Membrane Topology and Identification of Key Functional Amino Acid Residues of Murine Acyl-CoA:Diacylglycerol Acyltransferase-2*

Scott J. Stone†§, Malin C. Levin†§, and Robert V. Farese, Jr.‡§¶

From the §Gladstone Institute of Cardiovascular Disease, San Francisco, California 94158 and the ¶Cardiovascular Research Institute, the †Departments of Medicine and of Biochemistry & Biophysics, and the ¶Diabetes Center, University of California, San Francisco, California 94143

Triacylglycerols are the predominant molecules of energy storage in eukaryotes. However, excessive accumulation of triacylglycerols in adipose tissue leads to obesity and, in nonadipose tissues, is associated with tissue dysfunction. Hence, it is of great importance to have a better understanding of the molecular mechanisms of triacylglycerol synthesis. The final step in triacylglycerol synthesis is catalyzed by the acyl-CoA:diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2. Although recent studies have shed light on metabolic functions of these enzymes, little is known about the molecular aspects of their structures or functions. Here we report the topology for murine DGAT2 and the identification of key amino acids that likely contribute to enzymatic function. Our data indicate that DGAT2 is an integral membrane protein with both the N and C termini oriented toward the cytosol. A long hydrophobic region spanning amino acids 66–115 likely comprises two transmembrane domains or, alternatively, a single domain that is embedded in the membrane bilayer. The bulk of the protein lies distal to the transmembrane domains. This region shares the highest degree of homology with other enzymes of the DGAT2 family and contains a sequence HPHG that is conserved in all family members. Mutagenesis of this sequence in DGAT2 demonstrated that it is required for full enzymatic function. Additionally, a neutral lipid-binding domain that is located in the putative first transmembrane domain was also required for full enzymatic function. Our findings provide the first insights into the topography and molecular aspects of DGAT2 and related enzymes.

Triacylglycerols are the predominant molecules of energy storage in eukaryotic cells and organisms. However, excessive accumulation of triacylglycerols in adipose tissue leads to obesity and, in nonadipose tissues, such as skeletal muscle, liver, heart, and pancreatic β cells, is associated with tissue dysfunction (1–3). With the current epidemics of human obesity and type 2 diabetes, it is of great importance to have a better understanding of the molecular mechanisms of triacylglycerol synthesis. This knowledge may aid in developing strategies for preventing or reversing obesity and related metabolic disorders.

The final step in triacylglycerol synthesis, the esterification of sn-1,2-diacylglycerol with a long-chain fatty acyl-CoA, is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT)2 (4–6). We identified two different mammalian DGAT enzymes, DGAT1 and DGAT2 (7, 8), and demonstrated that both are membrane-associated proteins that catalyze the biochemical reaction that produces triacylglycerol. They have similarly broad fatty acyl-CoA substrate specificities in in vitro assays (8), and both are ubiquitously expressed in tissues, with the highest levels of expression in tissues that play prominent roles in triacylglycerol metabolism, such as white adipose tissue, liver, mammary gland, and small intestine (7, 8).

Although the enzymes share similar biochemical functions and tissue expression patterns, DGAT1 and DGAT2 also have striking differences. The genes encoding the two enzymes belong to different gene families that do not share sequence homology (7, 8). DGAT1 is a member of a family of enzymes that includes the acyl-CoA:cholesterol acyltransferase enzymes, ACAT1 and ACAT2, which catalyze cholesterol ester biosynthesis (7). In contrast, DGAT2 family members include acyl-CoA:monoacylglycerol acyltransferase enzymes (9–14) and wax synthases (15, 16), including one that is a multifunctional acyltransferase (17).

The two enzymes also have different biochemical properties. In overexpression studies in intact cells, DGAT2 was a more potent enzyme than DGAT1, yielding a much larger increase in intracellular triacylglycerol, which accumulated in large, centrally located cytosolic droplets (3). Moreover, DGAT1, but not DGAT2, is a multifunctional acyltransferase, catalyzing the synthesis of diacylglycerol, retinyl esters, and waxes in addition to triacylglycerol, in in vitro assays (34).

The enzymes have different physiological functions, as demonstrated by the strikingly different phenotypes after targeted gene disruptions. Mice lacking DGAT1 (Dgat1−/− mice) are viable, have modest reductions in tissue triacylglycerols, are

---

* This work was supported by an American Heart Association Scientist Development grant (to S. J. S.), a Hillblom postdoctoral fellowship (to M. C. L.), National Institutes of Health Grant 5R01-DK065599 (to R. V. F.), and the J. David Gladstone Institutes. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: The Gladstone Institute of Cardiovascular Disease, 1650 Owens St., San Francisco, CA 94158. Tel.: 415-734-2000; Fax: 415-355-0960; E-mail: sstone@gladstone.ucsf.edu.

‡ The abbreviations used are: DGAT, acyl-CoA:diacylglycerol acyltransferase; PBS, phosphate-buffered saline; ACAT, acyl-CoA:cholesterol acyltransferase; ER, endoplasmic reticulum; WT, wild type; PDI, protein disulfide isomerase.

Received for publication, August 21, 2006, and in revised form, October 2, 2006. Published, JBC Papers in Press, October 10, 2006, DOI 10.1074/jbc.M607986200

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 52, pp. 40273–40282, December 29, 2006
© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Murine DGAT2: Membrane Topology and Functional Amino Acids

TABLE 1
Primers used for mutagenesis of DGAT2 (modifications are underlined)

| Forward | Reverse |
|---------|---------|
| Myc90-DGAT2 | 5'-GAAGAAAACCTTATTCTGAAAGATAGCTGCTCAAGTACAC | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| Myc143-DGAT2 | 5'-GAAGAAAACCTTATTCTGAAAGATAGCTGCTCAAGTACAC | 5'-CTATATCTTTGGATACCACGGACATGGCATCATGGGCCTG-3 |
| Myc203-DGAT2 | 5'-GAAGAAAACCTTATTCTGAAAGATAGCTGCTCAAGTACAC | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| H161A | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| P162G | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| H163A | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| AGA | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| L83A | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| L81A | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |
| L83A | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 | 5'-CTATATCTTTGGATACGCCCCCCATGGCATCATG-3 |

resistant to diet-induced obesity, and are more sensitive to insulin and leptin (18–20). In contrast, mice lacking DGAT2 (Dgat2−/− mice) have severe (~95%) reductions in whole body triacylglycerols and die shortly after birth (3). DGAT1 cannot compensate for the absence of DGAT2, highlighting the functional differences between the enzymes.

Despite the advances in knowledge of these key lipid-synthesis enzymes, virtually nothing is known about their molecular structures and functions. Analysis of their respective amino acid sequences suggests that DGAT1 and DGAT2 are likely to be structurally dissimilar. DGAT1, which is ~500 amino acids in length, is predicted to be very hydrophobic, with 6–12 possible transmembrane domains (7). In contrast, DGAT2 is smaller (<400 amino acids), less hydrophobic, and is predicted to have fewer transmembrane domains (8).

In this study, we investigated topological and structural aspects of DGAT2. Specifically, we used both protease protection assays and immunofluorescence microscopy to determine the membrane topology of murine DGAT2. We also used directed mutagenesis to examine the functional importance of two highly conserved regions of the DGAT2 protein: a 4-amino acid sequence (HPHG) that is conserved in all members of the DGAT2 gene family and a putative neutral lipid-binding acid sequence (HPHG) that is conserved in all members of the DGAT2 protein family.

MATERIALS AND METHODS

Cell Culture and Transfection—COS-7 cells from the American Type Tissue Culture Collection were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a 37 °C incubator with 5% CO2. For transfections, 3 µg of plasmid DNA was incubated with 9 µl of FuGENE 6 transfection reagent (Roche Diagnostics) in 200 µl of Dulbecco's modified Eagle's medium for 30 min at room temperature. The transfection mixture was then added to a 100-mm culture dish containing 9 ml of medium and cells at ~75% confluence. After 24 h, transfected cells were harvested and used for experiments.

Construction of DGAT2 Mutant Plasmids—N-terminal FLAG-tagged murine DGAT2 (NFLAG-DGAT2) in the eukaryotic expression vector pCDNA3.1 was used as a template for all mutagenesis reactions. The various mutations were generated with the primer pairs listed in Table 1 and the QuikChange II site-directed mutagenesis kit (Stratagene), following the manufacturer's directions. All plasmids were sequenced to confirm the presence of the desired mutations.

Membrane Preparation—Twenty-four hours after transfection, COS-7 cells expressing the various DGAT2 constructs were washed twice with ice-cold PBS, harvested by scraping, and collected by centrifugation (1000 × g). Cells were resuspended in 200 µl of PBS and disrupted by 15 passages through a 27-gauge needle. Cell debris and nuclei were pelleted by centrifugation at 600 × g for 5 min, and the supernatant was centrifuged at 100,000 × g for 30 min at 4 °C. The membrane pellet was resuspended in 200 µl of PBS.

Membrane Protein Extraction—Samples of total membranes (100 µg of protein) were adjusted to a volume of 100 µl with
PBS and were incubated with an equal volume of PBS alone or PBS containing 2% SDS, 2% Triton X-100, 2 M hydroxylamine (pH 10), or 0.2 M sodium carbonate (pH 11.5). Samples were placed on a rotator at room temperature for 20 min and centrifuged at 100,000 × g for 30 min at 4 °C. Pellets were resuspended in 200 μl of PBS. SDS loading buffer (6×) was added to a 50-μl aliquot of the pellet and supernatant fractions. Samples were boiled for 5 min, separated by SDS-PAGE, and analyzed by immunoblotting.

**TABLE 2**

| Analysis software | Number of TMD* | Location of TMD |
|-------------------|----------------|-----------------|
| TMHMM             | 1              | 73–95           |
| SOSUI             | 2              | 66–88, 93–115   |
| MEMSAT3           | 2              | 70–93, 100–116  |
| TMpred            | 2              | 68–95, 229–244  |
| TMpred            | 2              | 85–105, 233–257 |
| TopPred           | 2              | 76–96, 230–250  |
| HMMTOP            | 1              | 75–99           |

* TMD, transmembrane domain.

**Protease Protection Assays**—Aliquots of total membranes (50 μg of protein) in PBS were incubated with 0.8 mg/ml proteinase K (Roche Applied Science) or 20 μg/ml trypsin (Sigma) with or without 1% Triton X-100 (total reaction volume = 60 μl) at room temperature for 30 min. Proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride (5 mM final concentration) for proteinase K and soybean trypsin inhibitor (4 μg/μl final concentration, Sigma) for trypsin. SDS loading buffer (6×) was added to the samples, which were then boiled for 5 min, separated by SDS-PAGE, and analyzed by immunoblotting.

**DGAT Activity Assays**—Transfected COS-7 cells were washed twice with ice-cold PBS, harvested by scraping, and collected by centrifugation (1000 × g). To lyse cells, the pellet was resuspended in 200 μl of 50 mM Tris–HCl (pH 7.4) and 250 mM sucrose and passed through a 27-gauge needle 15 times.

Cell debris and nuclei were pelleted by centrifugation at 600 × g for 5 min. The supernatant (10–50 μg of protein) was used for in vitro DGAT activity assays, performed as described (3).

**Immunoblot Analyses**—Samples were separated by SDS-PAGE on 10% or 12% polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Bio-Rad), and incubated with antibodies at the following dilutions: mouse anti-FLAG-M2 (Sigma), 1:2000; rabbit anti-FLAG (Sigma), 1:2000; anti-DGAT2, 1:1000; anti-PDI (Stressgen), 1:1000; anti-c-Myc (9E10, Calbiochem), 1:1000; anti-glycerolaldehyde-3-phosphate dehydrogenase (Covance), 1:2000; mouse anti-IgG (Amersham Biosciences), 1:2000; and anti-rabbit IgG (Bio-Rad), 1:2000. The anti-DGAT2 antibody is a rabbit antiserum that recognizes the C terminus of DGAT2 (amino acids 374–388). Protein-antibody complexes were visualized using the Supersignal West Pico kit (Pierce). Membranes were exposed to Fuji RX film. Bands were quantified by scanning densitometry using ImageJ image analysis software (rsb.info.nih.gov/ij/).

**Immunofluorescence Microscopy**—COS-7 cells were transiently transfected with either NFLAG-DGAT2 or the same construct bearing a Myc tag inserted between amino acids 90–91 (Myc90-DGAT2), amino acids 143–144 (Myc143-DGAT2), or amino acids 203–204 (Myc203-DGAT2). Twenty-four hours after transfection, cells were re-plated into 12-well dishes on glass coverslips and allowed to adhere over-

**FIGURE 3.** The N and C termini of DGAT2 have a cytosolic orientation as determined by protease protection analyses and indirect immunofluorescence microscopy. A, protease K protection analysis. Total membranes from COS-7 cells expressing NFLAG-DGAT2 were prepared as described under Materials and Methods. Aliquots of membranes (50 μg of protein) were incubated with or without 0.8 mg/ml proteinase K, in the presence or absence of 1% Triton X-100. Samples were analyzed by immunoblotting with antibodies that recognize the C terminus of DGAT2 and the FLAG epitope. The integrity of membrane vesicles was assessed by immunoblotting with an anti-PDI antibody. B, trypsin protection analysis. Total membranes were analyzed as in A except that 20 μg/ml trypsin (Sigma) was used. The integrity of membranes was assessed by immunoblotting with an anti-HSP70 antibody. C, immunofluorescence microscopy. COS-7 cells transiently expressing NFLAG-DGAT2 were fixed with methanol:acetone (1:1), which completely permeabilized all cellular membranes, or fixed with 4% paraformaldehyde and then treated with 3 μg/ml digitonin to selectively permeabilize the plasma membrane. Cells were then incubated with anti-FLAG and anti-DGAT2 antibodies and appropriate secondary antibodies to visualize the FLAG epitope of DGAT2 (red) and C terminus of DGAT2 (green) (left panel of four images) or anti-FLAG and anti-PDI antibodies to visualize DGAT2 (red) and PDI (green) (right panel of images) by fluorescence microscopy. Cells chosen for image comparison had similar levels of expression, as assessed by FLAG signal intensity.

**Murine DGAT2: Membrane Topology and Functional Amino Acids**
Murine DGAT2: Membrane Topology and Functional Amino Acids

night. Cells were then washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed three times with PBS, and plasma membranes were selectively permeabilized with 3 μg/ml digitonin in 0.3 M sucrose, 5 mM MgCl₂, 120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, and 25 mM Hepes/KOH (pH 7.6) for 10 min on ice. Alternatively, cells were fixed, and total cellular membranes were permeabilized with methanol/acetone (1:1) for 2 min at room temperature. After permeabilization, cells were washed three times with PBS and incubated with 3% bovine serum albumin in PBS for 5 min to block nonspecific antibody binding. Cells were then incubated at room temperature for 1 h with either mouse anti-FLAG-M2 (1:500 dilution) and rabbit anti-PDI (1:200 dilution) or rabbit anti-DGAT2 (1:200 dilution), mouse anti-c-Myc and rabbit anti-PDI. Cells were washed three times with PBS and incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1:200 dilution) and donkey anti-mouse 594 (Molecular Probes, 1:200 dilution) secondary antibodies for 30 min at room temperature. Cells were washed three times with PBS, and the coverslips were mounted on glass slides with a drop of Immuno-Fluore mounting medium (ICN). Images were acquired using the appropriate filter sets with an E-600 microscope (Nikon). For each signal, images were captured at the same wavelength, light intensity, and acquisition settings. Images shown are representative of results for the majority of transfected cells in each experiment.

RESULTS

DGAT2 Is an Integral Membrane Protein—We showed previously that DGAT2 expressed in insect cells is found in cell membranes (8). However, whether DGAT2 was a peripheral or integral membrane protein was unclear. To address this, membrane vesicles from COS-7 cells expressing NFLAG-DGAT2 were isolated and treated with chemicals that extract or solubilize proteins from membranes (Fig. 1). In samples incubated with PBS alone, DGAT2 was found exclusively in the pellet (membrane) fraction (Fig. 1, lane 2). In the presence of detergents (1% SDS or 1% Triton X-100), DGAT2 was completely solubilized (Fig. 1, lanes 3 and 5). In contrast, in the presence of 1 M hydroxyamine or 0.1 M sodium carbonate, which solubilize peripheral membrane proteins, DGAT2 remained in the pellet fraction (Fig. 1, lanes 8 and 10), demonstrating that it is an integral membrane protein. As a control for vesicle integrity, the luminal membrane protein, protein disulfide isomerase (PDI), was found in the pellet fraction of PBS-treated membranes (Fig. 1, lane 2). In contrast, when membranes were solubilized with SDS or Triton X-100 (Fig. 1, lanes 3 and 5), or disrupted with hydroxyamine and sodium carbonate (Fig. 1, lanes 7 and 9), PDI was present in the supernatant fraction.

Hydropathy Plot and Predicted Membrane Topology of DGAT2—A hydropathy plot of murine DGAT2 (Fig. 2) was generated by the method of Kyte and Doolittle (21) using a window parameter of 19 amino acids. This plot suggested that DGAT2 has two or three possible transmembrane regions. Further analysis of the amino acid sequence of DGAT2 by algorithms used to predict membrane topology also suggested that DGAT2 has two or three transmembrane domains (Table 2). Five of the seven algorithms indicated that a transmembrane domain was located near the N terminus in the region of amino acids 66–96. Three of the algorithms predicted a domain between amino acids 85 and 116, and three predicted a domain between amino acids 229 and 257.

The N and C Termini of DGAT2 Are Exposed to the Cytosol—To determine the orientation of the N and C termini of DGAT2, we performed protease protection assays. Membrane vesicles from COS-7 cells expressing DGAT2 with an N-terminal FLAG tag (NFLAG-DGAT2) were incubated with proteinase K and then immunoblotted with anti-FLAG, which recognizes the N terminus of the expressed protein, or with an anti-DGAT2 antibody, which recognizes the C terminus of DGAT2 (amino acids 374–388). In membrane samples not treated with either detergent or proteinase K, DGAT2 was detected by both anti-FLAG and anti-DGAT2 (Fig. 3A, lane 1). These epitopes were also present after detergent extraction of the protein (Fig. 3A, lane 4). In contrast, proteinase K treatment of membrane vesicles destroyed both the FLAG and DGAT2 epitopes, with or without detergent (Fig. 3A, lanes 2 and 3), suggesting that the N and C termini are both exposed to the cytosol. To verify the integ-

FIGURE 4. Expression of Myc-tagged DGAT2 constructs in COS-7 cells. A, cDNA expression constructs. Each DGAT2 construct contains an N-terminal FLAG epitope tag (blue circle). Myc tags (green diamonds) were inserted between amino acids 90–91 (Myc90-DGAT2), amino acids 143–144 (Myc143-DGAT2), and amino acids 203–204 (Myc203-DGAT2) by mutagenesis. Black boxes represent possible transmembrane domains. The red box indicates the epitope recognized by the anti-DGAT2 antibody. B, immunoblot demonstrating expression of epitope-tagged DGAT2 constructs in transiently transfected COS-7 cells. Cellular lysates (20 μg of protein) were immunoblotted with anti-DGAT2, anti-FLAG, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. C, DGAT activity in lysates from cells expressing NFLAG-DGAT2 or mutant proteins. Lysates from cells were assayed for in vitro DGAT activity as described under "Materials and Methods." The activities for untransfected and cells transfected with wild-type (WT) DGAT2 were 84 and 153 pmol/mg of protein/min, respectively. Data are mean ± S.D. for triplicate analyses from one experiment, which was repeated twice with similar results. *p < 0.05, compared with WT-transfected cells. Means were compared by analysis of variance followed by the Student-Newman-Keuls test.
From proteinase K, indicating that the membrane vesicles were intact (Fig. 3A, lanes 1 and 2). The immunoreactivity of PDI was destroyed when samples were treated with both detergent and proteinase K (Fig. 3A, lane 3).

To confirm the results obtained with proteinase K, we also performed protease protection assays with trypsin, a weaker protease that cannot digest proteins within a membrane bilayer. In membrane samples not treated with trypsin, DGAT2 was detected by both anti-FLAG and anti-DGAT2 antibodies (Fig. 3B, lane 1). Trypsin treatment of membrane vesicles destroyed both the FLAG and DGAT2 epitopes, with or without detergent (Fig. 3B, lanes 2 and 3), indicating that the N and C termini are both exposed to the cytosol. To verify the integrity of the membrane vesicles, samples were immunoblotted with antibodies for the ER luminal proteins PDI and grp78 (BiP). However, both of these proteins were resistant to trypsin in the presence of detergent (not shown). Therefore, samples were immunoblotted for HSP70, a mitochondrial matrix protein as a control for membrane integrity. In the absence of Triton X-100, HSP70 was protected against proteolysis by trypsin, indicating that organelle membranes were intact (Fig. 3B, lanes 1 and 2). When samples were treated with both detergent and trypsin, the immunoreactivity of HSP70 was destroyed (Fig. 3B, lane 3).

Indirect immunofluorescence was also used to map the orientation of DGAT2. In COS-7 cells expressing NFLAG-DGAT2, both the N-terminal FLAG and C-terminal DGAT2 epitopes were detected when cellular membranes were completely permeabilized with methanol/acetone and when the plasma membrane was selectively permeabilized with 3 μg/ml digitonin (Fig. 3C). In contrast, the ER luminal protein PDI was detected when cells were exposed to methanol/acetone but not to digitonin, verifying that the ER membrane was intact with the latter condition. The immunofluorescence results
therefore confirmed that both the N and C termini of DGAT2 are exposed to the cytosol.

Mapping the Location of the Transmembrane Domains with Epitope-tagged DGAT2 Expression Plasmids—The localization of the N and C termini of DGAT2 to the cytosol suggested that DGAT2 has an even number of transmembrane domains, most likely two, given the hydrophobicity data and prediction algorithms. To determine the identity of the two transmembrane domains, we engineered a series of epitope-tagged constructs by inserting a Myc epitope between amino acids 90–91 (Myc90-DGAT2), 143–144 (Myc143-DGAT2), and 203–204 (Myc203-DGAT2) of the NFLAG-DGAT2 expression protein (Fig. 4A). Each of the mutants was expressed in COS-7 cells as demonstrated by immunoblotting with either anti-FLAG or anti-DGAT2 antibodies (Fig. 4B). The Myc-tagged proteins exhibited a slightly higher molecular weight than NFLAG-DGAT2 due to the presence of the Myc epitope. Expression of the Myc-tagged DGAT2 constructs in COS-7 cells indicated that proteins containing the Myc epitope were functional (Fig. 4C). DGAT activity in lysates from cells expressing the various Myc constructs was increased compared with untransfected cells, although not to the same level as cells expressing NFLAG-DGAT2.

Based on the sequence analysis algorithms, we hypothesized that the first transmembrane domain resided between amino acids 66 and 96. To determine whether the second domain was between amino acids 85 and 116 or between amino acids 229 and 257, we transfected COS-7 cells with Myc143-DGAT2 and Myc203-DGAT2 expression plasmids. If the second transmembrane domain were in the region of amino acids 85–116, then the Myc epitopes of Myc143-DGAT2 and Myc203-DGAT2 would reside in the ER lumen. Membrane vesicles were isolated from transfected cells and treated with proteinase K, with or without 1% Triton X-100. DGAT2 was detectable with anti-DGAT2, anti-FLAG, and anti-Myc antibodies in untreated membranes from cells expressing both Myc143-DGAT2 and Myc203-DGAT2 (Fig. 5A, lanes 1 and 4). Treatment of these membranes with proteinase K destroyed all these epitopes, indicating that they were exposed to the cytosol (Fig. 5A, lanes 2 and 5). Proteinase K treatment did not affect the ability to detect the luminal protein PDI. However, PDI immunoreactivity was lost after the samples were treated with proteinase K and Triton X-100 (Fig. 5A, lanes 3 and 6).

Immunofluorescence microscopy was used to confirm the cytosolic orientation of the Myc epitopes in Myc143-DGAT2 and Myc203-DGAT2 (Fig. 5B, C). COS-7 cells were transfected with Myc143-DGAT2 and Myc203-DGAT2, completely permeabilized with methanol/acetone or selectively permeabilized with digitonin, and then incubated with primary and secondary fluorescent antibodies to detect Myc (red) and FLAG (green) epitopes or Myc (red) and PDI (green) epitopes. In cells that had all cellular membranes permeabilized with methanol/acetone, Myc143-DGAT2 and Myc203-DGAT2 were visualized. In contrast, PDI immunoreactivity was negative. These results indicated that the ER membrane was not compromised by digitonin treatment and that the Myc epitopes in Myc143-DGAT2 and Myc203-DGAT2 were exposed to the cytosol. These data suggested that the sec-
ond transmembrane domain of DGAT2 likely resided in the region of amino acids 85 and 116.

A Loop between the Transmembrane Domains Is Not Exposed to the Cytosol—A membrane topology model based on the algorithm predictions and the experimental data is shown in Fig. 6. This model predicts that murine DGAT2 has one transmembrane domain between amino acids 66 and 88, and a second one between amino acids 93 and 115, with a short loop between the two transmembrane domains (amino acids 89–93) located in the ER lumen. To test this prediction, COS-7 cells were transfected with Myc90-DGAT2, which has a Myc epitope inserted between amino acids 90 and 91 (Fig. 4A), and examined by immunofluorescence microscopy. When cells were completely permeabilized with methanol/acetone, both the FLAG and Myc epitopes were visualized (Fig. 7). However, in cells selectively permeabilized with digitonin, only the FLAG epitope of Myc90-DGAT2 was visualized. The Myc epitope was not visualized, indicating that the loop between the transmembrane domains of DGAT2 is not exposed to the cytosol and likely resides in the lumen of the ER. A control protein, PDI, was visualized only with methanol/acetone treatment, verifying the integrity of ER membranes of cells selectively permeabilized with digitonin. (Protease protection assays were not performed on cells expressing Myc90-DGAT2, because fragments protected from proteolysis would be too small to be detected by immunoblotting.)

A Highly Conserved Sequence “HPHG” of DGAT2 Is Important for DGAT Activity—Topological studies indicated that the majority of DGAT2 is composed of a C-terminal cytoplasmic domain. Included in this domain are the amino acids HPHG (161–164 of murine DGAT2), which are conserved in all DGAT2 gene family members (Fig. 8A), suggesting that this sequence plays a crucial role in the function of the enzyme. To test this hypothesis, we elected to mutate the histidine and proline residues of this sequence. Four DGAT2 mutants with conservative substitutions were generated: H161A, P162G, H163A, and a triple mutant in which HPH was changed to AGA (Triple). The mutant proteins and the NFLAG-DGAT2 “wild-type” (WT) protein were expressed in COS-7 cells, and expression was confirmed by immunoblotting with anti-FLAG antibodies (Fig. 8B). In vitro DGAT activity was reduced in cell lysates for each of the mutants (Fig. 8C). DGAT activity of the H161A and P162G mutants was decreased 50–60%, whereas the H163A and the triple mutant had retained <20% of activity.

Identification of a Putative Neutral Lipid-binding Domain in DGAT2—DGAT2 contains a consensus sequence (FLXLXXX), where ω is a nonpolar amino acid) for a putative neutral lipid-binding domain that is present in proteins that either bind to or metabolize neutral lipids (22). For murine DGAT2, the topological data indicated that this sequence (FLVLGAC) resides in the first transmembrane domain. The neutral lipid-binding sequence is most highly conserved among vertebrate DGAT2 and MGAT orthologues (Fig. 9A). To determine if this sequence was important for DGAT function, we expressed FLAG-DGAT2 proteins in COS-7 cells in which Phe-80, Leu-81, or Leu-83 were replaced with alanine (Fig. 9B). Each of these mutants exhibited reduced DGAT activity in cell lysates (Fig. 9C). Activity was reduced in the F80A and L81A mutants by 66% and 85%, respectively. The L83A mutant was completely devoid of DGAT activity, with no increase in DGAT activity above that of nontransfected cells. To exclude the possibility that these mutations in the first transmembrane domain markedly altered DGAT2 structure, we analyzed the membrane topology of the expressed proteins. In protease pro-
Murine DGAT2: Membrane Topology and Functional Amino Acids

The proper orientation of the protein in the ER membrane was maintained (data not shown).

The same region of DGAT2 also contains a possible membrane lipid attachment site (LGVAC, amino acids 83–87) (Fig. 9A) that is found in prokaryotes (Prosite PS00013) and is used to anchor proteins to membranes by a thioether covalent bond between the sulfhydryl group side chain of a cysteine residue and a diacylglycerol (23). We therefore hypothesized that Cys-87 in DGAT2 interacts with sn-1,2-diacylglycerol, a DGAT substrate. To test this hypothesis, a C87S DGAT2 mutant was generated and expressed in COS-7 cells (Fig. 9C). DGAT activity levels were normal in lysates from the C87S mutant. The activities for the mutants are normalized to that of the expressed WT protein. The same region of DGAT2 also contains a possible membrane lipid attachment site (LGVAC, amino acids 83–87) (Fig. 9A) that is found in prokaryotes (Prosite PS00013) and is used to anchor proteins to membranes by a thioether covalent bond between the sulfhydryl group side chain of a cysteine residue and a diacylglycerol (23). We therefore hypothesized that Cys-87 in DGAT2 interacts with sn-1,2-diacylglycerol, a DGAT substrate. To test this hypothesis, a C87S DGAT2 mutant was generated and expressed in COS-7 cells (Fig. 9C). DGAT activity levels were normal in lysates from the C87S mutant. The activities for the mutants are normalized to that of the expressed WT protein.

FIGURE 8. A highly conserved sequence (HPHG) of DGAT2 is required for normal enzymatic function. A, alignment of partial sequences of DGAT2 family members showing the invariably conserved HPHG sequence. B, expression of DGAT2 proteins containing mutations in the HPHG sequence. Immunoblots of lysates from COS-7 cells expressing NFLAG-DGAT2 (WT) and DGAT2 mutants (H161A, P162G, H163A, and an H161A/P162G/H163A triple mutant (Triplet)). C, in vitro DGAT activities of lysates from COS-7 cells expressing NFLAG-DGAT2 (WT) and DGAT2 mutants. The activities for untransfected cells and cells transfected with wild-type (WT) DGAT2 were 93 and 165 pmol/mg of protein/min, respectively. The activities for the mutants are normalized to that of the expressed WT protein.

DISCUSSION

In this study, we report the topology and the identification of key functional amino acids for murine DGAT2. Our data indicate that DGAT2 is an integral membrane protein with both the N and C termini oriented toward the cytosol. The protein most likely has two transmembrane domains, although it is also possible that this entire hydrophobic region is embedded within the bilayer. We also identified two sequences of DGAT2 that are highly conserved across species and show that they are required for the full enzymatic activity of DGAT2. Our findings provide the first insights into the molecular aspects of DGAT2, an enzyme of fundamental importance for triacylglycerol synthesis and energy storage in eukaryotic organisms.

Using a biochemical approach, we found that DGAT2 was not extractable from membrane preparations treated with agents that release peripheral proteins, but was extractable with detergents, indicating that DGAT2 is an integral membrane protein. A Kyte-Doolittle hydrophobicity analysis and various sequence analysis algorithms of DGAT2 sequences indicated that the enzyme is a polytopic protein with two or three possible transmembrane domains. We mapped the membrane topology of murine DGAT2 by protease protection assays and immunofluorescence microscopy, the latter in conjunction with epitope tagging and selective permeabilization of cellular membranes. Our data suggest that DGAT2 has two transmembrane domains located near the N terminus of the protein and that both the N and C termini of DGAT2 are exposed to the cytosol. A short loop (~5 amino acids) likely resides between the two transmembrane domains and is oriented toward the ER lumen. Because this loop is relatively short and hydrophobic, an alternative possibility is that it is imbedded within the lipid bilayer and that the protein does not fully span the bilayer. The bulk of the protein (~87%) is distal to the second transmembrane domain and faces the cytosol. Because this region is homologous with other DGAT2 family members, it likely contains regions of the protein that are crucial for catalysis. Although the nonpolar region of amino acids 230–250 is not a transmembrane domain, it has characteristics of an amphipathic α-helix, which could associate with the cytosolic face of the ER.

Our model suggests that the active site of DGAT2 resides on the cytosolic face of the ER. This is consistent with the idea that the substrates for DGAT2, diacylglycerol and fatty acyl-CoA, most likely reside in the cytosolic face of the ER lipid bilayer, where they encounter the enzyme and, in particular, may encounter the HPHG sequence, which may be important in catalysis. This model agrees with previous biochemical studies in which the active site of DGAT (and that of the family member acyl-CoA:monoaoylglycerol acyltransferase) was oriented toward the cytosol (24). A latent DGAT activity (present only
after treating membranes with disrupting agents) has been detected in rat liver microsomes (25–28), suggesting that there are active sites on both sides of the ER membrane. Our topology data suggest that DGAT2 is not responsible for this latent activity. DGAT1 may account for a portion of this latent activity, because DGAT1 deficiency decreased both overt and latent activities in microsomes of \( \text{Dgat1}^{-/-} \) mice by similar proportions.3

We speculate that other members of the DGAT2 family have a topography similar to that of DGAT2. DGAT2 belongs to a highly conserved acyltransferase gene family. In humans, there are seven known members of the DGAT2 family, including three monoacylglycerol acyltransferases (9–14), a wax synthase (15, 16), and a wax synthase/multifunctional acyltransferase (17). All of these enzymes catalyze reactions in which a fatty acyl-CoA substrate is covalently linked to an acceptor molecule (e.g., a monoacylglycerol for monoacylglycerol acyltransferase or a long-chain alcohol for wax synthase). All family members share homology in the C-terminal regions distal to the second transmembrane domain of DGAT2, and their hydrophobicity plots are similar in this region. A recent study of the membrane
topology of a DGAT2 orthologue in \( \text{Vernicia fordii} \) showed that the N and C termini are oriented toward the cytosolic side of the ER membrane, which is in agreement with our findings (33). Additionally, preliminary experiments have shown that monoacylglycerol acyltransferase 2 is also an integral membrane protein and has a membrane topology similar to that of DGAT2, with both the N and C termini oriented toward the cytosol.4 Although all members of this family exhibit homology, only DGAT2 has a long domain (~70 amino acids) proximal to the first transmembrane domain. The function of this domain is unclear.

All DGAT2 family members, from yeast to human, contain a sequence of four amino acids, HPHG, that is located ~45 amino acids after the transmembrane domains. Because this sequence is absolutely conserved, it is highly probable that it is functionally important. Indeed, we found that mutations in this sequence markedly reduced DGAT activity. These amino acids may be involved in acyl-CoA binding, which is common to all enzymes of the family. Interestingly, an invariant histidine residue of the DGAT1/ACAT family has been implicated as part of the active site of ACAT1 and ACAT2 (29–31). Although the DGAT1/ACAT and DGAT2 families have no homology, it is possible the HPHG domain plays a role in the active site of DGAT2.

DGAT2 also contains the consensus sequence (FLX\( LXX \), where \( n \) is a nonpolar amino acid) for a putative neutral lipid-binding domain that resides within the first transmembrane domain (amino acids 80–87). We found that mutations in key residues of this domain of murine DGAT2 greatly reduced DGAT activity. In particular, the L83A mutation resulted in an apparently nonfunctional enzyme. Because we were concerned that mutations in the transmembrane region of DGAT2 might disrupt the topology of the enzyme, we verified that the N and C termini of the expressed mutant proteins were cytosolic in orientation by performing protease digestion experiments. However, we cannot exclude the possibility that the mutations may have disrupted the secondary structure of DGAT2 in this region, resulting in a nonfunctional protein.

The neutral lipid-binding domain is present in other proteins that bind to or metabolize neutral lipids, such as cholesterol...
Murine DGAT2: Membrane Topology and Functional Amino Acids

ester transfer protein, hormone-sensitive lipase, lecithin:cholesterol acyltransferase, cholesterol 7α-hydroxylase, cholesterol esterase, and triacylglycerol hydrolase (22, 32). For cholesterol ester transfer protein, deletion of the C terminus, which contains the neutral lipid-binding domain, demonstrated that this region is required for the binding and transfer of neutral lipids (32). The function of the neutral lipid-binding domain in DGAT2 is unclear. It may utilize this domain to interact with diacylglycerol, the more hydrophobic of its substrates. However, if our model is correct, the domain would reside in close proximity to the ER lumen rather than near the cytosolic face of the ER. Another possibility is that this domain transiently binds the triacylglycerol product of the reaction, facilitating its transfer to proteins that traffic this lipid.

In summary, using a combination of biochemical and immunofluorescence microscopy techniques, we provide the first insights into the membrane topology and key functional amino acid residues of DGAT2. The data from the present study provide the groundwork for future structure/function studies of this key lipid synthesis enzyme. These new insights may also be useful in developing pharmaceuticals for inhibiting DGAT2 as a means to prevent or treat obesity and related metabolic disorders.

Acknowledgments—We thank S. Ordway and G. Howard for editorial assistance, M. Chang for manuscript preparation, and K. Weisgraber and Y. Huang for comments on the manuscript.

REFERENCES

1. Unger, R. H., and Zhou, Y. T. (2001) Diabetes 50, Suppl. 1, S118–S121
2. Unger, R. H., and Orci, L. (2002) Biochim. Biophys. Acta 1585, 202–212
3. Stone, S. J., Myers, H., Brown, B. E., Watkins, S. M., Feingold, K. R., Elias, P. M., and Farese, R. V., Jr. (2004) J. Biol. Chem. 279, 11767–11776
4. Bell, R. M., and Coleman, R. A. (1980) Annu. Rev. Biochem. 49, 459–487
5. Brindley, D. N. (1991) in Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. E., and Vance, J. E., eds) pp. 171–203, Elsevier, Amsterdam
6. Gunstone, F. D., Harwood, J. L., and Padley, F. B. (1994) The Lipid Handbook, 2nd Ed., pp. 646–651, Chapman & Hall, London
7. Cases, S., Smith, S. J., Zheng, Y.-W., Myers, H. M., Lear, S. R., Sande, E., Novak, S., Collins, C., Welch, C. B., Luis, A. J., Erickson, S. K., and Farese, R. V., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13018–13023
8. Cases, S., Stone, S. J., Zhou, P., Yen, E., Tow, B., Lardizabal, K. D., Voelker, T., and Farese, R. V. Jr. (2001) J. Biol. Chem. 276, 38870–38876
9. Yen, C.-L. E., Stone, S. J., Cases, S., Zhou, P., and Farese, R. V., Jr. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8512–8517
10. Yen, C.-L. E., and Farese, R. V., Jr. (2003) J. Biol. Chem. 278, 18532–18537
11. Cao, I., Lockwood, J., Burn, P., and Shi, Y. (2003) J. Biol. Chem. 278, 13860–13866
12. Cheng, D., Nelson, T. C., Chen, J., Walker, S. G., Wardwell-Swanson, L, Meegailla, R., Taub, R., Billheimer, J. T., Ramaker, M., and Feder, J. N. (2003) J. Biol. Chem. 278, 13611–13614
13. Cao, J., Burn, P., and Shi, Y. (2003) J. Biol. Chem. 278, 25667–25663
14. Lockwood, J. F., Cao, J., and Shi, Y. (2003) Am. J. Physiol. Endocrinol. Metab. 285, E927–E937
15. Cheng, J. B., and Russell, D. W. (2004) J. Biol. Chem. 279, 37798–37807
16. Turkish, A. R., Henneberry, A. L., Cromley, D., Padamsee, M., Oelkers, P., Bazzi, H., Christiano, A. M., Billheimer, J. T., and Sturley, S. L. (2005) J. Biol. Chem. 280, 14755–14764
17. Yen, C.-L. E., Brown, C. H., IV, Monetti, M., and Farese, R. V., Jr. (2005) J. Lipid Res. 46, 2388–2397
18. Smith, S. J., Cases, S., Jensen, D. R., Chen, H. C., Sande, E., Tow, B., Sanan, D. A., Raber, J., Eckel, R. H., and Farese, R. V., Jr. (2000) Nat. Genet. 25, 87–90
19. Chen, H. C., Smith, S. J., Ladha, Z., Jensen, D. R., Ferreira, L. D., Pulawa, L. K., McGuire, J. G., Pitas, R. E., Eckel, R. H., and Farese, R. V., Jr. (2002) J. Clin. Invest. 109, 1049–1055
20. Chen, H. C., Ladha, Z., and Farese, R. V., Jr. (2002) Endocrinology 143, 2893–2898
21. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
22. Alam, M., Gilham, D., Vance, D. E., and Lehner, R. (2006) J. Lipid Res. 47, 375–383
23. Hayashi, S., and Wu, H. C. (1990) J. Bioenerg. Biomembr. 22, 451–471
24. Coleman, R. A., and Lee, D. P. (2004) Prog. Lipid Res. 43, 134–176
25. Owen, M. R., Corstorphine, C. C., and Zammit, V. A. (1997) Biochem. J. 323, 17–21
26. Waterman, I. J., Price, N. T., and Zammit, V. A. (2002) J. Lipid Res. 43, 1555–1562
27. Waterman, I. J., and Zammit, V. A. (2002) Int. J. Obes. Relat. Metab. Disord. 26, 742–743
28. Abo-Hashema, K. A. H., Cake, M. H., Power, G. W., and Clarke, D. (1999) J. Biol. Chem. 274, 35577–35582
29. Hofmann, K. (2000) Trends Biochem. Sci. 25, 111–112
30. Guo, Z.-Y., Lin, S., Heinen, J. A., Chang, C. C., and Chang, T. Y. (2005) J. Biol. Chem. 280, 37814–37826
31. Lin, S., Lu, X., Chang, C. C., and Chang, T. Y. (2003) Mol. Biol. Cell 14, 2447–2460
32. Au-Young, J., and Fielding, C. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4094–4098
33. Shockey, J. M., Gidda, S. K., Chapital, D. C., Kuan, J.-C., Dhanoa, P. K., Bland, J. M., Rothstein, S. J., Mullen, R. T., and Dyer, J. M. (2006) Plant Cell 18, 2294–2313
34. Yen, C.-L. E., Monetti, M., Burri, B. J., and Farese, R. V., Jr. (2005) J. Lipid Res. 46, 1502–1511