BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 3237–3245 3237
DOI: 10.1021/bi9020499

Role of Cysteine Residues in Thiol Modification of Acyl-CoA:Diacylglycerol Acyltransferase 2 from Yeast[†]

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Received December 1, 2009; Revised Manuscript Received February 19, 2010

ABSTRACT: Acyl-CoA:diacylglycerol acyltransferase (EC 2.3.1.20, DGAT or DAGAT), which catalyzes the final step in triacylglycerol biosynthesis, has at least two discrete family members (DGAT1 and DGAT2) with different physiological roles. Here we report a systematic study of the local functional and structural role of seven cysteine residues present in DGAT2 from *Saccharomyces cerevisiae* (ScDGAT2, also known as Dga1p) using chemical modification in combination with site-directed mutagenesis. We demonstrate that although DGAT2 was susceptible to various thiol-modifying reagents, none of the cysteines were directly involved in the catalytic activity. Analysis of the accessibility of the sulfhydryl groups revealed that cysteines are also not involved in formation of intramolecular disulfide linkages. Inhibition of DGAT activity with thiol-specific reagents was localized to cysteine 314, which was found to be in the proximity of a highly conserved motif of DGAT2. Our work indicates that although this cysteine does not play a role in enzymatic catalysis, it may reside in a crucial position that is near a possible active site of DGAT2 or related to proper folding of the protein.

Triacylglycerol (TAG)[1] is a neutral lipid that primarily provides reservoirs for energy storage in most eukaryotic organisms. Disorders of TAG metabolism in different human tissues are associated with a variety of diseases such as obesity, type II diabetes, non-alcoholic fatty liver, and coronary heart disease (*1*−*3*). A better understanding of TAG biosynthesis and the roles of the enzymes involved in this metabolic pathway could hasten the development of therapeutic intervention for the treatment of these disorders.

Acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) is an enzyme associated with the endoplasmic reticulum (ER) that catalyzes the terminal step in TAG biosynthesis (*4*−*6*). Several lines of evidence suggest that the level of DGAT activity may have a substantial effect on the flow of carbon into TAG, indicating that DGAT is critical for regulating TAG metabolism (*7*−*9*). There are at least two distinct DGAT families, DGAT1 and DGAT2, with gene orthologues in animals, plants, and microorganisms (*5*). DGAT1 and DGAT2 have strikingly different physiological roles. Reverse genetic studies have shown that DGAT2 knockout mice are severely deficient in TAG and manifest a skin barrier dysfunction, leading to early death. In contrast, DGAT1-deficient mice are viable, although they have a reduced level of TAG accumulation in tissues (*1*, *10*). DGAT1 is ∼500 amino acid residues in length and is generally believed to have 8−10 putative transmembrane domains (*11*). DGAT2 has ∼400 amino acid residues, is less hydrophobic, and may have only one or two transmembrane domains (*12*). Further insights into the molecular mechanism and the assignment of functional roles to structural domains of DGATs, however, have been limited because of the complexities in the solubilization and purification of these membrane-bound enzymes (*13*). To date, the high-resolution structure of either DGAT member remains unknown.

The current understanding of DGAT function has been restricted to local functional information obtained by biochemical approaches using microsomal fractions. In vitro chemical modification studies have been used to characterize DGAT for many years. Sauro and Strickland (*14*) have shown that mammalian DGAT activity is sensitive to cysteine-specific reagents such as *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate. DGAT activity from the fungus *Umbelopsis ramanniana* (formerly *Mortierella*) has also been found to be inhibited by NEM (*15*). In addition, our previous results for the microsomal DGAT activity from muscle tissue of *Bos taurus* were in agreement with these findings (*16*). Since microsomal fractions are likely to contain both DGAT1 and DGAT2 polypeptides, it is difficult to ascribe the effects of inhibition to one specific DGAT isoform (*4*, *5*). Nevertheless, these data suggest that cysteine residues, which are frequently present in functionally important sites because of their high reactivity, may play a functional role in DGAT1 and/or DGAT2. Therefore, cysteine residues may represent valuable targets in the investigation of structure and function in these enzymes.

In this work, we aimed to probe the functional and structural roles of cysteine residues in DGAT2. We chose the DGAT2 orthologue from yeast (*Saccharomyces cerevisiae*), also known as

[†]This research was supported by Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs Program, and the Canada Foundation for Innovation. Q.L. was the recipient of the Provost Doctoral Entrance Award, Queen Elizabeth II Scholarship, and Donald Shaw Memorial and J. Brian McQuitty Graduate Scholarships from the University of Alberta.

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[1]Abbreviations: TAG, triacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; ER, endoplasmic reticulum; ACAT, acyl-CoA:cholesterol acyltransferase; MBOAT, membrane-bound O-acyltransferase; ORF, open reading frame; SDM, site-directed mutagenesis; NEM, *N*-ethylmaleimide; IA, iodoacetamide; DTNB, 5,5′-dithiobis-2-nitrobenzoate; PEG-mal, mPEG5000-maleimide; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol.

Published on Web 03/12/2010

Table 1: Mutagenic Oligonucleotides Used for Site-Directed Mutations within ScDGAT2[a]

mutation	forward	reverse
CC48−49AA	CTCAAACCACAACTAGAGTCAGCAGCTCCATTGGCGACC	GGTCGCCAATGGAGCTGCTGACTCTAGTTGTGGTTTGAG
C127A	CATTTGGAAGTGGTATGCTGATTATTTC	GAAATAATCAGCATACCACTTCCAAATG
C183A	TGACTATCGCAACCAGGAAGCTACAGGGCCAAC	GTTGGCCCTGTAGCTTCCTGGTTGCGATAGTC
C211S	GAGCGTTTGCAACAGAAGGTTCTAACTATTCCAAG	CTTGGAATAGTTAGAACCTTCTGTTGCAAACGCTC
C264A	TAAGCAAAAATCAGTCGATCGCCATTGTTGTTG	CAACAACAATGGCGATCGACTGATTTTTGC
C314A	TTGCATTTGGAGAGGTGGACGCTTATAATGTTC	GAACATTATAAGCGTCCACCTCTCCAAATGC
A314C	TTGGAGAGGTGGACTGTTATAATGTTCTGAGC	GCTCAGAACATTATAACAGTCCACCTCTCCAA

[a]Modification is indicated by an underline, and sequences are written from 5′ to 3′.

DAGAT or Dga1p (here termed ScDGAT2) as a model protein. This enzyme is the only DGAT characterized in *S. cerevisiae* and contributes most of the TAG production in this organism (*17*, *18*). We demonstrated that ScDGAT2 can be inactivated by different thiol-modifying reagents. We then undertook a detailed, systematic investigation of the mechanisms of thiol modification of yeast DGAT2 by probing the local structural and functional role of seven cysteine residues in this enzyme (Cys^{48}, Cys^{49}, Cys^{127}, Cys^{183}, Cys^{211}, Cys^{264}, and Cys^{314}). We explored the role of these cysteine residues in both enzyme catalysis and structural support using cysteine-specific modification and site-directed mutagenesis (SDM). Although none of the cysteine residues appear to be directly involved in DGAT activity, our results located a putative functional motif in DGAT2.

EXPERIMENTAL PROCEDURES

Materials. Yeast media and chemicals were purchased from BD Difco (Oakville, ON) or Fisher Scientific (Whitby, ON). Iodoacetamide (IA), NEM, SDS, and triolein were from Sigma. 5,5′-Dithiobis-2-nitrobenzoate (DTNB) was from Pierce. mPEG5000-maleimide (PEG-mal) was purchased from Sunbio (Orinda, CA). [^{14}C]Oleoyl-CoA was obtained from GE Healthcare (Baie d'Urfe, QC). Diolein was from Avanti (Alabaster, AL). Primers were synthesized by IDT (Coralville, IA). Anti-V5-HRP antibodies were from Invitrogen. The PVDF membrane (hybond-P) and the enhanced chemiluminescence kit (ECL advance) were obtained from GE Healthcare.

Yeast Strain and Cell Culturing. The *S. cerevisiae* stain with quadruple knockout of the *DGA1*, *LRO1*, *ARE1*, and *ARE2* genes (H1246, *MAT*α *are1*-Δ*::HIS3*, *are2*-Δ*::LEU2*, *dga1*-Δ*::KanMX4*, *lro1*-Δ*::TRP1 ADE2*) and the parental strain (SCY62, *MAT*α *ADE2*) were supplied by S. Stymne and U. Stahl (*19*). Wild-type strain SCY62 was used for cloning of ScDGAT2. H1246 was used as the host strain for protein expression experiments. Yeast transformation was performed according to the method of Gietz and Schiestl (*20*). Single colonies were inoculated into minimal medium with dextrose (YNBD) containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) dextrose, 20 mg/L adenine, arginine, tryptophan, methionine, histidine, and tyrosine, 30 mg/L lysine, and 100 mg/L leucine. Yeast cells in liquid medium were cultured at 30 °C and 250 rpm in an orbital shaker overnight.

Cloning and Expression of ScDGAT2. Manipulation of nucleic acids was performed using standard protocols (*21*). Yeast genomic DNA was isolated from SCY62 cells using the DNeasy tissue kit (Qiagen, Mississauga, ON). The open reading frame (ORF) encoding ScDGAT2 (YOR245C, designated DGA1) was PCR-amplified from yeast genomic DNA using the forward primer 5′-GCG**ATG**TCAGGAACGTTCAATGATATAAG-3′ and the reverse primer 5′-**CCC**AACTATCTTCAATTCTGCATCCGGTAC-3′. The first and last codons of DGA1 are in boldface, and the stop codon was excluded. Following PCR amplification and gel purification, ScDGAT2 was cloned into yeast expression vector pYES2.1-TOPO (Invitrogen) under the control of the GAL1 promoter and in frame with the C-terminal V5 epitope tag, resulting in the ScDGAT2 polypeptide with an additional KGELRGHPFEGKPIPNPLLGLDSTRTGHHHHHH sequence at the C-terminus. An untagged DGAT2 was constructed as described above, except that the reverse primer introduced a TAA stop codon after the last amino acid. The constructs were verified by sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and transformed into H1246 yeast cells. Yeast cells were also transformed with pYESLacZ or an empty plasmid as a control. Expression of recombinant protein was performed by cultivating the cell culture in YNBD overnight. Cells were harvested by centrifugation, washed with water, and resuspended in minimal medium YNBG containing 2% (w/v) galactose and 1% (w/v) raffinose at an OD_{600} of 0.4. The culture was incubated at 30 °C and 250 rpm to induce the expression of recombinant genes.

Preparation of ScDGAT2 Mutants. SDM within the ScDGAT2 ORF was conducted using PCR-driven overlapping extension described elsewhere (*22*). Briefly, *platinum* Taq DNA polymerase high fidelity (Invitrogen) was employed to amplify two overlapping PCR fragments. A list of ScDGAT2 mutants with different amino acid substitutions and corresponding primers used for mutagenesis are described in Table 1. The full coding region of each mutant was then reconstructed using these two fragments as templates. The mutant C0, in which all seven cysteines were converted to alanines, was synthesized by IDT and was used as the template for construction of mutant A314C. Other mutants were prepared using the template provided by ScDGAT2 in pYES2.1-TOPO. All mutations were confirmed by DNA sequencing.

DGAT Assay. Induced yeast cells were harvested by centrifugation, washed with water, and resuspended in 1 mL of cold extraction buffer [0.2 M Hepes-NaOH, 0.5 M sucrose, and 1 mM phenylmethanesulfonyl fluoride (pH 8.7)]. Yeast cells were lysed with 0.5 mm glass beads in a Bead Beater Cell homogenizer (Biospec, Bartlesville, OK). The lysate was centrifuged at 1500*g* for 15 min to remove the cell debris. Microsomes were recovered by ultracentrifugation at 100000*g* for 1 h at 4 °C. The resulting pellets were resuspended in 10 mM Hepes-NaOH (pH 7.4). Equivalent amounts of microsomal protein as determined by the BCA assay (Pierce) were used for in vitro DGAT activity assays which were performed using the method of Weselake et al. (*23*), with some modifications. The reaction mixture contained 0.15 M Hepes-NaOH (pH 7.0), 2.5 mM $MgCl_2$, 0.1 mg/mL BSA (fatty acid free), 308 μM *sn*-1,2-diolein, and 15 μM [^{14}C]oleoyl-CoA in a final volume of 55 μL. The assay was

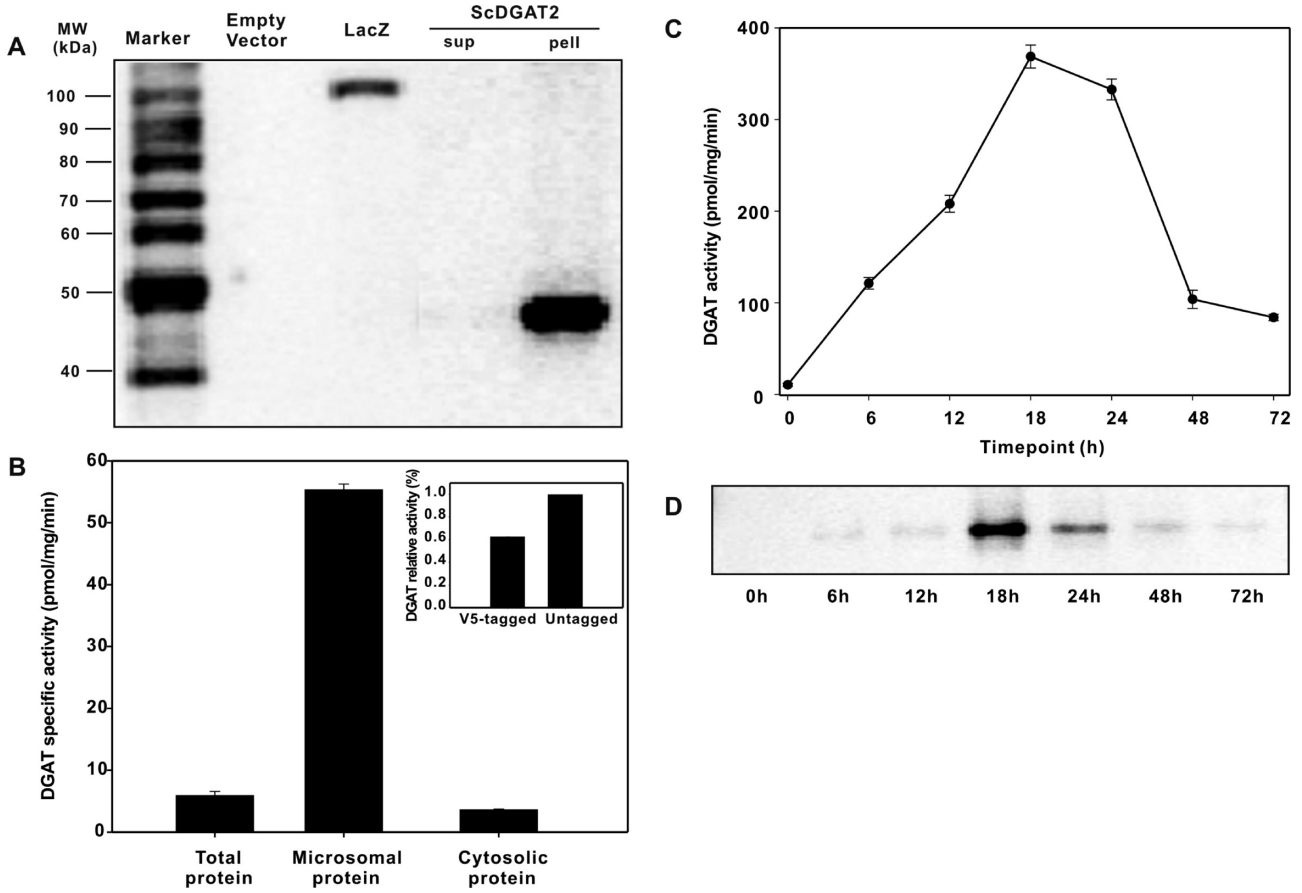

FIGURE 1: Functional expression of recombinant ScDGAT2 in the membrane of yeast cells. (A) Immunoblot of ScDGAT2 probed with anti-V5 antibodies. Membranes were isolated from yeast strain H1246 expressing V5-tagged ScDGAT2. The microsome pellet (pell) was separated from the cytosolic protein in the supernantant (sup) by 100000*g* centrifugation. Equal amounts of protein (10 μg) from each fraction were resolved by 10% SDS−PAGE and analyzed by immunoblotting. (B) In vitro DGAT activities in lysate (total protein), cytosolic (cytsosolic protein), and microsomal fraction (microsomal protein) from yeast cells expressing ScDGAT2. Different protein fractions from yeast cells expressing DGAT2 were incubated in the presence of diolein and [^{14}C]oleoyl-CoA and assayed as detailed in Experimental Procedures. All enzyme activities were expressed as picomoles of TAG formed per minute per milligram of yeast microsomal protein as means ± the standard deviation for biological triplicate analysis in one experiment. Enzyme activities of untagged DGAT2 compared to V5-tagged DGAT2 are shown in the inset. (C) Time course of ScDGAT2 expression. DGAT activity in microsome from yeast cells expressing V5-tagged ScDGAT2 harvested at different hours after the induction of expression. Data represent the average of three measurements in one experiment, with the error bars corresponding to the standard deviation. (D) Immunoblots of the microsomal protein of the same batch of cells harvested at different time points for the enzyme assay.

initiated with 10 μg of the microsomal protein in 10 μL. After incubation for 10 min at 30 °C, reactions were quenched by the addition of 10 μL of 10% SDS (w/v). Fifty microliters of the terminated reaction mixture was applied directly to silica gel TLC plates. Following extensive drying and application of 100 μg of carrier triolein, TAG produced in the reaction mixture was resolved using one ascension of an *n*-hexane/diethyl ether/acetic acid mixture (80:20:1, v/v). TAG spots were visualized with iodine vapor and scraped into scintillation vials. Radioactivity was quantified with a Beckman-Coulter LS6500 liquid scintillation counter. Assays were performed in three biological replicates. For enzyme inhibition assays, thiol protection and modification reagents dithiothreitol (DTT), IA, NEM, and DTNB were prepared individually as stock solutions in dimethyl sulfoxide (Me_2SO). For each reaction, 50 μL of microsomes at a concentration of 1 mg/mL was preincubated with 1 μL of a thiol-modifying reagent at 4 °C for 30 min prior to initiation of the assay. Enzyme activity based on incubation with 1 μL of Me_2SO served as the control with values of 100%.

Thiol-Specific Chemical Modification. Direct and indirect thiol-specific chemical modification with PEG-mal and IA under denaturing conditions was performed as reported previously (*24*, *25*) with the following modifications. Stock solutions of thiol-modifying reagents IA and PEG-mal were freshly prepared in the extraction buffer with 10% (w/v) SDS. For direct modification, 150 μg of cell homogenate was simultaneously added with different concentrations of IA and PEG as indicated in a final volume of 100 μL and incubated at 37 °C for 30 min. Under these conditions, ScDGAT2 was fully denatured in 5% SDS. Free cysteines were efficiently modified with either IA or PEG-mal, and an appropriate amount of incubation mixture was used for immunoblotting. For indirect modification, 100 μL of 20 mM IA in buffer with 10% SDS was first added to the same volume of cell homogenate (3 mg/mL) at 37 °C for 30 min to block the free cysteines in the protein. Excess IA was then removed by trichloroacetic acid precipitation. Recovered pellets were resuspended in 50 μL of extraction buffer (containing 5% SDS) with DTT (8 mM) or 2-mercaptoethnol (2-ME, 10 mM), and a sample without reducing reagents was used as a negative control. After incubation at 37 °C for 30 min, 50 μL of 8 mM PEG-mal was added to modify the free cysteines that would have been involved in a disulfide bond before reduction. Following incubation for an additional 30 min, the reaction mixture

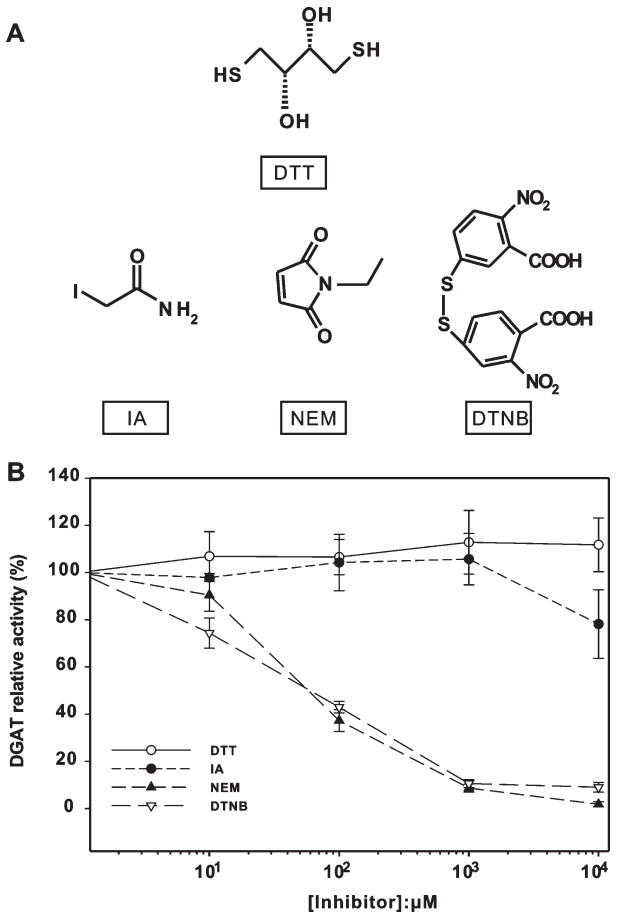

FIGURE 2: Susceptibility of ScDGAT2 enzyme activity to thiol-modifying reagents. (A) Chemical structures of thiol-specific reagents dithiothreitol (DTT), iodoacetamide (IA), *N*-ethylmaleimide (NEM), and 5,5′-dithiobis(2-nitrobenzoate) (DTNB). (B) Microsome fractions containing V5-tagged ScDGAT2 were treated with DTT, IA, NEM, and DTNB followed by a DGAT assay. Results are expressed as a percentage of residual activity, i.e., percentage of the activity in the presence of a modification reagent with respect to the control value (set to 100%) in which no thiol-specific reagents were added. Values of reagent concentration were log-normalized to evaluate their correlations to residual activities. Three independent treatments were assayed in one experiment. Error bars indicate the standard deviation of the mean. A four-parameter logistic model was used to fit to the data to calculate the IC_{50} values (http://bsmdb.tmd.ac.jp:3000/cbdb/ic50).

was then analyzed by SDS−PAGE and immunoblotting. The modification of free cysteines in these processes was monitored by quantifying the total free sulfhydryl groups using DTNB reagent. Cysteine hydrochloride monohydrate was used as the standard for quantification.

SDS−PAGE and Immunoblotting Analysis. SDS−PAGE was conducted via addition of loading buffer [125 mM Tris (pH 6.8), 2% SDS, 25% glycerol, 5% 2-ME, and 0.15% bromophenol blue] to microsomal protein and incubation at room temperature for 30 min. Protein samples were separated in a 10% SDS−PAGE gel. For samples used for mapping disulfide bonds, an appropriate amount of the modified homogenate was added to the loading buffer without 2-ME. Immunoblots were conducted by transferring proteins to a PVDF membrane. The recombinant ScDGAT2 was detected using anti-V5-HRP antibodies (Invitrogen) according to the protocol described by the manufacturer. Densitometry analysis of immunoblots was performed using ImageQuant TL (Amersham).

RESULTS

ScDGAT2 Is a Functional Membrane Protein. The scope of this study was to understand the role of cysteines in the structure and function of a type 2 DGAT. It was important to ensure that the enzyme could be functionally expressed, and therefore, we selected ScDGAT2, the only DGAT in *S. cerevisiae*. The cDNA encoding ScDGAT2 was expressed in quadruple knockout yeast strain H1246 which is devoid of DGAT activity and lipid droplet formation as a result of the knockout of four genes (*DGA1*, *LRO1*, *ARE1*, and *ARE2*) (*19*). To detect the polypeptide, we created a C-terminally V5-tagged version of ScDGAT2 (ScDGAT2-V5, abbreviated as ScDGAT2 for the following result). Immunoblotting indicated that ScDGAT2 was observed as a single band in the pellet fraction of a 100000*g* centrifugation, with a predicted molecular mass of ~48 kDa (Figure 1A). Enzyme assays showed that DGAT activity was predominantly in the microsomal fraction (Figure 1B). To examine the effect of C-terminal tag on protein function, we also constructed an untagged ScDGAT2. Assays of both versions of DGAT2 showed that V5-tagged DGAT2 had ~60% of the enzyme activity of the untagged protein (inset of Figure 1B). A time course expression analysis showed that optimal expression levels were achieved 18 h postinduction, with a good correlation between the level of protein accumulation and the level of DGAT2 activity (Figure 1C,D).

DGAT2 Activity Is Susceptible to Thiol-Modifying Reagents. To investigate the influence of cysteines on the enzyme activity of ScDGAT2, we evaluated the effects of thiol-protecting reagent DTT and thiol-modifying reagents IA, NEM, and DTNB which have different structures and properties (Figure 2A). Treatments of ScDGAT2 in yeast microsomes with DTT resulted in a marginal increase in enzyme specific activity because it could prevent formation of a disulfide bond between free cysteines in the protein as a potent reducing reagent. IA, NEM, and DTNB (commonly known as Ellman's reagent) represent three different classes of cysteine modifiers that are frequently used in protein biochemistry: halogen-β-ketone, maleimide, and aromatic disulfide. Modification with the NEM (membrane-permeant) and DTNB (membrane-impermeant) decreased ScDGAT2 activity in a concentration-dependent manner with IC_{50} values of 60 and 35 μM, respectively. NEM modifies the thiols by alkylation, while DTNB acts by cleaving its internal disulfide bond. IA, which is also membrane-permeant and reacts with free cysteines in the same way as NEM, inhibited the enzyme activity only at concentrations of >1 mM (Figure 2B). The only difference between IA and other two modifiers is that IA has a relatively small functional group. These results raised the hypothesis that certain reduced cysteines might be involved in the activity of ScDGAT2.

Cysteine Residues Are Not Essential for Enzyme Catalysis. To further explore the role of each of the seven cysteines in ScDGAT2 activity, we examined the hydrophobicity of the position corresponding to each cysteine residue by generating a Kyte−Doolittle (*26*) hydropathy plot (Figure 3A). We then applied an SDM approach to generate mutants C127A, C183A, C264A, and C314A in which cysteines Cys^{127}, Cys^{183}, Cys^{264}, and Cys^{314} were substituted with alanines, respectively. The two contiguous cysteines (Cys^{48} and Cys^{49}) were also replaced with alanines to produce the mutant CC48−49AA. Cys^{211} is situated in a hydrophobic segment (Figure 3A), and therefore, to minimize disruption to protein conformation, we

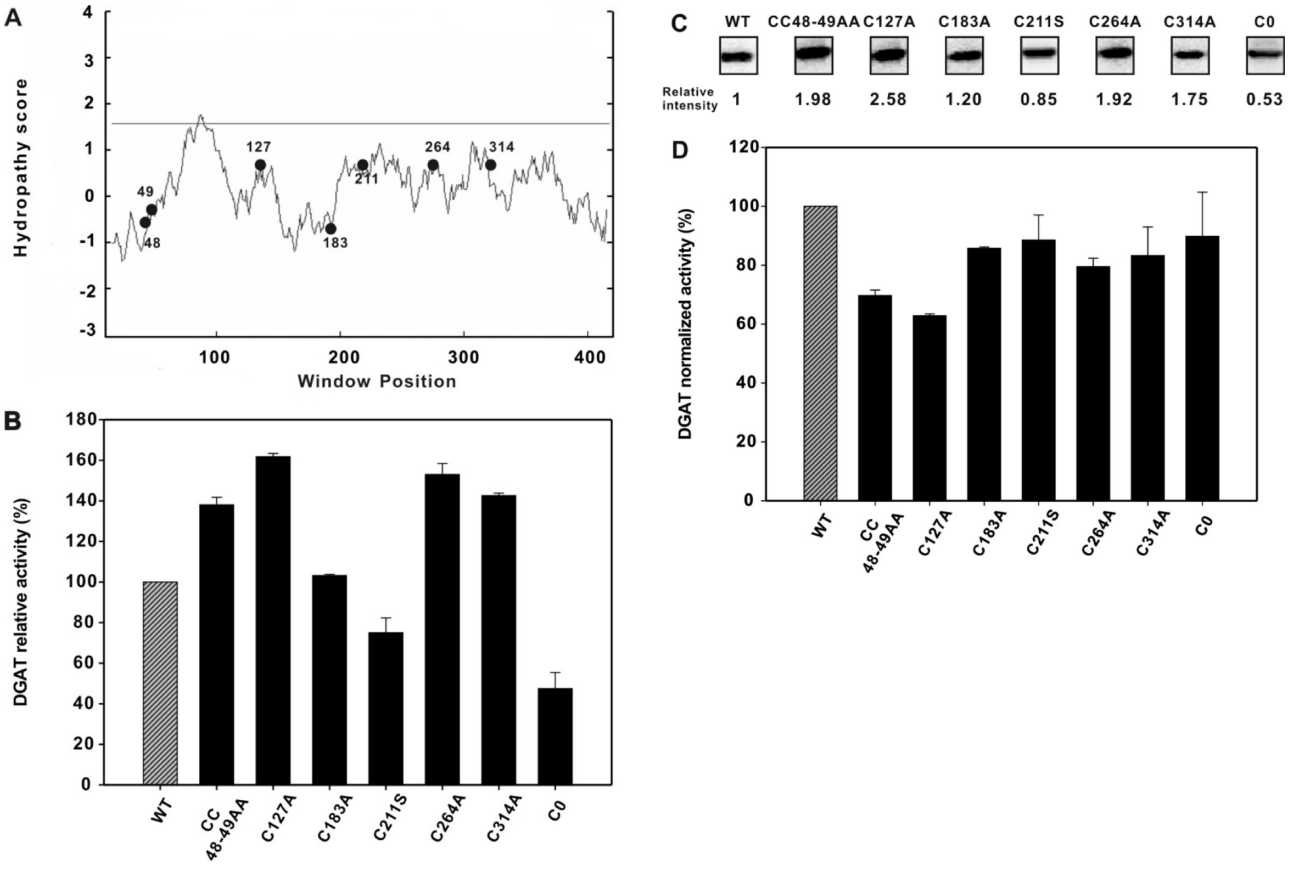

FIGURE 3: (A) Hydropathy plot of ScDGAT2 showing different hydrophobicity environments of each cysteine residue in ScDGAT2. The plot was generated by the method of Kyte and Doolittle (*26*) using a window size of 19. Cysteine residues in ScDGAT2 are represented by circles, and the positions were numbered. (B) DGAT relative activities of microsomes isolated from cells expressing mutants CC48−49AA, C127A, C183A, C211S, C264A, and C314A and Cys-less mutant C0. Details for designing these mutants are described in the Results. All activities are quoted relative to that of the wild type (WT) protein (defined as 100%). Data represent means ± the standard error of at least two independent experiments conducted in triplicate. (C) Expression levels of each mutant were examined by immunoblotting using the same amounts of protein from the same batch of cell microsomal fraction for the DGAT assay. The results shown are from one of three representative experiments. The relative band intensities compared to that of the wild type (set to 1) estimated by ImageQuant TL (GE Healthcare) are shown as the mean from three independent experiments. (D) Normalized activity of ScDGAT2 cysteine mutants. The DGAT relative activity was normalized by dividing the activity value by the relative intensity.

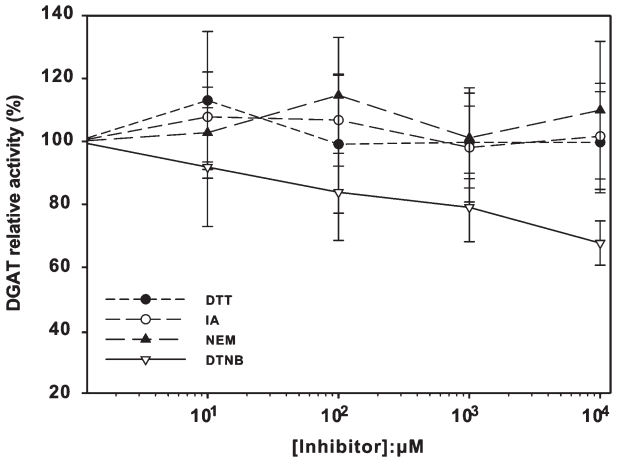

FIGURE 4: Mutant C0 is not susceptible to thiol-modifying reagents. Microsome fractions containing the V5-tagged C0 mutant were treated with IA, DTNB, DTT, and NEM, and a DGAT assay was performed under the same conditions described in the legend of Figure 2. Enzyme activity from microsomes not treated with thiol-specific reagents was set to 100%. The calculations were preformed as described in the legend of Figure 2. Values represent means ± the standard deviation for biological triplicate analysis.

converted Cys^{211} to serine in which only the thiol was replaced with a hydroxyl group yielding the mutant C211S. Each ScDGAT2 mutant was expressed in strain H1246 as previously described. The specific activity for each mutant was measured, and the results suggested that all mutants displayed specific activity equal to or higher than that of the wild type with the exception of C211S (Figure 3B). Immunoblots showed, however, that each recombinant protein accumulated at a different level as determined by densitometry analysis, where the intensity of wild-type ScDGAT2 was set to 1 (Figure 3C). After normalizing the specific activities with the immunobloting analysis, we found that the relative activity of each mutant was comparable to that of the wild type except for CC48−49AA and C127A which undergo a mild reduction in enzyme activities (Figure 3D). Therefore, single cysteine to alanine replacements might only have induced subtle alterations in the protein architecture. To investigate the possibility of a synergistic action of different cysteine residues, we also generated the mutant C0 in which all cysteines were converted to alanines. C0 was expressed in strain H1246 and analyzed as described for the previous mutants. Although C0 accumulated at lower levels compared to the wild type, it was functionally active (Figure 3C,D), demonstrating that cysteines are not essential for DGAT2 catalysis. To validate the role of the cysteine residues in

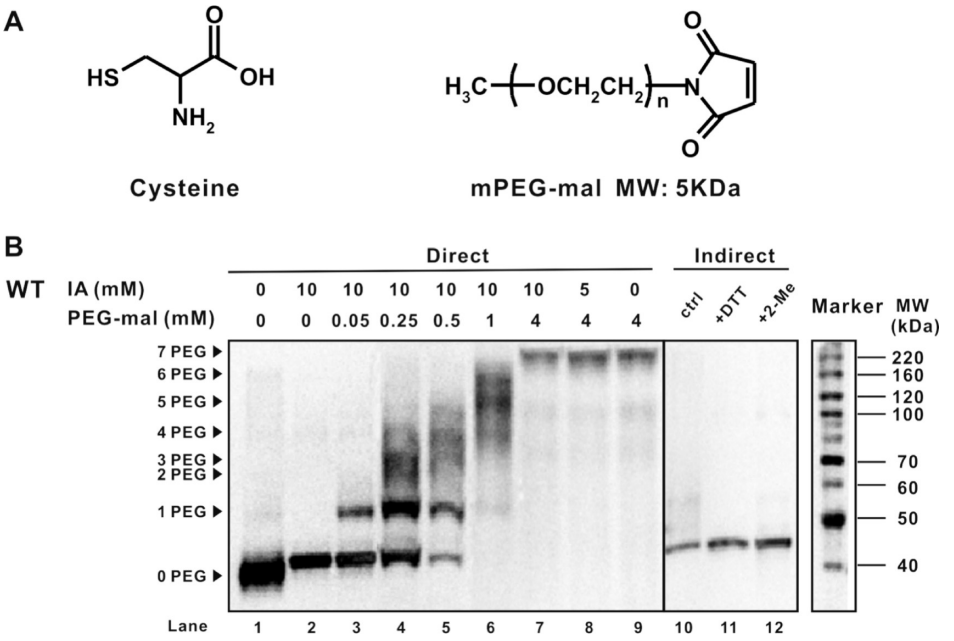

FIGURE 5: Analysis of disulfide bonds in ScDGAT2. (A) Chemical structures of a cysteine residue and mPEG-mal. (B) Direct and indirect thiol modification of ScDGAT2 under denaturing conditions. The details of modification are described in Experimental Procedures. After modification, suitable amounts of samples were used for further analysis by SDS−PAGE and immunoblotting. In lanes 1−9, the value reported at the top of each lane represented the concentration of IA and PEG-mal used in direct modification. Lanes 11 and 12 display the results of indirect modification, and lane 10 is the negative control for indirection modification. The results are from one of two representative experiments.

the inactivation of ScDGAT2, we repeated the treatment with the same thiol-modifying reagents using functional mutant C0. As shown in Figure 4, DTT, IA, or NEM did not produce any significant effect on mutant C0 activity. DTNB, however, decreased enzyme activity in a concentration-dependent manner, with more than 30% inhibition at a reagent concentration of 1 mM. These results confirmed that the decrease in enzyme activity observed with IA and NEM treatments were attributable to the modification of cysteine residues. The inhibitory effects of DTNB on the C0 mutant, however, suggested that this reagent has an unrelated nonspecific effect on enzyme activity.

ScDGAT2 Cysteines Are Not Involved in Disulfide Linkages. Disulfide linkage is a common structural element that facilitates proper folding of proteins to maintain structural integrity (*27*). To investigate if cysteine residues in ScDGAT2 participate in protein structure and folding, we attempted to map possible disulfide linkages using thiol-specific modification which is a variant of the substituted-cysteine accessibility method (*24*). The application of this methodology has been reported for acyl-CoA:cholesterol acyltransferase 1 (ACAT1, EC 2.3.1.26), another enzyme involved in neutral lipid biosynthesis (*25*). This strategy takes advantage of the mass-tagging competition between a large and a small thiol-alkylating reagent, which is manifested in different band shifts of the protein on an immunoblot. Under SDS denaturing conditions, the free cysteines of ScDGAT2 were modified by combinations of different concentrations of PEG-mal (molecular mass of 5000 Da) and IA (molecular mass of 185 Da) (Figure 5A). When ScDAGT2 was modified by only IA, the mobility rate of the modified protein on SDS−PAGE did not exhibit a significant change (Figure 5B, lane 2). Along with the increasing ratio of PEG-mal to IA, a ladder of modified ScDGAT2 species appeared (Figure 5B, lanes 3−8) until only PEG-mal was incubated with the protein (Figure 5B, lane 9). The protein band reached the maximum shift when all the free cysteines were linked to PEG-mal molecules. A total of seven extra bands in addition to the band representing unmodified ScDGAT2 were identified, which indicated the presence of seven free cysteines. To verify this, we performed an indirect modification as a complementary test. In this case, IA was incubated with ScDGAT2 first to block the free cysteine residues. DTT or 2-ME was then used to reduce the possible bonded cysteines followed by modification with PEG-mal. As shown in Figure 5B (lanes 11 and 12), a band shift did not occur as a result of treatments with DTT or 2-ME compared to a control implying that no cysteines were modified by PEG-mal. As an additional control, the same experiment was also conducted using mutant C0. As expected, no band shift appeared on immunoblots (Supporting Information), which confirmed that the additional bands visualized in Figure 5A corresponded to the modification of cysteines in ScDGAT2. These results signified that the cysteine residues in ScDGAT2 are not involved in the disulfide linkage.

Cys^{314} Is the Locus of NEM-Mediated Inhibition. Since the cysteines of ScDGAT2 were not directly involved in enzyme catalysis, we sought to further investigate the origin of the observed thiol-specific inhibition by characterizing the cysteine residue(s) responsible for mediating the inhibition of ScDGAT2 by NEM. Because there was no disulfide bond in ScDGAT2, we performed the inhibition assay with all cysteine mutants (CC48−49AA, C127A, C183A, C211S, C264A, and C314A) individually. Among all mutants, only C314A retained significant DGAT activity even at the highest NEM concentration that was utilized (Figure 6A). These data demonstrate that Cys314 is the major target of NEM-induced inhibition of ScDGAT2 activity. To confirm these results, we generated the mutant A314C using C0 as the template where a single cysteine residue was present at position 314 in ScDGAT2. Mutant A314C possessed a substantial enzyme activity and expression level compared to those of wild-type ScDGAT2 (inset of Figure 6B). Mutant A314C was inactivated by NEM in a fashion similar to that of the wild-type enzyme with an IC_{50} value of 160 μM.

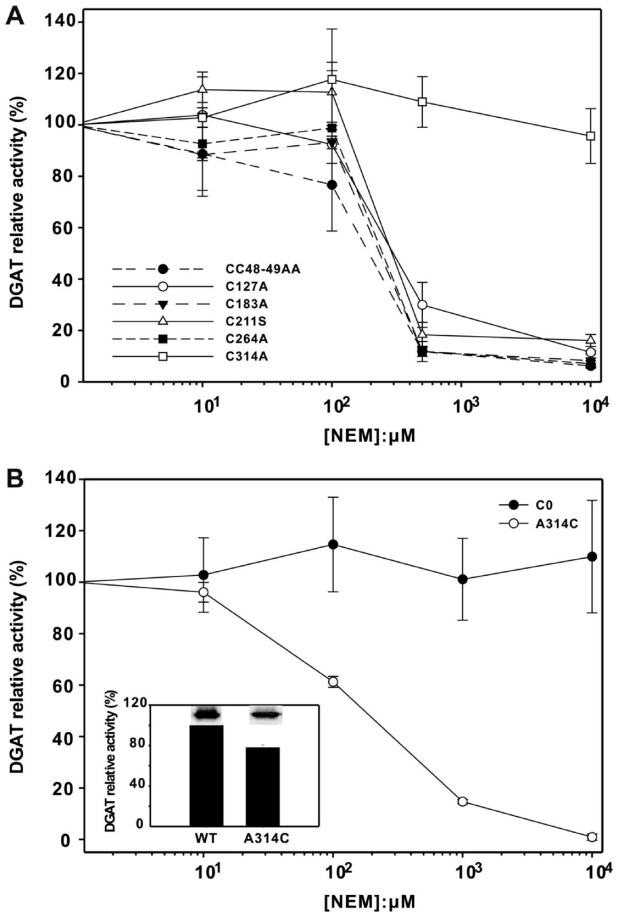

FIGURE 6: Sensitivity of cysteine mutants on NEM-mediated inhibition. (A) DGAT activity inhibition of ScDGAT2 cysteine mutants by NEM. Enzyme activities for each mutant without NEM treatment were assigned as 100%. Values represent means ± the standard deviation for triplicate analysis in one experiment. The results represent one of two representative experiments. (B) Inhibition of DGAT activity in mutant A314C by NEM. The values were compared with data for mutant C0. The enzyme activity and expression level of mutant A314C in contrast to those of the wild-type protein are shown in the inset. The IC_{50} value was determined as described in the legend of Figure 2.

In contrast, C0 was not sensitive to NEM, supporting the data obtained with the C314A mutant (Figure 6B).

DISCUSSION

In this study, we report a systematic survey of the local functional and structural role of seven cysteine residues in a DGAT2 using chemical modification in conjunction with SDM. For membrane proteins such as DGAT2, it is a valuable approach for obtaining structural and functional information by investigating the contribution of cysteine residues to the protein conformation and/or catalytic mechanism such as described for ACAT1 (*28*), carnitine/acylcarnitine carrier (*29*, *30*), and dopamine receptor (*31*).

We revealed that ScDGAT2 was functionally expressed and accumulated in the microsomal fraction of *S. cerevisiae*. These findings are consistent with previous reports on other DGAT2 family members such as DGAT2 from *U. ramanniana*, castor bean (*Ricinus communis*), and tung tree (*Vernicia fordii*), supporting the hypothesis that DGAT2 is associated with the ER and might not undergo apparent post-translational modifications (*32*−*34*). A previous study demonstrated that the yeast DGAT2 is also active in the lipid droplet (*17*). Although expression of ScDGAT2 in H1246 resulted in formation of endogenous TAG (data not shown), lipid droplets were not formed at a level that allowed their isolation. Thus, we used the microsomal vesicles as the source of recombinant ScDGAT2 for our studies. The DGAT activity of this fraction was susceptible to inhibition by thiol modification using various reagents. Genetic modifications of ScDGAT2 through SDM, however, showed that none of the cysteine residues is essential for enzyme catalysis. Thus, the catalytic mechanism of DGAT2 does not require the formation of a covalent acyl−thioester intermediate as reported for other acyltransferases such as chalcone synthase (*35*).

During the investigation of the chemical reactivity of the thiol group of each cysteine residue, we found that disulfide bonds were not formed among these cysteine residues. These bonds are typically found in the lumen of the ER due to a favorable oxidative environment, thus indicating that the thiols of ScDGAT2 are probably facing the cytosol, which has a more reduced environment (*36*, *37*). Moreover, DTNB, which cannot pass through biological membranes, efficiently inhibited ScDGAT2 activity, suggesting that this reagent exerted its action via modification of cysteine on the cytosolic side of the ER. It is possible that the isolated microsomal vesicles might not retain the original orientation during sample preparation. Therefore, to ensure that our microsome vesicles represent the topology in the cells, we performed a control immunoblot against DnaJ, an ER lumen protein marker (Supporting Information). Our results indicated that the vesicles were right-side-out. Thus, our findings corroborate the models proposed for DGAT2 from murine and tung tree in which the active site of the enzyme was proposed to reside on the cytosolic side of the ER and the bulk of the enzyme is exposed to the cytosol (*11*, *34*). This also supports the model in which DGAT2 may be involved in catalyzing the synthesis of TAG destined for deposition into cytosolic lipid droplets (*5*).

Although the cysteine residues in ScDGAT2 were not directly involved in enzyme catalysis or disulfide bond formation, the covalent attachment of the bulky group to the free sulfhydryl group of these residues triggered steric hindrance leading to protein inactivation. Therefore, it is possible to explain inhibition in terms of the size of the attached group and concentration of the thiol-specific reagent. In addition, modification on Cys^{314} was at the center of the inhibition. Cys^{314} probably resides in a critical position that is near the active site of the enzyme. It is interesting to note that cysteine residues in ACAT1, an enzyme structurally and functionally related to DGAT1, were also nonessential (*28*). Cys^{467} in the C-terminal region of ACAT1 was the major target for inhibition, but in this case, the cysteine residue resided in a hydrophobic region and was close to the putative active site His^{460} (*25*, *28*). This polar histidine was proposed to be in the active site of a superfamily of membrane-bound O-acyltransferases, including enzymes such as DGAT1, ACAT, and wax synthase (MBOAT, NCBI domain entry pfam03062), which are involved in transferring fatty acids onto membrane-embedded targets (*38*). The cluster of DGAT2 family proteins in the phylogenetic tree, however, is clearly different from that of the MBOAT family, suggesting that DGAT2 might not share similar catalytic sites with these enzymes (*39*). A closer look at the DGAT2 protein alignment showed that an RXGFX(K/R)XAXXXGXX(L/V)VPXXXFG(E/Q) consensus sequence (residues 288−311 of ScDGAT2) in the C-terminal region precedes Cys^{314} (Figure 7). This motif is the most conserved region in 36 DGAT2 sequences from a wide range of organisms,

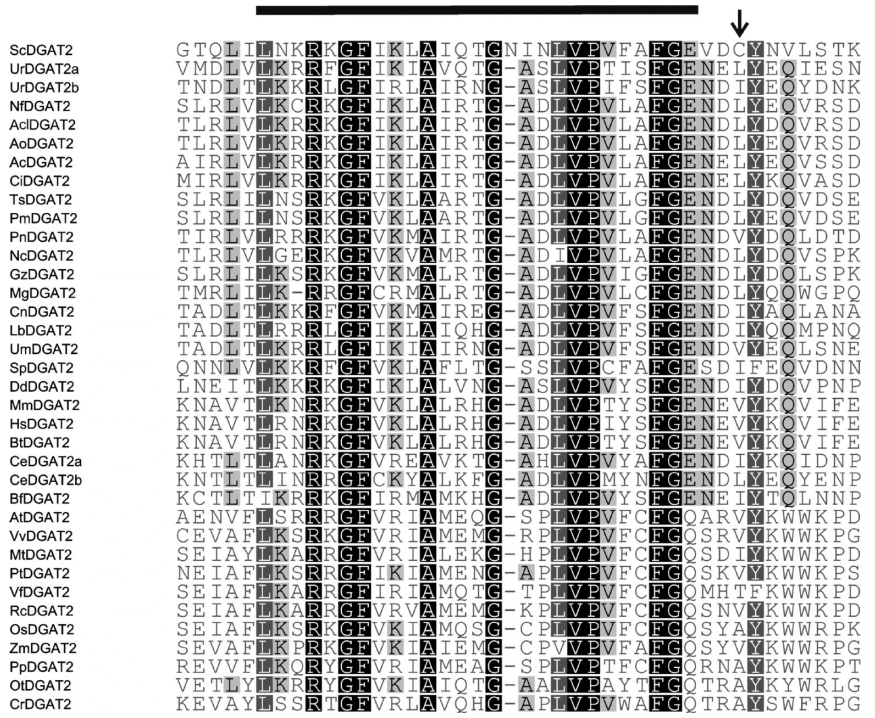

FIGURE 7: Sequence alignment of DGAT2 family members. Thirty-six DGAT2 polypeptides from animals, plants, and fungi were aligned using the Genius (Biomatters Ltd.) with the ClustalW algorithm, showing the relationship between the most conserved domain (indicated by the bar at the top) and Cys^{314} (represented by the arrow). Gray shades denote the polarity of blocks of conserved residues. Abbreviations for each DGAT2 polypeptide indicating the organism of origin and corresponding accession numbers are as follows: Ac, *Ajellomyces capsulatus*, XP_001540241; Acl, *Aspergillus clavatus*, XP_001273210; Ao, *Aspergillus oryzae*, XP_001822244; At, *Arabisopsis thaliana*, NP_566952; Bf, *Branchiostoma floridae*, XP_002208225; Bt, *B. taurus*, CAD58968; Ce, *Caenorhabditis elegans*, CeDGAT2a, CAB04533, CeDGAT2b, AAB04969; Ci, *Coccidioides immitis*, XP_001240299; Cn, *Cryptococcus neoformans*, EAL20089; Cr, *Chlamydomonas reinhardtii*, XP_001693189; Dd, *Dictyostelium discoideum*, XP_635762; Gz, *Gibberella zeae*, XP_381525; Hs, *Homo sapiens*, AAK84176; Lb, *Laccaria bicolor*, EDR14458; Mg, *Magnaporthe grisea*, XP_368741; Mm, *Mus musculus*, AAK84175; Mt, *Medicago truncatula*, ACJ84867; Nc, *Neurospora crassa*, CAE76475; Nf, *Neosartorya fischeri*, XP_001261291; Os, *Oryza sativa*, NP_001057530; Ot, *Ostreococcus tauri*, CAL58088; Pm, *Penicillium marneffei*, XP_002146410; Pn, *Phaeosphaeria nodorum*, EAT89076; Pp, *Physcomitrella patens*, XP_001777726; Pt, *Populus trichocarpa*, XP_002317635; Rc, *R. communis*, AAY16324; Sc, *S. cerevisiae*, NP_014888; Sp, *Schizosaccharomyces pombe*, XP_001713160; Ts, *Talaromyces stipitatus*, EED21737; Um, *Ustilago maydis*, XP_760084; Ur, *U. ramanniana*, UrDGAT2a, AAK84179, UrDGAT2b, AAK84180; Vf, *Vernicia fordii*, ABC94474; Vv, *Vitis vinifera*, CAO68497; Zm, *Zea mays*, ACG38122.

including animals, plants, and fungi. Residues such as arginine, lysine, and glutamic acid in this motif could serve as ideal molecules to facilitate protonation and deprotonation, which are important steps in many catalytic reactions as exemplified by the proposed catalytic mechanism of histone acyltransferase (*40*). It is possible that covalent attachment of thiol-specific groups to Cys^{314}, which might be near the active site(s) in this molecular signature, triggers steric interference within the protein conformation. In future experiments, it will be valuable to validate the functional significance of these multiple residues using site-saturated mutagenesis in conjunction with a high-thoughput screening system that has been applied in our studies of DGAT1 (*41*, *42*). Although our evidence for Cys^{314} being near a putative active site is compelling, the possibility still exists that Cys^{314} may be involved in protein folding in a way that is not related to disulfide bonding. Modification of Cys^{314} may have hampered the proper folding state within local regions or domains which involve interactions among some key residues in the proximity of each other (*43*). Structural transitions occurring during protein folding can be investigated in detail by many biophysical techniques ranging from optical methods to NMR spectroscopy (*44*). These methods, however, are still in their infancy for membrane proteins. Moreover, it is necessary to find effective strategies for overcoming the difficulties in DGAT purification before utilizing these methodologies.

In summary, we have provided insights into the local structural and functional role of cysteine residues in ScDGAT2. Although these cysteines were not directly involved in enzyme-mediated catalysis and structural support through disulfide bonding, we have demonstrated that ScDGAT2 could be inactivated by thiol-specific modification of a single cysteine in the C-terminus which might be near the active site or related to protein folding. Our insights into the role of cysteine residues in ScDGAT2 have created a foundation for further work in elucidating the details of the structure and function of this enzyme. In addition, our approach may prove useful for investigating the role of cysteine residues in other membrane-bound enzymes.

ACKNOWLEDGMENT

We thank Drs. Sten Stymne and Ulf Stahl for the yeast strains. In addition, we thank Drs. Martin Truksa and Zhanyun Guo for useful discussion and Crystal Snyder for critical evaluation of the manuscript.

SUPPORTING INFORMATION AVAILABLE

A figure displaying direct and indirect thiol-modification of mutant C0, and a figure depicting an immunoblot of ScDnaJ. This material is available free of charge via the Internet at http://pubs.acs.org.

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