Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis

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Abstract Stearoyl-coenzyme A desaturase (SCD) is an endoplasmic reticulum (ER) protein that catalyzes the Δ9-*cis* desaturation of saturated fatty acids. Mice with targeted disruption in SCD1 (Scd1$^{-/-}$) have significant reduction in the tissue content of triglycerides, suggesting that monounsaturated fatty acids endogenously synthesized by SCD1 are important for triglyceride synthesis. Acyl-coenzyme A: diacylglycerol acyltransferase (DGAT) is the enzyme that catalyzes the final reaction in the synthesis of triglycerides. The lack of DGAT2, one of the two DGAT isoforms, results in almost a complete loss of tissue triglycerides. We hypothesize that SCD1 participates in triglyceride synthesis by providing a more accessible pool of monounsaturated fatty acids through substrate channeling. In this study, we test whether SCD1 is proximal to DGAT2 by colocalization study with confocal microscopy, coimmunoprecipitation, and fluorescence resonance energy transfer using HeLa cells as the model of study. All of the results suggest that SCD1 and DGAT2 are located very close to each other in the ER, which is a very important criterion for the channeling of substrate. By performing subcellular fractionation using mouse livers, we also show, for the first time, that SCD is present in the mitochondria-associated membrane.—Man, W. C., M. Miyazaki, K. Chu, and J. Ntambi. **Colocalization of SCD1 and DGAT2: implying preference for endogenous monounsaturated fatty acids in triglyceride synthesis. *J. Lipid Res.* 2006.** 47: **1928–1939.**

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Triglycerides are the major energy store in eukaryotic organisms. However, excessive deposition of triglycerides in the white adipose tissue results in obesity, which has evolved into a serious health problem owing to its medical complications. Therefore, understanding the molecular basis of triglyceride biosynthesis is essential.

Stearoyl-coenzyme A desaturase (SCD) is an endoplasmic reticulum (ER) enzyme that is responsible for the critical committed step in the de novo synthesis of monounsaturated fatty acids (1–4). The preferred substrates are palmitoyl- and stearoyl-CoAs, which are converted into palmitoleoyl- and oleoyl-CoAs (5). These two monounsaturated fatty acids are the major fatty acids found in membrane phospholipids, triglycerides, and cholesteryl esters (6). Given that monounsaturated fatty acids are readily obtainable from diets, it is a surprising finding that the disruption of SCD1 in mice results in a significant reduction in the content of liver triglycerides and white adipose depots (7, 8). This suggests that SCD and its endogenous products are crucial in the regulation of lipid accumulation. Even when given a high-fat diet, mice with targeted disruption in SCD1 (Scd1$^{-/-}$) are resistant to diet-induced obesity and liver steatosis (7).

The final step in the synthesis of triglyceride is the addition of the third acyl chain to diacylglycerol, and this reaction is catalyzed by the membrane-bound enzyme acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) (9). Two DGAT genes, DGAT1 and DGAT2, have been identified (10, 11). Although they catalyze similar reactions, they share neither nucleotide nor amino acid sequence similarity (12). Studies performed using mice lacking DGAT1 (Dgat1$^{-/-}$) have suggested that DGAT1 does not have a profound effect on triglyceride metabolism in general and is not essential for life (13). In contrast, mice with a disruption in the DGAT2 gene (Dgat2$^{-/-}$) have severely reduced triglyceride content in their tissues and die in early postnatal periods, demonstrating that DGAT2 is essential for the fundamental synthesis of triglyceride in mammals and is crucial for survival (12).

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Abbreviations: CFP, cyan fluorescent protein; DGAT, acyl-coenzyme A:diacylglycerol acyltransferase; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; MAM, mitochondria-associated membrane; SCD, stearoyl-coenzyme A desaturase; YFP, yellow fluorescent protein.

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Suzuki et al. (14) predicted the subcellular localization of DGAT1 and DGAT2 based on their amino acid sequences. This prediction suggested that DGAT1 is targeted mainly to the plasma membrane and DGAT2 to the ER (14). In fact, overexpression of mouse DGAT2 results in the accumulation of lipid droplets in the cytosol, whereas lipid droplets around the cell periphery are observed when DGAT1 is overexpressed (12). It was proposed that the proximity of DGAT2 to the lipogenic enzymes on the ER allows it to make use of the fatty acids synthesized de novo around the ER (14).

A lipogenic high-carbohydrate diet induces SCD1 expression as well as other lipogenic genes in rodents, resulting in an increase in triglyceride synthesis (1). However, when the same diet is administered to mice with SCD1 deficiency, induction of triglyceride synthesis fails (6). Moreover, it is noteworthy that the basal mRNA expression and activity of DGAT in $Scd1^{-/-}$ mice is not lower than that in the wild-type counterpart, suggesting that SCD1 may serve as a key point of regulation in the synthesis of triglyceride. Despite the fact that DGAT1 and DGAT2 can use a wide range of fatty acids as substrates, oleate is still the most abundant fatty acid in tissue triglycerides, which suggests that a preference for oleate may exist in triglyceride synthesis. Therefore, we hypothesize that SCD1 is proximal to DGAT2 and that SCD1 participates in the synthesis of triglycerides by providing a more easily accessible pool of monounsaturated acyl-CoAs.

In this study, we evaluated whether SCD1 is proximal to DGAT2 in vivo and in vitro. DGAT and SCD are proteins in the ER. However, it has also been shown that DGAT is enriched in a subcellular compartment known as the mitochondria-associated membrane (MAM). MAM is a special ER domain enriched with enzymes responsible for the synthesis of phospholipids and triglycerides, including DGAT, and thus has been proposed to potentially serve as a component of the secretory pathway that supplies lipids for assembly into very low density lipoproteins. By subcellular fractionation of the mouse liver, we demonstrate for the first time that SCD1 is present in MAM, which may imply that SCD1 works intimately with other enzymes in the synthesis of triglycerides and phospholipids. In vitro, we investigated whether SCD1 and DGAT2 are closely located in the ER by colocalization analysis using confocal microscopy, coimmunoprecipitation, and fluorescence resonance energy transfer (FRET) using flow cytometry. Each of these individual approaches suggested that SCD1 and DGAT2 are located in close proximity to each other in the ER.

MATERIALS AND METHODS

Animals and diets

Six to 8 week old mice on a 129 SV background were used in all experiments. The mice were given ad libitum access to food and water and were housed in the animal facility of the Biochemistry Department at the University of Wisconsin-Madison. The breeding and care of the animals were according to the protocols approved by the Animal Care Research Committee of the University of Wisconsin-Madison.

Materials

HeLa cells were a generous gift from Dr. Ann Palmenberg at the University of Wisconsin-Madison. Tissue culture medium and reagents were purchased from Life Technologies-Invitrogen. Monoclonal antibodies against the epitopes were obtained as follows: anti-myc and Alexa-Fluor 488 goat anti-rabbit IgG was a gift from Dr. Anant Menon at Cornell University; anti-KDEL was from Stressgen (Victoria, British Columbia, Canada); anti-FLAG was from Sigma (St. Louis, MO); and Alexa-Fluor 594 goat anti-mouse IgG was from Molecular Probes (Eugene, OR). Protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [^{14}C]stearoyl-CoA and [^{14}C]oleoyl-CoA were purchased from American Radiolabeled Chemicals (St. Louis, MO). TransIT-LT1 was purchase from Mirus (Madison, WI). All other reagents were from Fisher or Sigma.

Plasmids

The pcDNA3 plasmid expressing SCD1 with the myc epitope tagged to the C terminus (SCD1-myc-C) was provided by Dr. Travis Knight (Iowa State University). The FLAG-tagged DGAT2 cloned into a pcDNA plasmid was a gift from Dr. Robert Farese (Gladstone Institute of Cardiovascular Disease, University of California-San Francisco). pECFP-N1 and pEYFP-C1 were generous gifts from Dr. Tom Martin (University of Wisconsin-Madison). The constructs cyan fluorescent protein (CFP)-SCD1 and yellow fluorescent protein (YFP)-DGAT2 were synthesized by cloning a PCR fragment encoding the open reading frame of SCD1 or DGAT2 into pECFP-N1 and pEYFP-C1, respectively. The primers used to synthesize the SCD1 PCR fragment were as follows: forward primer, 5′-ATT CTC GAG ATG CCG GCC CAC ATG CTC-3′; reverse primer, 5′-AAT AAG CTT GCT ACT CTT GTG ACT CCC-3′. For DGAT2, the primers used were as follows: forward primer, 5′-ATT CTC GAG CTA TGA AGA CCC TCA TCG CC-3′; reverse primer, 5′-TAA TCT AGA TCA GTT CAC CTC CAG CAC-3′. The SCD1 PCR fragment was cloned into pECFP-N1 using restriction sites *Xho*I and *Hin*dIII, whereas the DGAT2 PCR fragment was inserted into pEYFP-C1 at the restriction sites *Xho*I and *Xba*I.

Isolation and characterization of MAM, mitochondria, and ER from mouse livers

The procedures for preparing the subcellular fractions were based on those described by Vidugiriene et al. (15) and Vance (16). The mice were euthanized by carbon dioxide asphyxiation, and the livers were quickly removed and immersed in ice-cold isolation buffer (250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EGTA, and 0.1% BSA). Livers were minced and then homogenized using a Potter-Elvehjem motor-driven homogenizer. The homogenate was then centrifuged at 600 *g* twice to remove the large debris. The supernatant was then centrifuged at 10,000 *g* to obtain the crude mitochondria, and the resultant supernatant was layered on a discontinuous sucrose gradient (2 ml of 30% sucrose and 4 ml of 38% sucrose prepared in 10 mM HEPES, pH 7.4) and subjected to centrifugation at 100,000 *g* for 2 h to obtain the ER, which precipitated as a red pellet. For the isolation of MAM and mitochondria, the crude mitochondrial pellet was washed and resuspended in isolation medium. A small amount (0.5 ml) of the suspension was layered on top of each of the 30% Percoll gradients in polycarbonate ultracentrifuge tubes. The tubes were then centrifuged at 95,000 g_{max} for 33 min. The band corresponding to mitochondria was approximately

two-thirds down the ultracentrifuge tube, with the MAM band lying immediately above it. The two bands were removed by transfer pipette, diluted with isolation buffer, and subjected to further centrifugation at 6,300 *g*. The mitochondria pelleted after centrifugation as a yellowish brown pellet. The supernatant from the mitochondrial fraction was combined with the supernatant from the MAM fraction and further centrifuged at 100,000 *g* to obtain MAM.

Characterization of the subcellular fractions

To characterize the subcellular fractions, the following marker enzyme assays were performed. Cytochrome C oxidase and NADPH-cytochrome C reductase were chosen as the marker enzymes for mitochondria and ER, respectively. For the cytochrome C oxidase assay, 100 μl of 0.1 M phosphate buffer, pH 7.0, 30 μl of 0.5% Triton X-100, 800 μl of water, and subcellular fraction proteins (25 μg or less) were combined and incubated at 37°C for 5 min. Seventy microliters of reduced ferrocytochrome C at a concentration of 20 mg/ml was then added to the mixture to start the reaction. A decrease in optical density at 550 nm was measured for 3 min. For the NADPH-cytochrome C reductase assay, the subcellular fraction proteins were added to 100 μl of 0.5 M phosphate buffer and 45 μl of 1% cytochrome C and incubated at room temperature for 3 min before 100 μl of 10 mM NADPH was added to start the reaction. An increase in optical density at 550 nm was measured for 3 min. In both assays described above, the rate of change in optical density was used to calculate the specific activity of the marker enzyme.

Cell culture and transient transfection using electroporation

HeLa cells were maintained in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. The transfection of the different constructs into the HeLa cells was performed as described (17). Briefly, HeLa cells were cultured to 80–100% confluence. The cells were harvested and pelleted. The cell pellet was then washed by resuspending in 50 ml of cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 25 mM HEPES/KOH, pH 7.6, 25 mM EGTA, and 25 mM $MgCl_2$). The cells were then pelleted, and 500 μl of cytomix was added to resuspend the cells. The cell suspension was then transferred to an electroporation cuvette. Twenty-five micrograms of DNA plasmid was added to the cell suspension, and the mixture was exposed to a single electric pulse of 300 V with a capacitance of 1,000 μF using the Bio-Rad pulse system. The cell mixture was transferred back to a 100 mm culture plate, and the cells were incubated in culture medium as described above for 24 or 48 h as specified before analysis was performed. For confocal microscopy study, cells were plated onto polylysine-coated cover slips.

Analysis of triglyceride synthesis

HeLa cells were transfected with pcDNA, SCD1-myc only, DGAT2-FLAG only, or SCD1-myc and DGAT2 using TransIT-LT1. The cells were incubated with serum-free medium containing [^{14}C]stearate coupled to BSA. Forty-eight hours after transfection, the cells were lysed with lysis buffer and total lipids were extracted and separated by thin-layer chromatography, with petroleum hexane-diethyl ether-acetic acid (80:30:1) as the developing solvent. The counts in the triglyceride fractions (cpm) were determined by autoradiography using Packard Instant Imager. To determine the ratio of oleate (18:1) to stearate (18:0), the triglyceride fraction was scraped from the TLC plate. The lipids were extracted according to the method of Bligh and Dyer (18). Fatty acids were released from the triglyceride and separated on a silver nitrate-impregnated silica gel as described below for SCD activity assay.

Oil Red O staining

The cells were fixed with 10% buffered neutral formalin and then stained with the Oil Red O solution containing Oil Red O (1.5 mg/100 ml solution), 50% (v/v) alcohol and 50% (v/v) acetone.

Confocal microscopy

Approximately 24 h after transfection, the cover slips were taken from the culture plate and washed with PBS three times. The cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, followed by an additional three washes with PBS. For visualization of the distribution of SCD1-myc and DGAT2-FLAG, the cells expressing these recombinant proteins were permeabilized with 0.3% Triton X-100 in PBS for 25 min at 4°C. After permeabilization with detergents, the cells were washed with PBS three times and blocked with 10% (v/v) fetal bovine serum in PBS for 1 h at room temperature. The permeabilized cells were then incubated with rabbit anti-myc antibody at 1:500 dilution and anti-FLAG monoclonal antibody at 2 μg/ml for 1 h at room temperature. After incubation with primary antibodies, the cells were washed three times with PBS and incubated with Alexa-Fluor 594 goat anti-mouse IgG or Alexa-Fluor 488 goat anti-rabbit IgG at a dilution of 1:250 for 1 h at room temperature. After three more washes with PBS, the cover slips were mounted on microscopy slides with a drop of Vectorshield (Vector Laboratories) added between the slides and the cover slips. Confocal microscopy was performed to visualize the staining pattern. For cells expressing pECFP-SCD1 and pEDGAT2-YFP, after washing with PBS and fixation with 4% paraformaldehyde, the cover slips were mounted onto the slides and analyzed. Images were obtained using a confocal microscope (Nikon Eclipse TE 2000-U).

Coimmunoprecipitation

HeLa cells were transfected with SCD1-myc and DGAT2-FLAG by electroporation. Forty-eight hours after transfection, the cells were harvested and lysed with lysis buffer [40 mM HEPES-KOH, pH 7.4, 150 mM NaCl, and 0.5% (w/v) Nonidet P-40] containing protease inhibitors at the following concentrations: 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, and 2 mM PMSF. The lysis was allowed to proceed for 40 min on ice, after which the lysate was spun at 16,000 *g* for 20 min. The supernatant was transferred to a new microcentrifuge tube. Protein A/G bead slurry was added and incubated at 4°C for 1 h with gentle rotation. After centrifugation at 2,000 *g* for 1 min, monoclonal myc antibody or monoclonal FLAG antibody was added to the supernatant and incubated at 4°C for 1 h with gentle rotation. Protein A/G bead slurry was then added and incubated at 4°C overnight with rotation. The next day, the protein A/G beads were washed with lysis buffer three times and subjected to boiling for 5 min to release the proteins, which were then separated by SDS-PAGE.

Preparation of microsomes

The cells expressing the desired recombinant constructs were harvested 48 h after transfection and washed with 0.1 M potassium phosphate buffer, pH 7.2. The cells were then disrupted with a variable-speed tissue disruptor and centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was then subjected to further

centrifugation at 100,000 *g* for 1 h at 4°C. The pellet obtained was the microsomes.

DGAT activity assay

The DGAT activity assay was performed as described (19) by incubating the subcellular fractions or microsomes with 10 μl of 40 mM DTT, 5 μl of 8 mM diacylglycerol in acetone, 5 μl of 1.5 mM oleoyl-CoA, 10 μl of 2.5 mg/ml BSA, 2 μl of [^{14}C]oleoyl-CoA, and reaction buffer to give a total reaction volume of 200 μl for 5 min at 37°C. The reaction buffer was composed of 250 mM sucrose, 1 mM EDTA, and 100 mM Tris-HCl, pH 7.5. Chloroform-methanol (0.75 ml; 1:2, v/v) was added to the mixture and vortexed. Chloroform (0.25 ml) and 0.25 ml of water were added, vortexed, and centrifuged at 13,000 *g* for 2 min. A total of 250 μl of the chloroform (bottom) layer was taken out and dried by nitrogen. The lipid was redissolved in 50 μl of chloroform-methanol (2:1, v/v). The lipid was then separated by thin-layer chromatography (hexane-ether-acetic acid, 90:20:1 or heptane-isopropyl ether-acetic acid, 60:40:3). The TLC plate was then subjected to autoradiography. The ratio of the cpm corresponding to the triacylglycerol band to the total cpm was used to determine the DGAT activity of the subcellular fraction.

SCD activity assay

The SCD activity in subcellular fractions/microsomes was estimated by measuring the conversion of stearoyl-CoA (18:0 CoA) to oleoyl-CoA (18:1 CoA) (20). The assay was performed by combining the subcellular fractions/microsomes with 6 nmol of stearoyl-CoA, 0.03 μCi of [^{14}C]stearoyl-CoA, 2 mM of NADH, and 0.1 M potassium phosphate buffer, pH 7.2, to give a total reaction volume of 200 μl. For assaying SCD activity of microsomes expressing recombinant SCD1 construct, cold stearoyl-CoA was omitted from the reaction. The reaction was carried out at room temperature for 15 min and was stopped by the addition of 200 μl of a mixture of 2.5 ml of 10 M KOH, 500 μl of 5 mg/ml butylhydroxytoluene, and 7.0 ml of ethanol. The reaction mixture was then incubated at 80°C for 45 min. Formic acid (280 μl) was then added to protonate free fatty acids. Hexane (700 μl) was added to extract fatty acids, followed by vigorous vortexing. Two hundred to 500 μl of the hexane (upper) layer was taken out and dried under nitrogen gas. The lipid was resuspended in 50 μl of hexane and spotted on a 10% silver nitrate-impregnated TLC plate. The lipid was separated using chloroform-methanol-acetic acid-water (90:8:1:0.8). The plate was then subjected to autoradiography using Packard Instant Imager. The ratio of the cpm in the band corresponding to the oleic acid to the total cpm was used to calculate the SCD activity.

Flow cytometry

Flow cytometry was performed based on the protocol described by Dye et al. (21). Briefly, HeLa cells were transfected with the constructs using electroporation. Twelve hours after transfection, the cells were harvested for flow cytometry analysis. The data were collected with an LSRII flow cytometer (Becton Dickinson, San Jose, CA). Propidium iodide was added to the cell suspension to differentiate live cells from dead cells, as live cells would not pick up propidium iodide. Laser line 488 nm was used to excite propidium iodide, the fluorescence of which was detected using a 630/30 band-pass filter. Two lasers tuned to 488 and 403 nm were used for flow cytometry analysis. The 403 laser line was used for CFP, and the cyan fluorescence was detected using a 470/40 band-pass filter. The yellow fluorescence resulting from direct excitation by the 488 laser line was measured using a 560/10 band-pass filter, to differentiate from the yellow fluorescence resulting from FRET, which was detected using a 530/30 band-pass filter. Data analysis was performed using FlowJo (Tree Star, Inc., Ashland, OR).

Western blot analysis

For immunoblot analysis, protein samples were mixed with SDS loading buffer, loaded, and separated on a 10% SDS-PAGE gel, after which the proteins were transferred to Immobilon-P transfer membranes at 4°C. The membrane was blocked in 10% nonfat dry milk in TBS-T overnight at 4°C. It was then washed with TBS-T and incubated with the primary antibody. The membrane was again washed with TBS-T and incubated with the secondary antibody. Visualization of the SCD1 protein was performed using the Super Signal West Pico kit (Pierce Biotechnology, Rockford, IL).

RESULTS

SCD1 is present in the MAM

MAM is the region of the ER that is in close proximity to the outer membrane of the mitochondria (22). MAM has been shown to be enriched with many enzymes responsible for the synthesis of phospholipids and neutral lipids, including DGAT. SCD is known to be present in the bulk ER, but we were curious whether SCD is also present in the MAM, a compartment that is actively involved in lipid synthesis.

To determine whether SCD is present in MAM, SCD activity assay and Western blot analysis were performed using the subcellular fractions. The MAM, mitochondria, and ER fractions were isolated from mouse livers as described in Materials and Methods. Both the Western blot and specific activity data show that SCD is present in both MAM and ER at a similar level but absent in mitochondria (**Fig. 1A, B**). DGAT activity is present in all three fractions, with the specific activity being highest in MAM (Fig. 1C), which agrees with the observation by Rusinol et al. (22) that the specific activity of DGAT is higher in MAM compared with the bulk ER. As a control, NADPH-cytochrome C reductase and cytochrome C oxidase were used as ER and mitochondrion marker enzymes, respectively, to characterize the subcellular fractions. Figure 1D shows the specific activity of the ER marker NADPH-cytochrome C reductase in MAM, mitochondria, and ER. As expected, no activity was detected in mitochondria. The specific enzyme activity was ~2-fold higher in ER than in MAM, which is in accordance with the characteristic 2-fold difference reported by other groups (16, 17, 22). Figure 1E shows the specific activity of the mitochondrion marker cytochrome C oxidase in the different subcellular fractions. These results were in agreement with the expectation of the specific activity being highest in mitochondria and lowest in the ER.

Coexpression of SCD1 and DGAT2 further promotes triglyceride synthesis

Listenberger et al. (23) have shown that the level of desaturase activity correlates with triglyceride storage, suggesting that endogenously synthesized monounsaturated

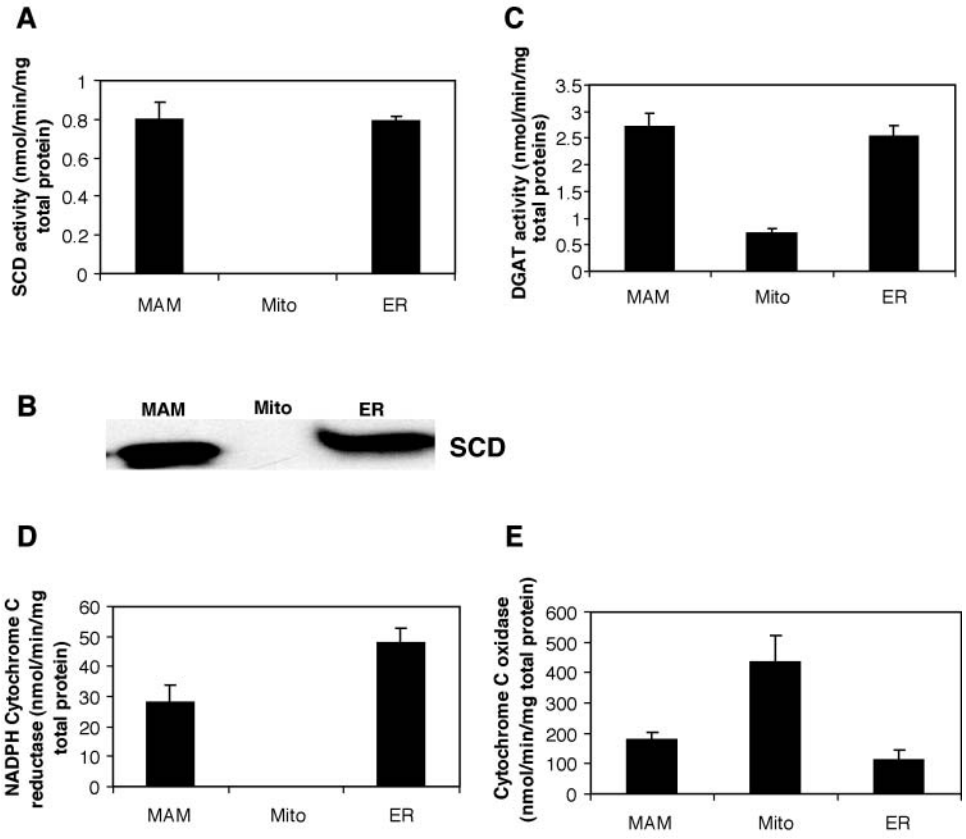

Fig. 1. Distribution of stearoyl-coenzyme A desaturase (SCD) among mitochondria-associated membrane (MAM), mitochondria (Mito), and endoplasmic reticulum (ER). A, B: Distribution of SCD activity and protein among MAM, mitochondria, and ER. C: Distribution of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity. D, E: Distribution of marker enzyme activity among the subcellular fractions. NADPH-cytochrome C reductase is the ER marker enzyme (D), and cytochrome C oxidase is the mitochondrial marker enzyme (E). Values shown are means ± SD (n = 3).

fatty acids promote triglyceride production. The importance of DGAT2 in triglyceride synthesis has been revealed in the significant reduction in triglyceride content in *Dgat2*$^{-/-}$ mice (12) and in primary mouse hepatocytes in which DGAT2 expression is reduced by antisense oligonucleotide (24). To test whether triglyceride synthesis can be induced by overexpressing SCD1 or DGAT2, we transfected HeLa cells with the SCD1-myc construct (SCD1 with myc epitope) or the DGAT2-FLAG construct (DGAT2 with FLAG epitope). Although HeLa cells may not be reflective of the major triglyceride-synthesizing cells, they were chosen as the model of study because of their low basal level of SCD activity (25). The transfected cells were incubated with serum-free medium containing [^{14}C]18:0 conjugated to BSA for 16 h. Total lipids were then extracted from the cells and separated by thin-layer chromatography. Triglyceride (cpm/mg total protein) is increased in HeLa cells overexpressing SCD1 or DGAT2 by 2.0- and 2.6-fold, respectively, compared with cells transfected with pcDNA (**Fig. 2A**). The observation that the mere overexpression of SCD1 increases triglyceride synthesis supports the notion that endogenously synthesized monounsaturated fatty acids promote triglyceride production. We then hypothesized that when we coexpressed SCD1 in cells expressing DGAT2, the triglyceride synthesis would further increase compared with cells expressing DGAT2 alone, as a result of the increased availability of this pool of endogenously synthesized monounsaturated fatty acids. Figure 2A shows that the triglyceride (cpm/mg total protein) is highest in cells coexpressing SCD1 and DGAT2. The expression of SCD1 or DGAT2 does not affect the transport of labeled fatty acids into the cells (data not shown). Figure 2B shows the ratio of oleate to stearate (18:1/18:0) in the triglyceride fractions. The ratio is higher in samples in which SCD1 is overexpressed, confirming that the desaturase activity is higher in these groups. Figure 2C–F show the results of Oil Red O staining on the four groups of cells described above. The accumulation of triglycerides as represented by the red staining is increased in cells expressing SCD1 and/or DGAT2.

SCD1 and DGAT2 colocalize in the same subcellular compartment, as shown by confocal microscopy

The results presented in the previous sections suggested that SCD1 and DGAT2 may be linked in triglyceride synthesis. We hypothesized that endogenously synthesized monounsaturated fatty acids promote triglyceride accumulation because SCD1 and DGAT2 are located in close

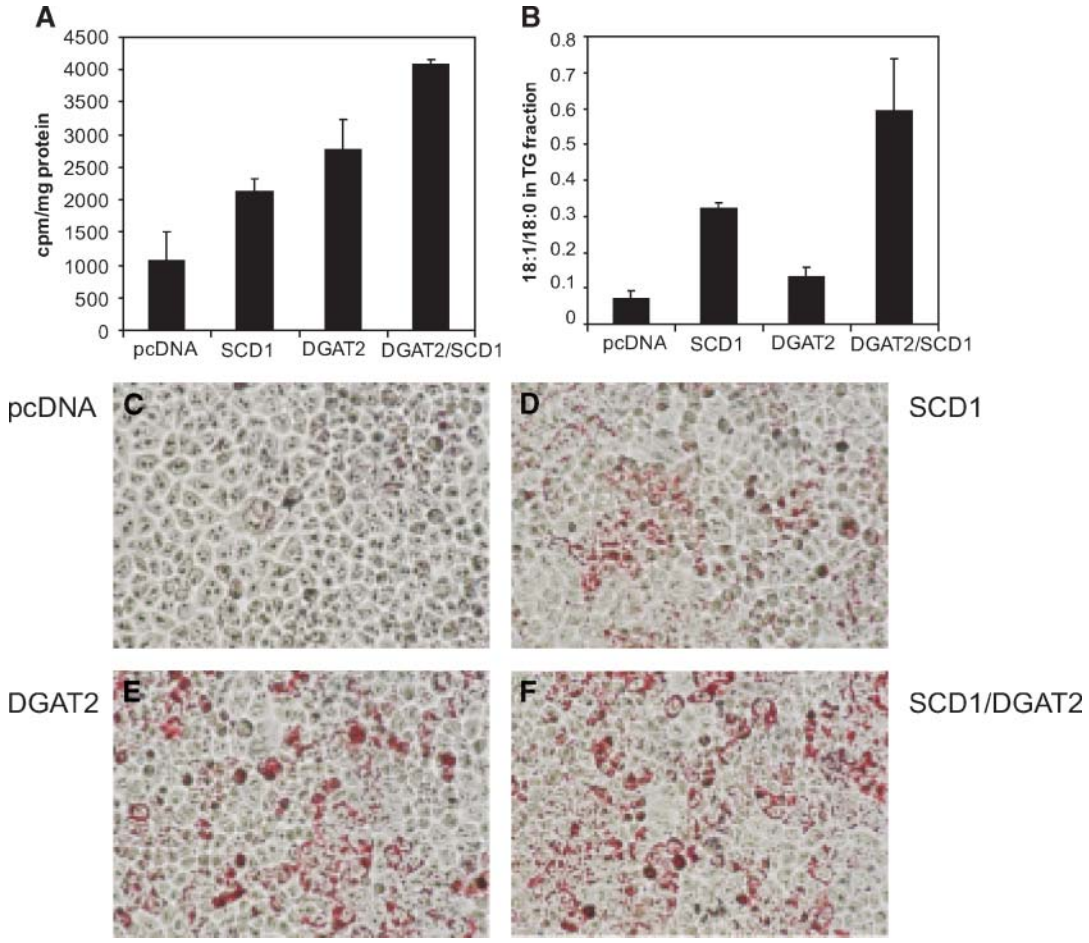

Fig. 2. Triglyceride synthesis in HeLa cells expressing SCD1-myc and DGAT2-FLAG and the ratio of 18:1/18:0 in the triglyceride fraction. A: HeLa cells were transfected with *1*) pcDNA, *2*) SCD1-myc only, *3*) DGAT2-FLAG only, or *4*) SCD1-myc and DGAT2 using TransIT-LT1. The cells were incubated with serum-free medium containing [^{14}C]stearate coupled to BSA. Forty-eight hours after transfection, the cells were lysed with lysis buffer and total lipids were extracted and separated by thin-layer chromatography with petroleum hexane-diethyl ether-acetic acid (80:30:1) as the developing solvent. The counts in the triglyceride (cpm) were determined by Packard Instant Imager. Values shown are means ± SD (n = 3). *$P < 0.05$ versus SCD1 $P < 0.05$ versus DGAT2. B: The triglyceride (TG) band on the TLC plate was scraped, and the free fatty acids in the fraction were released. The free fatty acids were then spotted onto a silica gel impregnated with silver nitrate, and stearate and oleate were separated using the solvent system chloroform-methanol-acetic acid-water (90:8:1:0.8). The counts corresponding to the ^{14}C-labeled stearate and oleate were determined by autoradiography using Packard Instant Imager. Values shown are means ± SD (n = 3). * $P < 0.05$ versus SCD1, $P < 0.05$ versus DGAT2. C–F: Accumulation of triglycerides as represented by Oil Red O staining of the cells expressing the four groups of cells described above.

proximity, thus allowing some substrate channeling to occur. As a first step to evaluate whether SCD1 is proximal to DGAT2, we performed immunofluorescence confocal microscopy on HeLa cells transfected with the constructs SCD1-myc and DGAT2-FLAG. Approximately 24 h after transfection, the cells were fixed with 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100. The permeabilized cells were then incubated with anti-myc and anti-FLAG antibodies for 1 h, followed by incubation with the corresponding Alexa-Fluor-conjugated secondary antibodies. SCD1 and DGAT2 are ER proteins (1, 14). **Figure 3A, B** show the signature meshwork-like ER distribution of SCD1 and DGAT2, respectively. Extensive colocalization of the two proteins is represented by yellow color (Fig. 3C) when Fig. 3A, B are superimposed. We also evaluated the extent of colocalization of SCD1 with another ER protein, Bip, which is not known to be directly involved in the lipid synthesis pathway. The distribution of SCD1 and Bip is shown in Fig. 3D, E, respectively. Figure 3F represents the merged images of Fig. 3D, E.

SCD1 coimmunoprecipates with DGAT2

HeLa cells were transfected with SCD1-myc and DGAT2-FLAG. Forty-eight hours after transfection, the cells were harvested and treated with lysis buffer. The cell extract was then immunoprecipitated with either anti-myc antibody or anti-FLAG antibody. The protein A/G agarose beads were boiled to release the precipitated proteins, which were then separated by SDS-PAGE and analyzed by Western blotting. Cell lysate containing SCD1-myc and DGAT2-FLAG was immunoprecipitated with FLAG (**Fig. 4A, B**, lane 1) or myc (Fig. 4A, B, lane 3) antibodies. Cell lysates from cells transfected with pcDNA were immunoprecipitated similarly to serve as negative controls to test whether

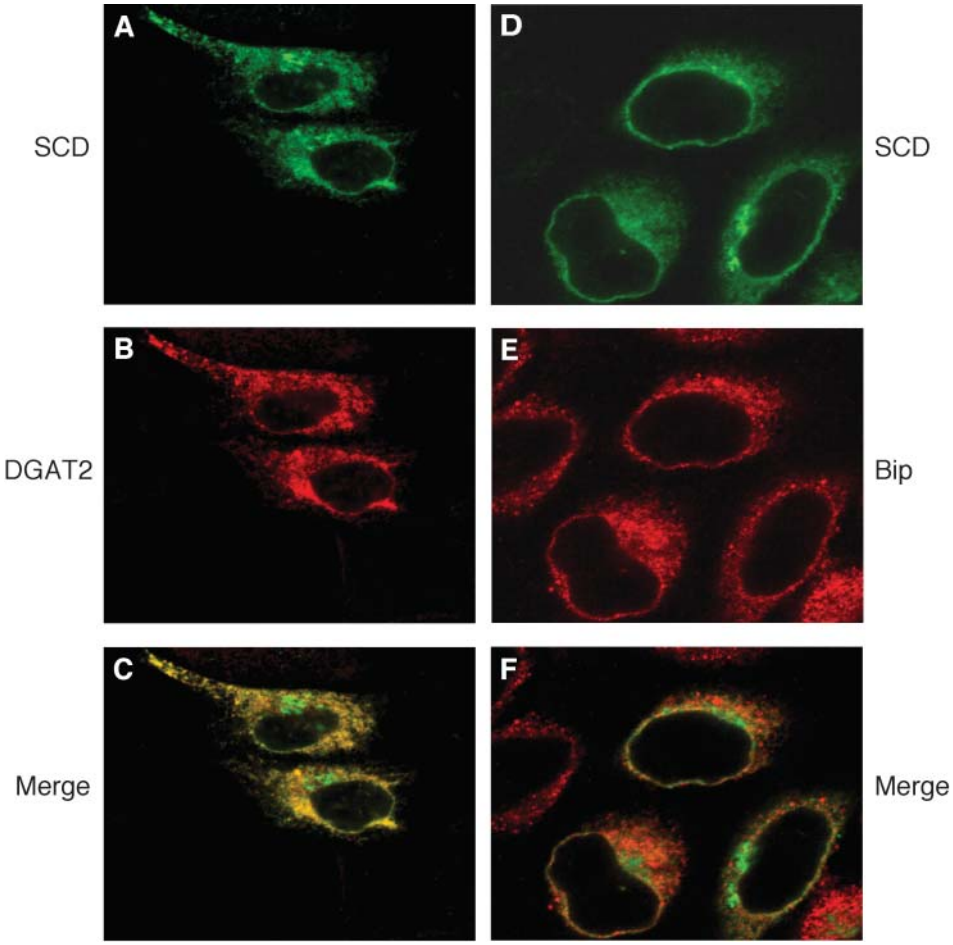

Fig. 3. Colocalization study of SCD1-myc with DGAT2-FLAG using confocal microscopy. A, B: Distribution of SCD1-myc (green) and DGAT2-FLAG (red). C: Merged image. D, E: Distribution of SCD1-myc (green) and Bip (red). F: Merged image.

any contaminants in the cells could be precipitated by the antibodies. SCD1-myc and DGAT2-FLAG coimmunoprecipitated with each other whether anti-myc or anti-FLAG antibodies were used for the immunoprecipitation procedure, suggesting that interaction may occur between SCD1-myc and DGAT2-FLAG (Fig. 4A, B). As another negative control, we tried to immunoprecipitate SCD1-myc with anti-FLAG antibody and DGAT2-FLAG with anti-myc antibody. Lane 3 in Fig. 4C represents cell lysate containing SCD1-myc immunoprecipitated with FLAG antibody, and the results show that FLAG antibody cannot immunoprecipitate SCD1-myc. Lane 4 in Fig. 4D represents cell lysate containing DGAT2-FLAG immunoprecipitated with myc antibody, and no DGAT2-FLAG was detected in the precipitated proteins. These results indicate that the antibodies are specific for the corresponding epitopes and that the results observed in Fig. 4A, B are not attributable to nonspecific binding of the antibodies to the tagged proteins.

FRET by flow cytometry suggests protein-protein interaction between SCD1 and DGAT2

As an alternative way to examine whether there is interaction between SCD1 and DGAT2, we tested whether FRET occurred when SCD1-CFP and DGAT2-YFP were coexpressed in HeLa cells. FRET is a radiationless transfer of energy (21). It occurs when the energy emitted by the donor chromophore upon excitation is transferred to and excites an acceptor chromophore with lower energy absorption (21, 26). One important criterion for FRET to take place is that the donor and acceptor chromophores have to be very close to each other, thus allowing FRET to be used as a reliable indicator for protein-protein interaction (21, 26). The efficiency of energy transfer is inversely proportional to the sixth power of the distance between the chromophores (27), hence a slight increase in the distance may render FRET indistinguishable from the background. Förster radius is defined as the distance at which the energy transfer efficiency reaches 50%; for a CFP/YFP pair, the radius is ~50 Å.

As a first step for the FRET experiment, we cloned SCD1 into pECFP vector and DGAT2 into pEYFP vector, giving rise to the constructs SCD1-CFP and DGAT2-YFP. These constructs were expressed in HeLa cells, and confocal microscopy data indicate that the recombinant proteins are targeted to the ER membrane (**Fig. 5A, B**). The white color in Fig. 5C represents colocalization of the two proteins.

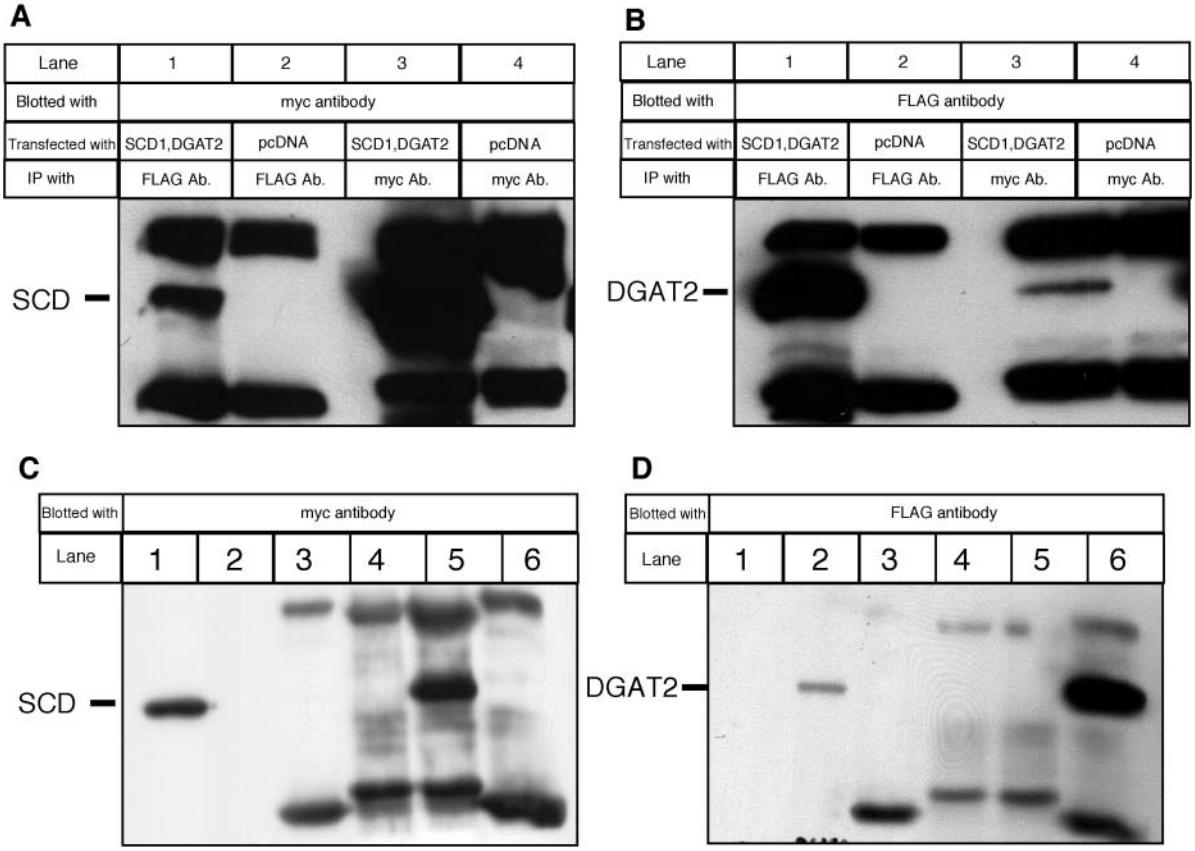

Fig. 4. Coimmunoprecipitation of SCD1-myc and DGAT2-FLAG. SCD1 and DGAT2 have very similar molecular weights. A: The blot was probed with anti-myc antibody. Cell lysate containing SCD1-myc and DGAT2-FLAG was immunoprecipitated (IP) with FLAG (lane 1) or myc (lane 3) antibodies. Cell lysate from cells transfected with pcDNA was immunoprecipitated similarly to serve as a negative control. B: The immunoprecipitation procedure was carried out as described for A except that the blot was probed with anti-FLAG antibody. C: The blot was probed with anti-myc antibody. Cell lysate from cells expressing SCD1-myc only (lane 1) or DGAT2-FLAG only (lane 2) was loaded. Lane 3 represents cell lysate containing SCD1-myc immunoprecipitated with FLAG antibody. Lane 4 represents cell lysate containing DGAT2-FLAG immunoprecipitated with myc antibody. Lane 5 represents postimmunoprecipitation supernatant obtained from the sample in lane 3 and immunoprecipitated with myc antibody. Lane 6 represents postimmunoprecipitation supernatant obtained from the sample in lane 4 and immunoprecipitated with FLAG antibody. D: Same as in C except that the blot was probed with anti-FLAG antibody.

Generally, attachment of green fluorescent protein and its derivatives, such as CFP and YFP, to a protein does not affect its activity or the subcellular distribution (21). However, to ensure that the detection of FRET, if any, is attributable to the interaction of functional proteins, we performed SCD activity and DGAT activity assays on microsomes expressing SCD1-CFP or DGAT2-YFP. Figure 5D, E indicate that the two recombinant proteins are functional. Compared with the cells expressing the empty pcDNA vector, cells expressing SCD1-CFP and DGAT2-YFP show 5.8-fold and 1.6-fold increases in SCD and DGAT activities, respectively.

Four groups of HeLa cells were transfected with the constructs as follows: *a*) SCD1-CFP; *b*) DGAT2-YFP; *c*) SCD1-CFP and YFP; and *d*) SCD1-CFP and DGAT2-YFP. The fluorescence properties of each group of live samples were analyzed by flow cytometry. A dual-laser flow cytometer was used in this experiment, and three fluorescence measurements were used for FRET analysis: CFP fluorescence, YFP fluorescence, and the FRET channel, which represents the YFP fluorescence produced by CFP excitation. Because free cytosolic YFP is not expected to interact with SCD1-CFP, HeLa cells coexpressing SCD1-CFP and YFP serve as a negative control. If HeLa cells coexpressing SCD1-CFP and DGAT2-YFP show a significantly higher FRET intensity than the negative control group, interaction between SCD1 and DGAT2 occurs.

Because the HeLa cells were transiently transfected, not every cell would express both CFP-SCD1 and DGAT2-YFP. However, FRET will only occur in cells that express both recombinant proteins. Therefore, we transfected two groups of cells that expressed either CFP-SCD1 or DGAT2-YFP, which would serve as the reference for setting the gate (CFP+ YFP+) that would include cells coexpressing both fusion proteins. Flow cytometry data were plotted in log scale dot plots. **Figure 6A, B** represent live cells expressing CFP-SCD1 and DGAT2-YFP, respectively. The gate (CFP+ YFP+) was derived and is shown in both panels. Figure 6C, D show the data obtained from the negative control group, CFP-SCD1 and YFP. The CFP+ YFP+ gate shown in Fig. 6C crops the cells that coexpress CFP-SCD1 and YFP. CFP fluorescence was plotted against the yellow fluorescence

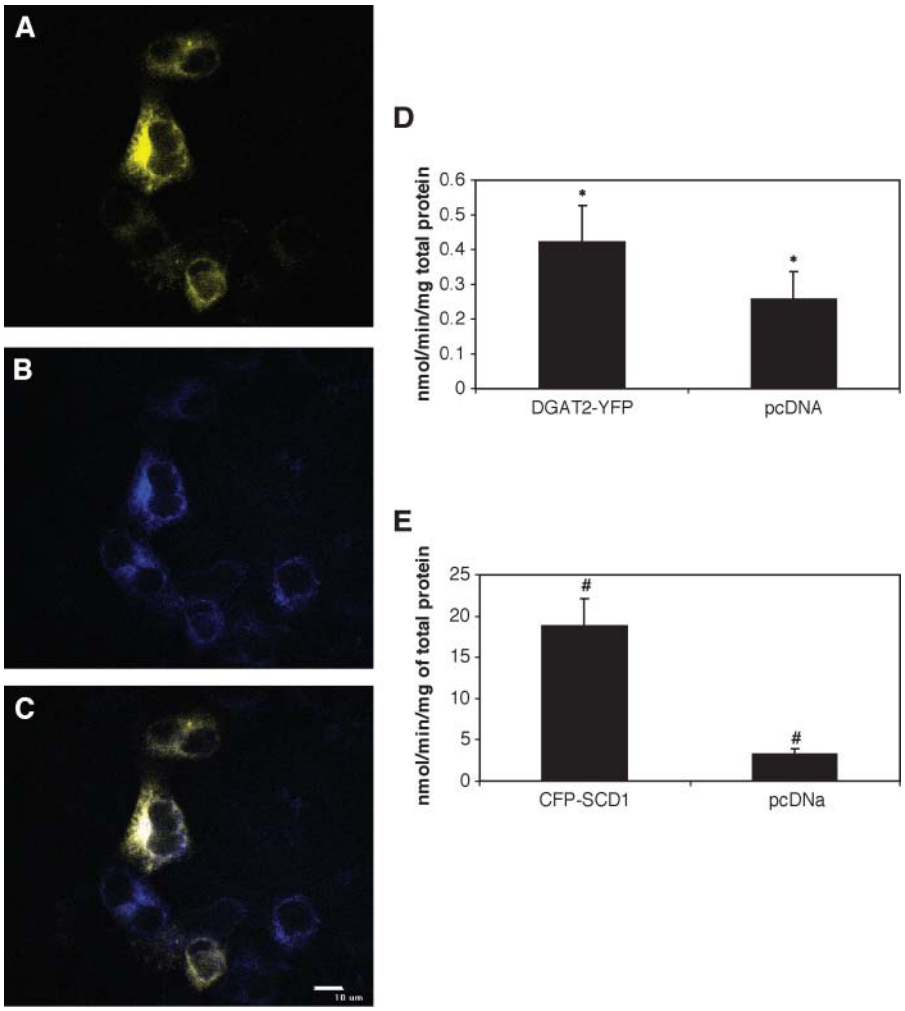

Fig. 5. Subcellular distribution and functional analysis of the recombinant proteins cyan fluorescent protein (CFP)-SCD1 and yellow fluorescent protein (YFP)-DGAT2. A, B: Distribution of CFP-SCD1 and YFP-DGAT2, respectively. C: Superimposed images from A and B. D: Results of DGAT activity assay (means ± SD, n = 3; * $P < 0.05$). E: Results of SCD activity assay (means ± SD, n = 4; # $P < 0.05$).

from the FRET channel, and these data are displayed in Fig. 6D. Because CFP-SCD1 and free YFP will not interact, a gate (FRET) was drawn in Fig. 6D to identify the region on the plot that will only include cells exhibiting FRET intensities greater than the negative control. The left boundary of the gate represents the threshold of FRET. Figure 6E, F represent the data obtained from the experimental group, cells that express both CFP-SCD1 and DGAT2-YFP. Only cells that fell into the CFP+ YFP+ gate in Fig. 6E were analyzed for FRET. A total of 18.2% of the cells coexpressing CFP-SCD1 and YFP-DGAT2 fell into the FRET gate in Fig. 6F. It has been reported in another study that the occurrence of FRET was indicated by the observation that 15% of the cells coexpressing T70-YFP and T70-CFP recombinant proteins fell into a FRET gate (21). This suggests that the 18.2% that we observed was not attributable to noise signal. Furthermore, the histogram in Fig. 6G indicates that the experimental sample shows overall higher fluorescence intensities in the FRET channel than the negative control, as represented by the shift of the peak. Together, these results indicate the occurrence of FRET and suggest protein-protein interaction between SCD1 and DGAT2.

DISCUSSION

SCD is the enzyme catalyzing the desaturation of saturated fatty acids (1). Significant reduction in lipid accumulation, especially triglycerides, is observed in Scd1$^{-/-}$ mice, suggesting that the loss of SCD1 function protects mice against adiposity (1, 4, 7, 28). Studies have suggested that this protection is achieved from changes in two pathways: the increase in fatty acid oxidation and the reduction in lipogenesis (1, 28). In Scd1$^{-/-}$ mice, the enzymes responsible for lipogenesis/lipid synthesis are downregulated, whereas the expression of the enzymes involved in fatty acid oxidation is increased. The focus of the current study was to gain a deeper understanding of the role of SCD in the lipid synthesis pathway. Similar to SCD,

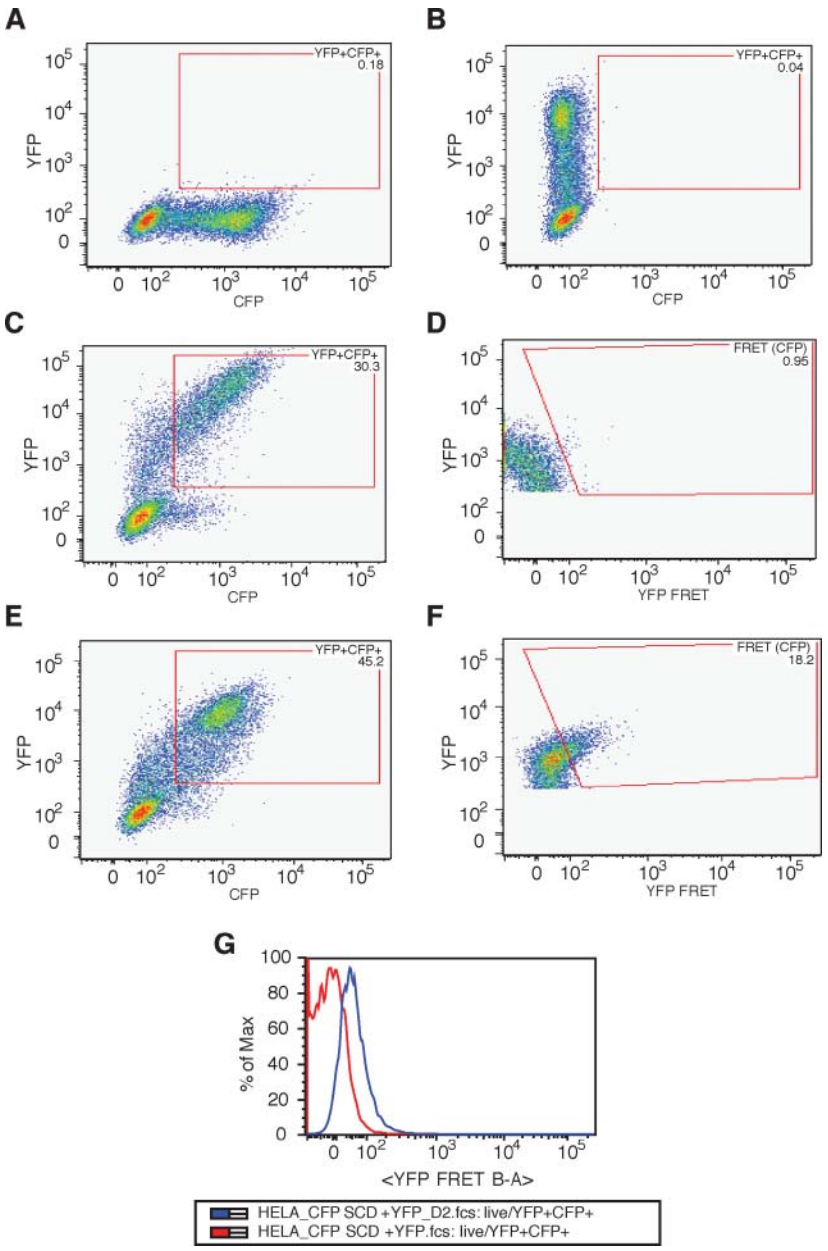

Fig. 6. Fluorescence resonance energy transfer (FRET) analysis using flow cytometry on live cells expressing CFP-SCD1 and YFP-DGAT2. HeLa cells were transfected transiently with the corresponding constructs and harvested for analysis 12 h after transfection. The data from live cells are displayed in log-scale dot plots. A, B: Live cells expressing CFP-SCD1 or YFP-DGAT2. The gate (CFP+ YFP+) including cells coexpressing both recombinant proteins was derived and is shown in both panels. C, D: Data obtained from cells coexpressing CFP-SCD1 and free YFP, which served as the negative control. The CFP+ YFP+ gate shown in C crops the cells that coexpress CFP-SCD1 and YFP. The FRET gate in D identifies the region on the plot that only includes cells exhibiting FRET intensities greater than the negative control. E, F: Data derived from cells coexpressing CFP-SCD1 and YFP-DGAT2. Only cells that fell into the CFP+ YFP+ gate in E were analyzed for FRET. A total of 18.2% of the cells coexpressing CFP-SCD1 and YFP-DGAT2 fell into the FRET gate in F. G: The experimental sample shows an overall higher fluorescence intensity in the FRET channel than does the negative control, as represented by the shift of the peak.

another key player in the triglyceride synthesis pathway is DGAT, which links a fatty acyl-CoA with a *sn*-1,2-diacylglycerol (29, 30). Between the two isoforms of DGAT, DGAT2 is crucial for survival, as the deficiency of DGAT2 results in death soon after birth (12). Although mice lacking DGAT1 or DGAT2 show decreased production of triglycerides, the disruption of DGAT2 results in an almost complete absence of triglyceride in tissues compared with the wild type (12). Interestingly, despite the dramatic reduction in hepatic triglyceride content, the DGAT activity and the diacylglycerol content in the liver of Scd1$^{-/-}$ mice are not significantly different compared with those of wild-type

mice (31). This strongly suggests that monounsaturated fatty acids are crucial in the production of triglyceride. A positive correlation has been observed between the level of desaturase activity and triglyceride accumulation, suggesting that the endogenously produced unsaturated fatty acids promote the storage of triglyceride (23). It is possible that organisms have adopted this system to prevent the accumulation of saturated fatty acids, which can induce apoptosis (32).

It is interesting that the lack of an enzyme that supplies substrates for the production of triglyceride leads to a dramatic decrease in triglyceride synthesis, as in animals deficient in the enzyme that catalyzes the final reaction in the triglyceride synthesis pathway. This observation led us to the hypothesis on which the current study is based: SCD1 is proximal to DGAT2, and it participates in the synthesis of triglycerides by producing a more easily accessible pool of monounsaturated fatty acids. In this study, we provide evidence for the first time that SCD is present in a subcompartment of the ER called the MAM, which has been proposed to play a role in supplying lipids for packaging VLDL in the secretory pathway (22). The colocalization of SCD and DGAT in a compartment that is involved in active lipid synthesis suggests that SCD and DGAT work together in the synthesis of triglycerides. This observation led us to perform in vitro studies to test whether SCD1 and DGAT2 are very close to each other physically. We used various approaches, such as confocal immunofluorescence, coimmunoprecipitation, and FRET using flow cytometry, to test the hypothesis, and the data independently suggest that SCD1 and DGAT2 are in close proximity. Results from confocal immunofluorescence display an extensive colocalization of the two proteins in the ER, as represented by yellow images that result from superimposing images representing SCD1-myc (green) and DGAT2-FLAG (red) subcellular distribution. Coimmunoprecipitation studies show that in HeLa cells coexpressing SCD1-myc and DGAT2-FLAG, SCD1-myc coprecipitates with DGAT2-FLAG whether anti-myc antibody or anti-FLAG antibody is used in the procedure, suggesting a possible interaction between the two proteins. Because the key requirement for FRET to occur is the extreme close proximity between the chromophores, we made use of this characteristic to analyze FRET between CFP-SCD1 and YFP-DGAT2 using flow cytometry as a marker of interaction between the two proteins. These data suggest that FRET occurs between the two fusion proteins. Fusing CFP or YFP to the proteins of interest, in this case SCD1 and DGAT2, allows us to analyze the possible interaction between them in live cells. As for flow cytometry, apart from the advantage that a large number of cells can be analyzed in a short period of time, this technique also allows us to minimize the effect of spectral bleedthrough when assessing FRET by compensation.

The major question asked in this study is, what is a possible mechanism through which endogenously synthesized fatty acids affect the synthesis of triglyceride? We proposed that the monounsaturated fatty acids synthesized by SCD are more accessible to DGAT. In fact, an increase in the accumulation of hepatic triglyceride is observed in SCD1-deficient mice only after long-term feeding of a diet containing 20% triolein (6), but the level of triglyceride accumulated is still lower than that in wild-type animals. However, when the percentage of fat in the diet is reduced to 5% triolein, the increase in hepatic triglyceride content is absent, suggesting that exogenous fatty acids exert an effect only when they are administered at a nonphysiologically high level.

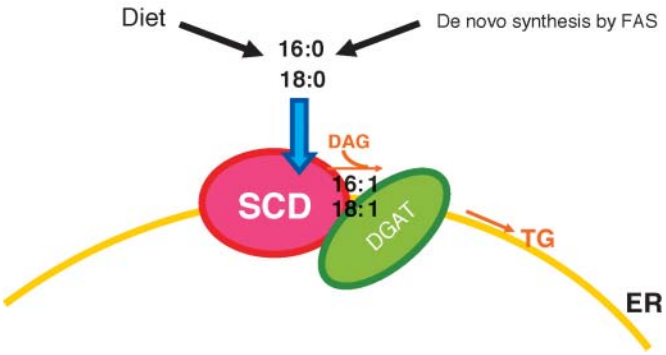

Fig. 7. Proposed model for the channeling of endogenously synthesized monounsaturated fatty acids from SCD1 to DGAT2 in the synthesis of triglycerides (TG). Palmitate (16:0) and stearate (18:0) from the diet or de novo synthesis of fatty acids are desaturated by SCD and channeled to DGAT for the final step in triglyceride synthesis in the ER. DAG, diacylglycerol.

Several observations have suggested that SCD1 is more tightly linked with DGAT2 in the synthesis of triglyceride. The *ob/ob* mouse is morbidly obese. In *ob/ob* mice, SCD1 expression is increased significantly (33). However, when ob/+ mice are intercrossed with heterozygous asebia ($ab^{J/+}$) mice, which contain a natural mutation in the SCD1 gene, the ab^{J}/ab^{J}; *ob/ob* mice become significantly less obese and have dramatic reductions in hepatic triglyceride levels (33). Similar to SCD1, DGAT2 is also highly expressed in *ob/ob* mice, whereas the expression level of DGAT1 is not significantly different compared with wild-type mice, suggesting that DGAT2 is more responsible for the accumulation of fat in *ob/ob* mice (34). When mice are fasted and refed with a high-carbohydrate/low-fat diet, the mRNA levels of lipogenic enzymes, including fatty acid synthase, acetyl-CoA carboxylase, SCD, and DGAT2, increase drastically, whereas the expression DGAT1 does not change significantly (34). This again suggests that DGAT2 may be more closely related to the endogenously synthesized fatty acids (34).

In conclusion, this study has provided evidence that SCD1 and DGAT2 are located in close proximity to each other in the ER, as depicted in the model shown (**Fig. 7**). Palmitate (16:0) and stearate (18:0) from the diet or de novo synthesis of fatty acids are desaturated by SCD and channeled to DGAT for the final step in triglyceride synthesis in the ER. The close association of SCD and DGAT thus increases the efficiency of triglyceride synthesis. Our findings may provide another explanation for why endogenously synthesized monounsaturated fatty acids and hence SCD1 are important in triglyceride metabolism.

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