and is responsible for a large portion of triglyceride synthesis in this model organism (16–18). This activity is supplemented by that of LRO1 (an ortholog of mammalian lecithin cholesterol acyltransferase), which also esterifies diacylglycerol but uses phospholipids as the acyl donor (19). While their relative contribution varies with culture con

DGAT, acyl-CoA:diacylglycerol acyltransferase; MGAT, acyl-CoA monoacylglycerol acyltransferases; AWAT, acyl-CoA wax alcohol acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; TG, triglyceride.
ditions, together these enzymes are responsible for about 98% of triglyceride synthesis in yeast. Deletion of ARE2, a steryl ester synthase, in conjunction with DGA1 and LRO1 eliminates all detectable TAG synthesis in yeast (17). As described here, we have utilized this “triple” deletion strain to study mammalian mediators of triglyceride metabolism such as the DGA2 gene family.

The human DGA2 gene family consists of seven members: DGA2 (10), 3 acyl-CoA monoacylglycerol acyltransferases (MGATs 1, 2, and 3; Refs. 21–23), and the three genes characterized by our study. Based on expression patterns, MGAT2 and MGAT3 were proposed as the major mediators of the intestinal MAG pathway. This pathway predominates in the intestine, whereby 2-monoacylglycerol, a product of partial lipolysis of triglyceride, is re-esterified using a fatty acyl-CoA to produce diacylglycerol. Moreover, the substrate preference of MGAT3 (49% sequence identity with human DGAT2) for produce diacylglycerol. However, the preferred alcohol substrates for two of three DGAT2 family members were expressed cDNAs for these three genes and defined the substrate specificity, tissue expression patterns, and genealogic relationships of the enzymes, relative to human DGAT2. Markedly, all three enzymes are capable of esterifying DAG. However, the preferred alcohol substrates for two of them are long chain alcohols leading to the production of wax esters. We therefore rename these enzymes acyl-CoA wax alcohol acyltransferase (AWAT) 1 and 2.

**EXPERIMENTAL PROCEDURES**

**General**—Molecular biology and yeast procedures were performed according to conventional protocols (27, 28). Complete (yeast extract, peptone, dextrose, YPD), synthetic complete (SC), and selective media (with 5-FOA for DNA expression) were prepared as described (27). DNA-modifying reagents were purchased from New England Biolabs. (with galactose for cDNA expression) were prepared as described (27).

**Identification, Isolation, and Construction of Expression Plasmids with Human DGA2, hDC3, and hDC4—**Human expressed sequence tags, genomic sequences, and predicted mRNA sequences for DGA2, hDC3, and hDC4, with Human DGA2, hDC3, and hDC4—performed at the Columbia University Cancer Center sequencing nol was obtained from Sigma. Automated DNA sequencing was performed on IMAGE cDNA clones in the pOTB7 (clone ID 4778300) and pCMV-SPORT6 (clone ID 4778300) vectors, respectively, and sequenced. Expression plasmids for the full-length human DGAT2 and hDC4 were engineered by subcloning the cDNA fragments into the EcoRI and XmaI, and EcoRI sites, respectively, of the yeast galactose inducible vector, pRS423-GP, downstream of the GAL 11/10 promoter (35). Using predicted cDNA-flanking regions, oligo primer pairs (Supplementary Table I) were designed to amplify the full-length coding region of DGA2 and hDC4 from thymus and lung cDNA, respectively. Amplification was performed with nested PCR using 2 ng of human cDNA, 10× PCR buffer, 1.75 mM MgCl2, 1.5 units of Taq polymerase, (TaqDNA polymerase, Invitrogen), 1 μM oligo-primers, and 0.4 units of Pfu DNA polymerase (Stratagene) in the Gene Amp PCR System 2400 (Applied Biosystems). PCR reactions were held at 94 °C for 5 min followed by 28 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1.5 min. PCR products were gel-isolated, purified, TA-cloned into the EcoRI and NotI sites of the pCR 2.1-TOPO vector (Invitrogen), and transformed into chemically competent Top10 Escherichia coli (Rapid One Shot Chemical Transformation, Invitrogen). The full-length hDC4 was then subcloned into the EcoRI site of the pRS423-GP vector downstream of the GAL 11/10 promoter. The full-length DGA2 was subcloned into the EcoRI and NotI sites of the pRS423-GP vector, also downstream to the GAL 11/10 promoter. The full-length cDNA of DGAT2 and hDC4 was completely sequenced.

**Yeast Strains and Transformation—**Strains of S. cerevisiae with triple deletions of the DGA1, LRO1, and ARE2 (SCY2056, dpa1::URA3 lro1::::Ura3 ars::::LEU2) genes have been previously described to possess almost 99% depletion of triglyceride synthesis capabilities (17). These strains were transformed with the human DGAT2, DGA2, hDC3, and hDC4 expression plasmids or pRS423-GP using lithium acetate followed by prototrophic selection (36).

**Expression of DGA2, hDC3, and hDC4—**RNA was prepared from transformed yeast strains grown in SC-His + 2% galactose + 1% raffinose or SC-His + 2% dextrose (control). 10 μg of RNA from each culture was resolved on a 1.2% agarose, formaldehyde gel, transferred to nylon membrane (Hybond-N, Amersham Biosciences), and hybridized in Quik-Hyb buffer (Stratagene) at 65 °C for 1 h with a random hexamer-primer. [32P]cDNA labeled gene specific probe, generated with PCR (right), Molecular weight markers were purchased from LifeTech.

**Lipid Metabolism—**Molecular biology and yeast procedures were performed as previously described (17, 19). A dilute inoculation (1:20–1:30) of a saturated culture grown in SC-His + 2% dextrose, followed by washes and resuspension in SC-His + 2% galactose + 1% raffinose, was grown to 30 °C to early logarithmic phase (A600 = 0.3–0.4). For in vivo DGAT assays, 10 ml of cells were added to 0.01 M CuCl2 or 0.25 M CuCl2 medium containing 20 μM of [14C]oleic acid. Protein deuterium incorporation was monitored by HPLC analysis. Lipids were extracted from dried cell pellets by cell wall hydrolysis (lyticase) and organic extraction (37) and were resolved by TLC in petroleum ether/diethyl ether/acetic acid (84:15:1) and the two solvent resolution system as described (38). For in vivo AWAT assays, cell pellets were resuspended in 5 ml of 10 mM Tris/37 °C. Media were added as indicated and cells were harvested by centrifugation at 37 °C for 1 h with shaking, washed twice with 0.5% tergitol and once with diH2O, and lyophilized. Lipids were extracted from dried cell pellets by cell wall hydrolysis (lyticase) and organic extraction (37) and were resolved by TLC in petroleum ether/diethyl ether/acetic acid (84:15:1) and the two solvent resolution system as described (38). For in vivo AWAT assays, cell pellets were resuspended in 5 ml of 10 mM Tris/37 °C. Media were added as indicated and cells were harvested by centrifugation at 37 °C for 1 h with shaking, washed twice with 0.5% tergitol and once with diH2O, and lyophilized. Lipids were extracted from dried cell pellets by cell wall hydrolysis (lyticase) and organic extraction (37) and were resolved by TLC in petroleum ether/diethyl ether/acetic acid (84:15:1) and the two solvent resolution system as described (38). For in vivo AWAT assays, cell pellets were resuspended in 5 ml of 10 mM Tris/37 °C. Media were added as indicated and cells were harvested by centrifugation at 37 °C for 1 h with shaking, washed twice with 0.5% tergitol and once with diH2O, and lyophilized. Lipids were extracted from dried cell pellets by cell wall hydrolysis (lyticase) and organic extraction (37) and were resolved by TLC in petroleum ether/diethyl ether/acetic acid (84:15:1) and the two solvent resolution system as described (38).

**Lipid Accumulation—**Assessment of the steady-state accumulation of lipids was performed by labeling a dilute inoculation (~1:1000 of a saturated culture) of each strain into 10 ml of SC-His + 2% galactose + 1% raffinose with 0.01 μM [3H] or [14C]oleic acid or 0.25 μCi/ml of [3H]palmitate and overnight growth at 30 °C. Cells were washed, and lipids were extracted and analyzed as described above.

**In Vitro Assay of DGAT and AWAT Activity and Substrate Specificity in Yeast Microsomes—**A dilute inoculation of each strain into 500 ml of SC-His + 2% galactose + 1% raffinose was grown overnight at 30 °C into log phase. Cells were washed and lysed, and microsomes were prepared from a 100,000 × g spin, as previously described (39). Protein concentrations were determined (40) and diacylglycerol esterification assays were performed as described previously (17, 19, 41). In brief, microsomes (equivalent for 5 min at 37°C in 100 mM Tris/20 mM NaCl, pH 7.5 containing 0.25% sucrose, 1 mM EDTA, 10 mM MgCl2, 20 mM fatty acid free bovine serum albumin, 160 μM dioleoylglycerol (in 5 μl acetone, final volume 200 μl). The reaction was initiated by the addition of [14C]oleoyl-CoA (10–20,000 dpm/ml, 40 μM) and stopped after 10 min by the addition of 4 ml of chloroform/methanol (2:1, v/v). Carrier TAG (10 μg) and internal standard [1H]triolein, 30,000 dpm) were added followed by 0.8 ml of water to separate phases. The lower chloro-
X-linked Acyltransferases and Neutral Lipid Biosynthesis

14574

roform layer containing the lipids was removed, evaporated to dryness, resuspended in 50 μl of chloroform, and individual lipid classes separated on Varian chromatography paper using a hexane/diethyl ether/acytolic acid (170:30:1, v/v) mobile phase. The spot corresponding to TG was cut out and counted. Similar assays were performed in triplicate for AWAT activity using at least three independent yeast microsome preparations. Initially (Table II), conditions were similar to those used for DGAT activities (i.e. 100 μg microsomes, assayed for 20 min in a final volume of 250 μl) with the substitution of 200 μg cetyl alcohol (unlabeled or 14C-labeled 10,000 dpm/μg) for DAG. After separation by TLC, the spot corresponding to wax ester was cut out and counted. The AWAT assays were subsequently optimized to be linear with time and so that the reaction velocity was proportional to the amount of protein (Table III). For AWAT1 (DGAs), microsomes (10 μg) were preincubated for 5 min at 30 °C in 100 mM Tris, pH 7.5 containing 0.25 mM sucrose, 1 mM EDTA, 20 mM MgCl2, 20 μM fatty acid-free bovine serum albumin, 200 μM wax alcohol in 2 μl of acetic acid). The reaction (final volume 250 μl) was initiated by the addition of [14C]oleoyl-CoA (40 μM) and stopped after 10 min by the addition of chloroform/methanol. Cholesteryl ester (15 μg) and [3H]cholesterol ester (30,000 dpm) were added as carrier and internal standard, respectively and lipids extracted and separated as described above. Cholesteryl ester and wax ester have the same solubility in the mobile phase. The assay for AWAT2 (hDC4) was performed as AWAT1 except there was no preincubation and the assay length was 20 min. In some AWAT assays [14C]cetyl alcohol was used to follow esterification. Kill reactions and vector controls were included in all experiments. To assess substrate specificities, the various wax alcohols and acyl-CoAs were substituted for cetyl alcohol or oleoyl-CoA at the concentrations specified above. Activity is presented as pmol/min/mg protein.

Glycerol acyltransferase activity was assayed according to the procedure of Lee et al. (42) using [14C]oleoyl-CoA and 5% glycerol. Monoacylglycerol acyltransferase (MGAT) was assayed as previously described (23) using [14C]oleoyl-CoA and 200 μM sn-2-monooacylglycerol. Mouse microsomes were used as a positive control in both assays.

**RESULTS**

The Human DGAT2 Gene Family, Conserved Motifs and Evolutionary Relationships—There are six paralogous genes to human DGAT2 whose chromosomal locations based on the human genome databases at NCBI, Ensembl, UCSC, and Celera are listed in Table I. In addition to DGAT2 and MGATs 1–3, we used the previously termed hDC3 and hDC4 (human DGAT candidate genes 3 and 4), and DGA2 (diacylglycerol acyltransferases) as acronyms for the uncharacterized family members, upon the assumption that these genes direct the synthesis of triglyceride. The predicted polypeptides of these six genes exhibit 27–34% and 46–51% sequence identity to the primordial yeast DAG1 and human DGAT2 proteins, respectively, with marked conservation toward the C terminus of the protein (Fig. 1A). All seven human sequences and the yeast Dga1p are predicted transmembrane proteins (Fig. 1A). An iterative algorithm (PSI-BLAST, Ref. 30), suggests these gene products to be derived from an ancestor with lysophosphatidic acid acyltransferase (LPAAT) or glycerol-3-phosphate acyltransferases (GPAT) activity. These enzymes and its relatives mediate the final steps in phosphatidic acid biosynthesis. This conservation includes those residues implicated by site-directed mutagenesis as forming the active sites of bacterial GPAT (40).

**TABLE I**

| Gene     | Chromosome | Amino acids | % Identity to DGAT2 |
|----------|------------|-------------|---------------------|
| hDGAT2   | 11q13.5    | 388         | 100                 |
| DGA2     | Xq13.1     | 329         | 51                  |
| hDC3     | Xp13.1     | 338         | 50                  |
| hDC4     | Xq13.1     | 334         | 48                  |
| MGAT1    | 2q36.3     | 335         | 50                  |
| MGAT2    | 11q13.5    | 335         | 46                  |
| MGAT3    | 7q22.1     | 342         | 49                  |

The MGAT Arm of the DGAT2 Gene Family—The MGAT branch of the DGAT2 gene family has been extensively characterized (21–26), primarily in murine tissues. We performed nested PCRs using cDNA from a variety of tissues to confirm and further discern the tissue expression of human MGATs 1–3 (Fig. 2A). Similar to mouse MGAT1, the human paralog is transcribed in stomach, uterus, kidney, adipose, and liver. However, unlike mice, human brain, lung, thymus, prostate, testes, colon, and notably, small intestine had significant expression. Two splice variants of human MGAT1 exist, and the larger of the two is the predominant transcript found in thymus and testes. Based on sequence analysis, the larger transcript results from the splicing out of the third exon of MGAT1 and an alternatively spliced-in portion of intron 4; this introduces an early stop codon at base pair 312, truncating the protein. The
FIG. 1. A, sequence alignments of the human DGAT2 family. Amino acid sequences of the DGAT2 family were aligned using the ClustalW multiple sequence alignment program. Dark and light shading indicate identity and similarity of residues, respectively. Conservation of putative active site residues based on mutagenesis of GPATs and LPAATs are indicated by an asterisk (*). Putative transmembrane domains are indicated by underline. B, dendrogram depicting the evolutionary relationship of the human DGAT2 gene family. A phylogenetic tree indicating the relatedness of DGAT2 family sequences was constructed using the ClustalW program. DGA2 (GenBank™ accession number AY947638), hDC3 (GenBank™ accession number AL357752), and hDC4 (GenBank™ accession number AY605053) appear to be derived from hDGAT2 and are more closely related to each other than to the MGAT subfamily. C, genomic structure spanning DGA2, hDC3, and hDC4 on the human X-chromosome. The representation is based on physical data from the NCBI, Ensembl, UCSC, and Celera genome datasets. Shaded boxes depict exons. The locus of three genes spans about 200 kbp with a significant density of predicted genes in the intergenic regions. Extended intergenic regions are indicated by the line breaks. ATG represents the likely translational starts, with the direction of transcription indicated by the arrows.
The functional significance of the human MGAT1, MGAT2, and MGAT3 splice variants remains to be determined. MGAT activity has been considered a reaction that predominates in the intestine but also in liver and fat, at least in rodents (44). The expression patterns of this branch of the human DGAT2 family are inconsistent with this concept. However, if the aforementioned conservation with the LPAAT enzymes is significant, then the majority of these splice variants would be anticipated to be inactive because of premature truncation. This may implicate these proteins as having alternate activities or regulatory roles in neutral lipid metabolism. For example, why is the larger MGAT1 message the sole transcript found in thymus and testes? Interestingly, in humans, all of the MGATs, including the full-length transcripts, are expressed in adipose tissue (Fig. 2C).

**DGAT2 and the Sex-linked Arm of the DGAT2 Gene Family**

Human and murine DGAT2 have been characterized extensively (9, 10). By contrast the X-linked hDC4-hDC3-DGA2 cluster (Xq13.1) of this gene family has not been investigated previously with regard to function or expression. We therefore focused our efforts on defining the role of this subfamily in neutral lipid biosynthesis. All three genes, DGA2 (amino acids 34–328), hDC3 (amino acids 38–337), and hDC4 (amino acids 38–333), possess DAGAT (diacylglycerol acyltransferase) domains that essentially are regions of amino acid sequence similarity common to the DGAT2 family. DGA2 is previously undescribed and comprises seven exons covering 6.1 kb on the direct strand, ∼190-kb downstream from hDC4 and 29-kb downstream from hDC3. It encodes a 329-amino acid protein with 51% amino acid identity to hDGAT2 and a predicted molecular mass of 37.9 kDa. The initial 44 amino acids of DGA2 are predicted to be a signal peptide. DGA2 has one potential N-glycosylation site and one potential protein kinase C phosphorylation site. Interestingly, DGA2 possesses a region with remote similarity to the IGF (insulin-like and insulin growth factor) domain, which belongs to a family of proteins that include insulin-related growth factors.

hDC4 (10), encodes a 334-amino acid protein with extensive similarity to the phosphate acyltransferase domains (PlsC, COG0204) of GPAT (amino acids 37–226), and LPAAT (amino acids 103–227). hDC4 has a predicted molecular mass of 38.2 kDa, and is 48% identical to hDGAT2 and 51% identical to DGA2 (Table I). The initial 35 amino acids of hDC4 are predicted to be a signal peptide and the remaining sequence predicts two transmembrane regions (amino acids 39–58 and 127–149) as well as one potential N-glycosylation site, one potential tyrosine sulfation site, and two potential protein kinase C phosphorylation sites. hDC4 covers 8.5 kb on the reverse strand of the X chromosome and comprises seven exons.

hDC3 (10) encodes a 338-amino acid protein also with a phosphate acyltransferase domain similar to the GPAT (amino acids 45–233) and LPAAT (amino acids 115–227) family of acyltransferases. hDC3 is 50% identical to hDGAT2, 51% identical to hDC4, and 51% identical to DGA2. It has a predicted molecular mass of 38.7 kDa and possesses one transmembrane region (amino acids 21–43) in addition to one potential N-glycosylation site and three potential protein kinase C phosphorylation sites. hDC4 is comprised of seven exons covering 28.22 kb on the direct strand of the X chromosome, ∼130-kb downstream from the DGA3 gene.

To examine the expression of DGA2, hDC3, and hDC4 in humans, nested PCR was performed using cDNA generated from a variety of tissues (Fig. 2B). DGA2 is expressed in all tissues except the spleen; interestingly, the strongest bands were found in thymus, prostate, and testes. hDC4 is expressed in all tissues surveyed except the placenta whereas hDC3 is

---

**Fig. 2. Tissue expression of the human DGAT2 gene family.**

A. Analysis of the DGAT subfamily tissue expression was performed via standard (MGAT3) or nested PCR (MGAT 1 and 2) using human cDNA obtained as part of a Quick Screen cDNA panel of human tissues obtained from Clontech and primers specific for each gene (Supplementary Table I). Splice variants for MGAT1 were gel-isolated, purified, and sequenced. B. Analysis of human DGA2, hDC3, and hDC4 tissue expression was performed via nested PCR using the same cDNA tissue panel and method as above. The splice variant of hDC4 was isolated, purified, and sequenced. C. Nested RT-PCR was performed on mRNA using primers specific for each gene (Supplementary Table I) to determine expression of the DGAT2 gene family in adipose tissue. Transcripts consistent with splice site variants of hDC4, MGAT1 (as in other tissues), and MGAT2 were also observed in this tissue.

A smaller transcript is a product of exon 3 being spliced out, also predicting a truncated protein due to an early stop at base pair 294.

Human MGAT2 transcripts were identified in liver, prostate, small intestine, colon, and adipose (Fig. 2, A and C), confirming previous findings (22, 26). In contrast to mMGAT2 (24, 25) and a previous report on hMGAT2 (22), no expression was found in kidney tissue. Consistent with previous reports (22, 26), splice variants were also exhibited by MGAT2, especially in adipose tissue.

Extensive searching of the available murine databases indicates that the MGAT3 gene is not present in mice. It has been previously reported that hMGAT3 expression is restricted to the gastrointestinal tract and the liver (23). In our studies (Fig. 2A) the full-length hMGAT3 is found in liver, small intestine, and colon. However, splice variants of hMGAT3 are ubiquitously found in many human tissues. The larger of these splice variants results from the splicing out of exon 5, leading to a truncated protein because of the creation of an early stop codon at base pair 774.
X-linked Acyltransferases and Neutral Lipid Biosynthesis

Microsomes from TG-null yeast strains transformed with human DGA2, DGA2, hDC3, or hDC4 were incubated in the presence of dioleoylglycerol or cetyl alcohol and [14C]oleoyl-CoA and assayed for esterification as described in “Experimental Procedures.” Data are expressed as pmol of neutral lipid (TAG or Wax ester) formed per min/mg protein as a mean ± S.D. DGAT activities are from three replicate experiments, and AWAT activities are from a representative experiment performed in triplicate. Background levels to the assay from strains transformed with vector control have been subtracted. DGAT activity values for all strains and AWAT activity from DGA2 and hDC4 were significantly elevated relative to the vector control (not shown).

Table II

| Nomenclature | DGAT activity | AWAT activity |
|--------------|---------------|---------------|
| DGA2         | 65.4 ± 3.4    | -11.5 ± 1.3   |
| DGA2         | 7.8 ± 3.2     | 31.7 ± 17.4   |
| hDC3         | 13.3 ± 4.6    | -25.7 ± 9.3   |
| hDC4         | 11.4 ± 3.4    | 1066 ± 18.6   |

*In vitro acyltransferase assays*

Expressed in all tissues except pancreas. A splice variant of hDC4 results from the excision of the 175-base pair exon 5. This creates an early stop codon at base pair 483 in the resultant transcript. The physiological function of this splice variant remains to be determined. All three genes are expressed in human adipose tissue (Fig. 2C).

Expression of DGAT2 in Yeast Cells Deficient in TG Synthesis—To examine the expression and biochemical activity of DGAT2, we expressed its cDNA in a diacylglycerol esterification-deficient yeast strain (SCY2056, (17)) in which the endogenous DGA1, LRO1, and ARE2 genes were deleted. Metabolic labeling experiments performed with [3H]/[14C]oleate and [3H]palmitate are consistent with a major role of DGAT2 in triglyceride synthesis (Fig. 4, A and B) and to a lesser but significant (p < 0.05) extent, diacylglycerol synthesis (not shown) (21). It has been previously shown that yeast deficient in triglyceride and sterol ester display minimal cytoplasmic lipid droplets when stained with the vital stain Nile Red and followed by fluorescence microscopy (17). Upon expression of human DGAT2, these strains exhibit a marked accumulation of cytoplasmic neutral lipid droplets (not shown). In addition, microsomes from null TAG background strains of yeast, transformed with an expression vector harboring no insert or the cDNA insert for DGAT2 were assayed in vitro for the incorporation of [14C]oleate and DAG into triglyceride. As shown in Table II, in an in vitro microsomal assay, DGAT2 forms triglyceride at a rate of 65.4 pmol/min/mg protein.

Expression of the Sex-linked Arm of the DGAT2 Gene Family in Yeast—The previous experiments with human DGAT2 demonstrate the utility of this heterologous system in the analysis of mammalian triglyceride synthesis as was demonstrated previously for sterol esterification by members of the ACAT gene family (35, 45). We thus performed similar investigations expressing the cDNA of DGA2, hDC3, and hDC4 in the null TAG background strains of yeast. Northern blot hybridization analysis confirmed the high level expression of DGA2, hDC3, and hDC4 transcripts of the predicted size (Fig. 3).

During pulse and steady state [3H]/[14C]oleate and [3H]palmitate metabolic labeling, DGA2, hDC3, and hDC4 exhibit significant triglyceride production, compared with vector-transformed control (VC) strains (Fig. 4, A and B). Notably, hDC3 demonstrated a marked incorporation of [3H]oleate into TAG. Microsomes from null TAG background strains of yeast, transformed with an expression vector containing DGA2, hDC3, and hDC4 were assayed in vitro for the incorporation of [14C]oleate and DAG into triglyceride. In each case significant synthesis of TAG was detected (Table II).

Substrate Specificity of Sex-linked Arm of the DGAT2 Gene Family—Although DGA2, hDC3, and hDC4 were capable of synthesizing TG in transformed yeast (Fig. 4, A and B) and demonstrate in vitro DGAT activity (Table II), the low specific activity, particularly compared with DGAT2, suggests that DAG is not their primary substrate. Under the conditions used here we demonstrated a significant accumulation of diacylglycerol in strains expressing DGAT2, consistent with the known MGAT activity of this enzyme. However, there was no detectable production of diacylglycerol, phospholipid or cholesteryl ester by DGA2, hDC3, or hDC4 above background strains (not shown). Similarly in vitro assays of microsomes (23, 42) prepared from these strains, did not indicate any increase in esterification of MAG or glycerol above vector control (not shown). This prompted us to search for other alcohols as substrates. Recently a novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase that shares no detectable similarity with the DGAT2 gene family was discovered in Actinobacter calcoaceticus (46). This, in combination with the fact that a mammalian AWAT sequence was unknown at the time of this study, led us to assay DGA2, hDC3 and hDC4 strains for this activity. Initial experiments were performed using cetyl alcohol (C16) and oleoyl-CoA as substrates in reaction conditions identical to those used for DGAT assays.

Both DGA2 (31.7 pmol/min/mg) and hDC4 (1066 pmol/min/mg) but not DGAT2 or hDC3 demonstrated significant AWAT activity using [14C]oleoyl-CoA as a monitor for wax ester formation (Table II). Wax esters co-migrate with sterol esters on thin layer chromatography. Although it is unlikely that the activity observed is due to sterol ester formation (yeast sterols are not preferred substrates for mammalian enzymes, Refs. 35, 47), a similar in vitro assay was performed using [14C]cetyl alcohol rather than radiolabeled oleoyl-CoA. Again, significant AWAT activity was observed with DGA2 (113 pmol/min/mg) and hDC4 (1232 pmol/min/mg) demonstrating that wax ester, not sterol ester, is the enzymatic product and leading us to rename these proteins AWAT1 and AWAT2, respectively.

We then performed in vitro AWAT assays of yeast cells expressing AWAT1 and AWAT2. AWAT1 and AWAT2 significantly (p < 0.05) produced wax ester above background strains during [14C]hexadecanol pulse metabolic labeling (Fig. 4C).

The substrate specificities of AWAT1 and AWAT2 were then investigated in detail in assays that were optimized so that the reaction velocity was proportional to the amount of microsomal protein. As shown (Table III, A) the alcohol substrate specificities of the two enzymes are strikingly dissimilar. Using oleoyl-CoA as the acyl donor, AWAT1 (DGA2) has a definite preference for decyl alcohol (C10), with less activity using C16 and C18 unsaturated alcohols. AWAT1 utilizes arachidyl alcohol about 20% as well as decyl alcohol, demonstrating its relatively poor activity using saturated long chain alcohols (C16, C18,
and C20). In contrast, AWAT2 exhibited no activity using decyl alcohol and significantly preferred the C16 and C18 alcohols. AWAT1 and AWAT2 also showed a difference in acyl-CoA preference (Table 3, B). Using cetyl alcohol as the acyl acceptor, AWAT1 shows a strong preference toward the saturated acyl group; it uses oleoyl-CoA (C18:1) only 40% as well as stearoyl-CoA (C18:0). In contrast, AWAT2 demonstrates significant activity using all four acyl-CoAs and utilizes unsaturated acyl-CoAs twice as well as saturated acyl-CoAs under the conditions employed.
**Expression of AWAT1, AWAT2, and hDC3 in Human Skin**—In addition to several other neutral lipids such as TAG, wax esters are major components of sebum, a production of the sebaceous gland. We therefore tested human skin cDNA preparations for the expression of these genes and discovered that transcripts from all seven members of the DGAT2 family were detectable, with particularly high levels of expression of the X-linked subfamily (Fig. 5A). To identify the cell types involved we performed in situ hybridizations of human skin sections with sense and antisense probes for AWAT1, AWAT2, and hDC3 (Fig. 5B). Control sense probes did not result in section staining. Expression of AWAT1 and AWAT2 was clearly limited to the sebaceous gland, with AWAT2 primarily restricted to the cytoplasm of undifferentiated peripheral sebocytes. AWAT1 was expressed in more mature, centrally located cells just before their rupture and sebum release. hDC3 transcripts were also localized to the cells of the sebaceous gland, however the predominant staining corresponded to nuclei, raising the possibility that the gene is transcribed but not translated in sebocytes.

**DISCUSSION**

Neutral lipids such as TAG and SE are commonly sequestered in the core of cytoplasmic lipid droplets until such time when hydrolysis allows their return to the metabolic fray. This efficient process of energy storage and release is widely used in nature to facilitate such diverse processes as hibernation in animals, embryo development in eggs and plant seeds, and perhaps sporulation in yeast. Neutral lipid biosynthesis therefore represents an advantageous response to overabundance and thus potential toxicity of membrane lipids and energy. Given this pivotal role in cellular metabolism, it is perhaps not surprising to discover so many distinct pathways (PDAT-, DGAT1- and DGAT2-mediated, to date) and genes for the production of TG. The complexity of the large gene family associated with the DGAT2 reaction is perhaps more surprising and likely reflects alternate substrate specificities. For example, although MGATs 1–3 appear to be genealogically closely related to DGAT2, they differ markedly in their substrate specificity and expression patterns. Similarly, we demonstrate here that the previously uncharacterized X-linked members of this family have only modest DGAT activity in vivo and in vitro. Instead, we demonstrate that two members of this subdivision of the DGAT2 gene family are responsible for the generation of acyl esters of long chain alcohols (fatty alcohols) in the synthesis of wax esters. Interestingly, plant orthologs of DGAT2 have been reported to mediate the synthesis of both TG and wax esters (49). Recently, the murine ortholog of AWAT2 has been demonstrated to also perform this reaction (50). Based on sequence conservation, the three X-linked members of this 7-component family are closer to DGAT2 than to the MGAT subfamily (Fig. 1B). Thus it is possible that the final member of this gene family, hDC3 is a bona fide DGAT enzyme. The enzyme was expressed in yeast and clearly conferred the ability to synthesize significant levels of triacylglycerol, particularly with oleate as a substrate. Alternatively, hDC3 may be an orphan waiting for an activity and substrate to be ascribed. Interestingly, the hDC3 mRNA appears to remain within the nucleus in in situ hybridizations of skin sections.

The chromosomal location of these genes may implicate them as candidates for several human syndromes ranging from obesity to skin disorders. For example, the hDC3, hDC4, and DGA2 genes comprise a cluster of 3 physically linked genes...
localizing to the X chromosome, in a contig of ~200 kb, in mice and humans (Fig. 1C). Interestingly there are several undefined sex-linked obesity (e.g. Borjeson-Forssman-Lehmann and Wilson-Turner syndromes, see Ref. 51 for review) and dermatological syndromes (52). Similarly, it is interesting to note that chromosome 11 (specifically 11q13) containing DGAT2, MGAT2, uncoupling protein UCP2, and UCP3 (53, 54), and BBS1, the gene most commonly involved in Bardet-Biedl syndrome (55), has been linked to obesity and hyperinsulinemia. Similarly, one genome scan also implicated the same region of 11q13 in childhood and adolescent obesity (56). Moreover, several linkage studies suggest that q22.1, a region flanking the leptin gene but also MGAT3, is closely linked to obesity and body mass index in humans (57, 58). Variation at these loci is clearly worthy of investigation as causative agents of several disease syndromes. In addition to their roles as safe harbingers for toxic fatty acids or alternative form of energy storage, molecules such as wax esters and TAG function as a hydrophobic permeability barrier to limit dehydration from tissue surfaces, molecules that are seen as usual.”

The sebaceous maturation cycle of wax ester hydrolysis and reesterification occurs, such that the wax esters are remodeled prior to their secretion. The relevance of this lack of clarity is not clear, however it may serve to homogenize the sebum in terms of fatty acid and alcohol saturation or chain length such that it is optimally hydrophobic and effective as a permeability barrier.

Acknowledgments—We thank Andrey Panteleev, Loan Phan, and Ying Liu for assistance and insight.

REFERENCES

1. Oelkers, P. M., and Sturley, S. L. (2004) in Lipid Metabolism and Membrane Biogenesis (Daum, G., ed) Vol. 6, pp. 281–311, Springer-Verlag, Berlin

2. Yu, Y. H., and Ginsberg, H. N. (2004) Ann. Med. 36, 252–261

3. Coleman, R. A., Lewin, T. M., and Muoio, D. M. (2000) Annu. Rev. Nutr. 20, 77–103

4. Subauste, A., and Burant, C. F. (2003) Curr. Drug Targets Immune Endocr. Metabol. Disord. 3, 263–275

5. Ross, R. (1995) in Molecular Cardiovascular Medicine (Haber, E., ed), pp. 11–30, Scientific American, New York

6. Krauss, R. M. (1998) Am. J. Med. 105, 585–626

7. Mulhall, B. P., Ong, Y. J., and Younes, Z. M. (2002) J. Gastroenterol. Hepatol. 17, 1136–1143

8. Cases, S., Smith, S. J., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C. W., Panas, E. A., Stahl, U., Lenman, M., and Farese, R. V., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13018–13023

9. Lardizabal, K. D., Mai, T. J., Wagner, N. W., Wyrick, A., Voelker, K., and Farese, R. V., Jr. (2001) J. Biol. Chem. 276, 38862–38869

10. Cases, S., Stone, S. J., Zhou, P., Yen, E., Tow, B., Lardizabal, K. D., Voelker, K., and Farese, R. V., Jr. (2000) Nat. Genet. 25, 87–90

11. Chen, H. C., Smith, S. T., Tow, B., Elias, P., and Farese, R. J. (2002) J. Clin. Investig. 109, 175–181

12. Chen, H., Smith, S., Ladha, Z., Jensen, D. F., Murphy, L., Gigueire, J., Pitas, R., Eckel, R., and Farese, R. J. (2002) J. Clin. Investig. 109, 1049–1055

13. Chen, H., Ladha, Z., and Farese, R. J. (2002) J. Endocriol. 143, 2893–2898

14. Stone, S. J., Myers, H. M., Watkins, S. M., Brown, E. F., Feingold, K. R., Elias, P. M., and Farese, R. V., Jr. (2004) J. Biol. Chem. 279, 11767–11776

15. Sandager, L., Dahlqvist, A., Panas, E. A., Stahl, U., Lenman, M., Gustavsson, M., and Stymne, S. (2000) Biochim. Biophys. Acta. 1476, 28, 700–702

16. Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J. T., and Sturley, S. L. (2002) J. Biol. Chem. 277, 8877–8881

17. Sorger, D., and Daum, G. (2004) J. Biol. Chem. 279, 15419–15424

18. McPherson, P., Tinkelenberg, A., and Sturley, S. L. (2000) J. Biol. Chem. 275, 15609–15612

19. Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., and Stymne, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6487–6492

20. Zinser, E., and Daum, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 13613–13616

21. Cao, J., Burn, P., and Shi, Y. (2003) J. Biol. Chem. 278, 25657–25663

22. Cao, J., Lockwood, J., Burn, P., and Shi, Y. (2003) J. Biol. Chem. 278, 25686–25690

23. Lockwood, J., Cao, J., Burn, P., and Shi, Y. (2003) J. Am. Physiol. Endocrinol. Metab. 285, E927–937

24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1998) Current Protocols in Molecular Biology, John Wiley & Sons, New York

25. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1987) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

26. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410

27. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, Z., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402

28. Mark, C. (1988) Nucleic Acids Res. 16, 1829–1836

29. Thomson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680

30. Bairoch A., Bucher P., and H. K. (1997) Nucleic Acids Res. 25, 217–221

31. Leutenig, A., Copley, R. R., Schmidt, S., Ciccimarelli, F. D., Doerke, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) Nucleic Acids Res. 32, D142–D144

32. Zinser, E., and Daum, G. (1995) Yeast 11, 493–506

33. Lowe, R. H., Rosebrugh, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

34. Cao, J., Burn, P., and Shi, Y. (2003) J. Biol. Chem. 278, 25567–25560

35. To, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168

36. Bairoch A., Bucher P., and H. K. (1997) Nucleic Acids Res. 25, 217–221

37. Leutenig, A., Copley, R. R., Schmidt, S., Ciccimarelli, F. D., Doerke, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) Nucleic Acids Res. 32, D142–D144

38. Zinser, E., and Daum, G. (1995) Yeast 11, 493–506

39. Lowe, R. H., Rosebrugh, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

40. Rustan, A. C., Nossen, J. O., Christiansen, E. N., and Dreven, C. A. (1988) J. Lipid Res. 29, 1417–1426

41. Lee, D. P., Deonnarre, A. S., Kienzle, M., Zhu, Q., Skrzympek, M., Chan, M.,
