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Monoacylglycerol Acyltransferase-2 Is a Tetrameric Enzyme That Selectively Heterodimerizes with Diacylglycerol Acyltransferase-1*

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Background: MGAT2 and DGAT1 play essential role in catalyzing the biosynthesis of triglyceride and mediating intestinal fat absorption.
Results: MGAT2 heterodimerizes with DGAT1, a process that is mediated by amino acids 35–80 of DGAT1 but not an N-signal peptide of MGAT2.
Conclusion: MGAT2 may be part of an enzyme complex.
Significance: Our findings provide direct molecular evidence of the interactions of essential enzymes for fat absorption.

Acyl-CoA:monoacylglycerol acyltransferases (MGATs) and diacylglycerol acyltransferases (DGATs) catalyze the two consecutive steps in the synthesis of triacylglycerol, a key process required for dietary fat absorption into the enterocytes of the small intestine. In this report, we investigated the tendency of MGAT2 to form an enzyme complex with DGAT1 and DGAT2 in intact cells. We demonstrated that in addition to the 38-kDa monomer of the MGAT2 enzyme predicted by its peptide sequence, a 76-kDa moiety was detected in SDS-PAGE without reducing agent and heat inactivation. The 76-kDa MGAT2 moiety was greatly enhanced by treatment with a cross-linking reagent in intact cells. Additionally, the cross-linking reagent dose-dependently yielded a band corresponding to the tetramer (152 kDa) in SDS-PAGE, suggesting that the MGAT2 enzyme primarily functions as a homotetrameric protein and as a tetrameric protein. Likewise, DGAT1 also forms a homodimer under nondenaturing conditions. When co-expressed in COS-7 cells, MGAT2 heterodimerized with DGAT1 without treatment with a cross-linking reagent. MGAT2 also co-eluted with DGAT1 on a gel filtration column, suggesting that the two enzymes form a complex in intact cells. In contrast, MGAT2 did not heterodimerize with DGAT2 when co-expressed in COS-7 cells, despite high sequence homology between the two enzymes. Furthermore, systematic deletion analysis demonstrates that N-terminal amino acids 35–80 of DGAT1, but not a signal peptide at the N terminus of MGAT2, is required for the heterodimerization. Finally, co-expression of MGAT2 with DGAT1 significantly increased lipogenesis in COS-7 cells, indicating the functional importance of the dimerization.

Dietary fat, mainly consisting of triacylglycerols (TAG)[4] and phospholipids, is a major energy source for humans in most developed countries. Under normal physiological conditions, the digestion and absorption of dietary lipids is a very efficient process, facilitated by a series of digestive and assimilating enzymes. Dietary TAG and/or phospholipids are first hydrolyzed by lipases in the lumen of stomach and intestine into monoacylglycerol and fatty acids. Through mechanisms that are still not completely understood, the fatty acids and monoacylglycerol are taken up into the enterocytes from mixed micelles (1, 2). Once inside the enterocytes, these products are transported to the endoplasmic reticulum, where they undergo consecutive re-esterification, first into *sn*-2,3(1)-DAG catalyzed by monoacylglycerol acyltransferases (MGATs) and then into TAG catalyzed by diacylglycerol acyltransferases (DGATs) before being packaged into chylomicrons for secretion into the lymphatic system for transport into the bloodstream and other organs (3, 4). An alternative pathway involved in the absorption process is the glycerol 3-phosphate pathway, a *de novo* triglyceride synthesis pathway that is believed to play a role in mediating TAG biosynthesis only in the resting state in the small intestine but accounts for the majority of TAG biosynthesis in most other tissues (1). Because of the importance of MGAT and DGAT in fat absorption and storage, considerable studies have been conducted to elucidate their molecular and enzymatic features. Facilitated by the recent advances in genomics and bioinformatics, we and other groups have identified three mammalian MGAT enzymes (designated MGAT1, MGAT2, and MGAT3) and two DGAT enzymes (DGAT1 and DGAT2) (5–10).

Consumption of a Western diet enriched with animal fat is one of the main contributing factors to the ongoing obesity epidemic. In industrialized countries, dietary fat intake contrib-

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[4] The abbreviations used are: TAG, triacylglycerol(s); MGAT, acyl-CoA:monoacylglycerol acyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; DSP, dithiobis(succinimidyl propionate); ER, endoplasmic reticulum; ORO, Oil Red O.

utes over 40% of the caloric content of daily food consumption, which is equivalent of 100 g of lipids and 4 g of phospholipids, such as phosphatidylcholine (lecithin). As an energy-dense fuel source, a high fat diet causes leptin and insulin resistance (11), thus contributing to the onset of obesity and its related metabolic complications. The enzymatic reactions catalyzed by MGATs and DGATs are believed to be an essential step for fat absorption in the small intestine, and thus have been subjected to intense investigation as novel drug targets for obesity (12–17). Among the three MGAT enzymes identified recently (7–9), MGAT2 is predominantly expressed in small intestine and plays a predominant role in dietary fat absorption. MGAT2 is a membrane protein associated with the endoplasmic reticulum (ER), and its expression and activity are induced by a high fat diet (18–20), thus providing a potential drug target for the treatment of obesity (12, 21). In support of a potential role of MGAT2 in obesity, mice with targeted deletion of MGAT2 exhibit resistance to the onset of diet-induced obesity and its related metabolic complications (22–24).

DGAT catalyzes the last step in the synthesis of TAG that merges the MGAT and glycerol 3-phosphate pathways. Although both DGAT enzymes (DGAT1 and DGAT2) identified thus far catalyze the same reaction, they share no sequence homology and exhibit quite different functional roles in lipid metabolism. In contrast to MGAT enzymes that show restricted expression in gastrointestinal tissues, both DGAT enzymes are widely expressed in various tissues due to their involvement in both TAG resynthesis in enterocytes for dietary absorption and *de novo* fat synthesis in lipogenic tissues. TAG is the major stored form of energy in mammals, and excessive accumulation of TAG in white adipose tissues leads to obesity. Thus, mice with targeted deletion of the *DGAT1* gene demonstrate a reduction in TAG levels, an increase in metabolic rate, and resistance to diet-induced obesity. Surprisingly, there is no major defect in overall dietary fat absorption in DGAT1-deficient mice, which could be compensated for by DGAT2 or MGAT enzymes that possess DGAT activity (25). In contrast, inactivation of DGAT2 in mice leads to neonatal lethality, lipopenia, and skin barrier abnormalities resulting from defective TAG synthesis and storage in lipogenic tissues. The lethal phenotype is apparently not compensated for by DGAT1, as evidenced by different subcellular localization of TAG catalyzed by the two enzymes in intact cells. It remains elusive whether a 50% reduction in DGAT2 expression impairs dietary fat absorption in heterozygous DGAT2 knock-out mice.

Enzymes involved in TAG synthesis are believed to form complexes both in yeast and mammals. The formation of enzyme complex might improve catalytic efficiency by increasing the proximity of substrates to corresponding enzymes and improving the turnover rate of substrate to product. Despite the important roles of MGAT and DGAT enzymes in regulating lipid absorption and metabolism, little is known about whether the two families of enzymes interact with each other in intact cells. In this study, we report characterization of MGAT2 for its biochemical features in dimer formation and its interactions with DGAT1 as well as the implications of such interactions for enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—*sn*-2-Monooleoylglycerol, *sn*-1,2-dioleoylglycerol, *rac*-1,2-dioleoylglycerol, *sn*-1,3-dioleoylglycerol, 1,2,3-trioleoylglycerol, oleic acid, and oleoyl-CoA were purchased from Doosan Serdary Research Laboratories (Toronto, Canada). [^{14}C]Oleoyl-CoA (50 mCi/mmol) was obtained from American Radiolabeled Chemicals Inc. All other chemicals and solvents used in this study were obtained from Sigma-Aldrich.

Generation and Expression of MGAT2 and DGAT1 Deletion Mutants—A deletion mutant of human MGAT2 with a truncation of the N-terminal signal peptide (Fig. 6*A*) was engineered by PCR amplification using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and the resulting PCR products were cloned into the pPCR-script Amp SK(+) vector (Stratagene) and sequenced. To identify the interaction domain of DGAT1 with MGAT2, several deletion mutants of DGAT1 were generated using the QuikChange II site-directed mutagenesis kit (Stratagene). Primer pairs used were as follows: Δ1F (5′-GGCTTCAGCAACTACCGTGGC-3′) and Δ1R (5′-GTAGTTGCTGAAGCCGGCGTCCCGCACCTCCTCTTC-3′) for Δ2–35 deletion mutant; Δ2F (5′-GGCTTCAGCAACTACCGTGGC-3′) and Δ2R (5′-GTAGTTGCTGAAGCCCTTGTCGTCATCGTCTTTGTAGTCCAT-3′) for Δ2–80 deletion mutant; Δ3F (5′-GCCACCATTCTGTGTTTCCCA-3′) and Δ3R (5′-ACACAGAATGGTGGCCTCAACCTGGAATGCAGCCAC-3′) for Δ152–172 deletion mutant; and Δ4F (5′-CGCCTCTGGGCGTTCACG-3′) and Δ4R (5′-GAACGCCCAGAGGCGTGTCCTGGCCATCCACTTGCT-3′) for Δ406–425 deletion mutant. N-terminal FLAG-tagged human DGAT1 in pcDNA3.1 was used as the template for all mutagenesis reactions. All plasmids were confirmed in the presence of the desired mutations by sequencing.

Oil Red O Staining—COS-7 cells were transiently transfected with MGAT2, DGAT1, or the two in combination. After 36 h of transfection, lipid droplet formation was stimulated by incubating cells with 0.5 mM sodium oleate complexed to 0.67% fatty acid-free bovine serum albumin (molar ratio, 4.7:1) for 12 h. After washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then subjected to Oil Red O (ORO) staining. Cell images were acquired using an Olympus IX71 inverted microscope. For quantification, isopropyl alcohol was added to cells to dissolve ORO after taking images, and then the absorbance at 510 nm was measured.

Western Blot Analysis—293E cells were transiently transfected with MGAT2, DGAT1, DGAT1 deletion mutants, or the three in combination. After 48 h of transfection, cells were lysed in Bug Buster HT reagent (Novagen, San Diego, CA). Cell lysates were dissolved in 2× sample buffer (Invitrogen) and then directly resolved on Novex 4–20% Tris-glycine PAGE (Invitrogen) and transferred to a PVDF membrane. The membrane was blocked in 5% nonfat milk in TBST for 2 h at room temperature and then incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma). After incubation with HRP-conjugated anti-mouse IgG (GE Healthcare) at room temperature for 1 h, the blots were visualized with ECL reagents (Pierce).

In Vitro Assays for MGAT Activity—MGAT activity was determined by measuring the incorporation of oleoyl moiety into *sn*-2-[^{14}C]monooleoylglycerol (acyl acceptors). The acyl acceptors were introduced into the reaction mixture by dissolving in ethanol (less than 1% in volume). The reaction mixture contained 100 mM Tris/HCl, pH 7.0, 5 mM $MgCl_2$, 1 mg/ml BSA free fatty acids (Sigma), 200 mM sucrose, 40 μM oleoyl-CoA, 20 μM *sn*-2-[^{14}C]monooleoylglycerol (50 Ci/mmol), and 100 μg of cell homogenate protein. The indicated concentration of detergent was delivered into reactions together with substrates. After a 10-min incubation at room temperature, lipids were extracted with chloroform/methanol (2:1, v/v). After centrifugation to remove debris, aliquots of the organic phase containing lipids were dried under a speed vacuum and separated by the Linear-K Preadsorbent TLC Plate (Waterman Inc., Clifton, NJ) with hexane/ethyl ether/acetic acid (80:20:1, v/v/v). The TLC plates were exposed to a Phosphor Screen to assess the incorporation of ^{14}C-acyl moieties into the respective lipid products. Phosphorimaging signals were visualized using a Storm 860 PhosphorImager (Amersham Biosciences) and quantitated using ImageQuant software.

Immunocytohistochemistry—For the subcellular co-localization study, a FLAG epitope (DYKDDDDK) tagged to the N terminus of DGAT1 and a Myc epitope-tagged DGAT2 were transiently transfected into COS-7 cells. Cells were grown and transfected on a coverslip (BD Biosciences). Forty-eight hours after transfection, cells were washed two times with PBS (2 min each) and fixed with freshly prepared 4.0% paraformaldehyde prewarmed at 37 °C. The samples were rinsed twice with PBS (5 min each) and permeabilized with 0.2% Triton X-100 in PBS, followed by incubation for 1 h in 5% normal donkey serum to block nonspecific binding. The samples were then incubated for 2 h at room temperature with mouse monoclonal or rabbit polyclonal anti-FLAG antibody (5.0 μg/ml; Sigma) and monoclonal anti-Myc antibody (5.0 μg/ml; Sigma). After a brief wash with PBS three times, the samples were incubated for 1 h at room temperature with Cy2-conjugated donkey anti-mouse IgG or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The samples were washed four times with PBS and analyzed with a confocal fluorescence microscope (BX61, Olympus, Nashua, NH).

DSP Cross-linking and Western Blot Analysis—COS-7 cells were transiently transfected with MGAT2, DGAT1, DGAT2, or a combination. After 48 h of transfection, the cells were treated with various doses of DSP for 30, 60, and 120 min, respectively, followed by Western analysis, as described previously (18).

Analytical Gel Filtration Column Chromatography—A sample of 100 μl of COS-7 cell lysate overexpressing MGAT2 and DGAT2 was applied to a Superdex gel filtration column (S-75, HR 1.0 × 30 cm) that was equilibrated with 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 1 mM DTT. The column was calibrated with protein molecular weight standards (Sigma). On the basis of the elution profiles of the molecular weight standards, a standard curve was generated, which was used to calculate the molecular weight of each protein according to its elution profiles. All procedures of chromatography were performed at 4 °C. Elution fractions were analyzed by Western blot analysis using anti-FLAG antibodies.

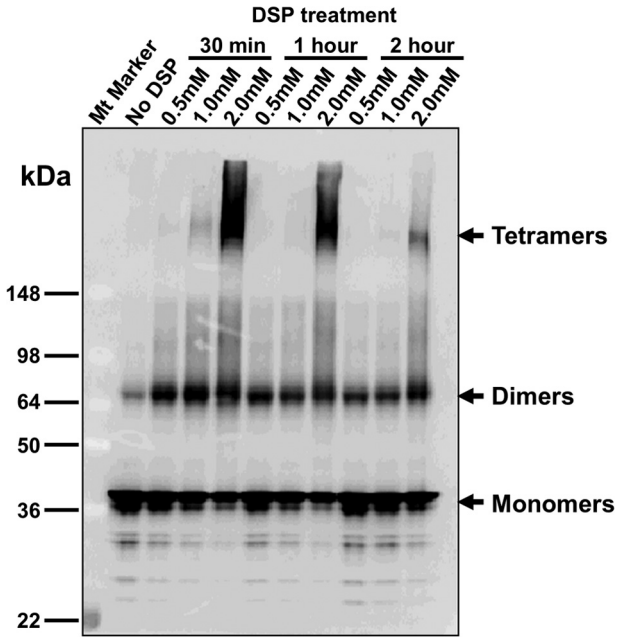

FIGURE 1. **MGAT2 forms homodimers and tetramers in intact COS-7 cells.** COS-7 cells were transiently transfected with expression vector for FLAG-tagged MGAT2. At 48 h post-transfection, the cells were treated with the indicated doses of DSP for 30 min, 1 h, and 2 h, respectively. Cell lysates were dissolved in a sample buffer without reducing reagents and boiling, resolved on SDS-PAGE, and analyzed by Western blot analysis using anti-FLAG antibodies. The *arrows* highlight monomers, dimers, and tetramers of MGAT2 protein.

Statistical Analysis—Data are given as mean ± S.E. The differences between two groups were analyzed by Student's *t* test.

RESULTS

Human MGAT2 Is a Homotetrameric Protein in Intact Cells—MGAT2 plays a major role in dietary fat absorption. To better understand its structural and catalytic properties, we examined its protein moiety by transient transfection of FLAG-tagged recombinant human MGAT2 protein in COS-7 cells, followed by Western blot analysis using anti-FLAG antibodies. In addition to the predicted molecular mass of human MGAT2 at 38 kDa, a 76-kDa moiety was also detected in SDS-PAGE without reducing agent and heat inactivation, suggesting that MGAT2 formed a dimer in intact COS-7 cells (Fig. 1). To test this hypothesis, we analyzed MGAT2 moieties after treating COS-7 cells with different concentrations of DSP, a cross-linking reagent, for different lengths of time. The results show that the DSP reagent dose-dependently enhanced the formation of higher molecular masses of proteins on SDS-PGAE, which correspond to the sizes of MGAT2 dimers (76 kDa) and tetramers (152 kDa). The intensity of the complexes appeared to be decreased with increased length of time for the treatment, probably caused by a toxic effect of DSP.

Human MGAT2 Heterodimerizes with DGAT1 but Not DGAT2—MGAT2 and DGAT enzymes catalyze the two consecutive steps in TAG resynthesis. We hypothesized that MGAT and DGAT enzymes may form an enzyme complex to facilitate the channeling of DAG, the product of the first MGAT reaction, to DGAT. To test this hypothesis, we co-expressed

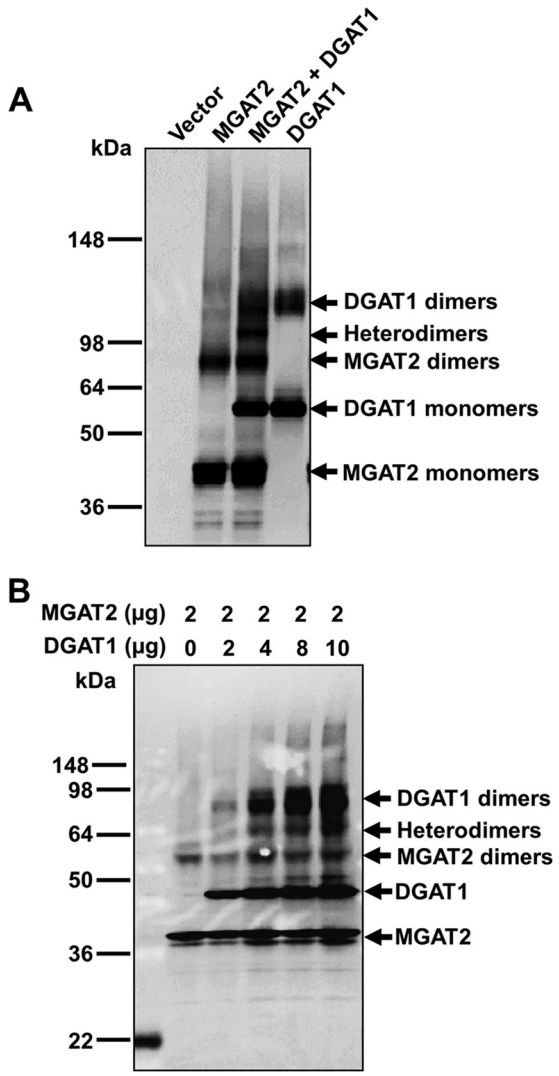

FIGURE 2. **MGAT2 heterodimerizes with DGAT1 in intact COS-7 cells.** *A*, COS-7 cells were transiently transfected with expression vector for FLAG-tagged MGAT2, FLAG-tagged DGAT1, or both. At 48 h of post-transfection, cell lysates were dissolved in a sample buffer without reducing reagents and boiling, resolved on SDS-PAGE, and analyzed by Western blot analysis using anti-FLAG antibodies. *B*, COS-7 cells were co-transfected with 2 μg of plasmid expression vector for FLAG-tagged MGAT2 with the indicated amounts of expression vector for FLAG-tagged DGAT1, followed by Western blot analysis for dimerization using anti-FLAG antibodies. The *arrows* highlight different complex moieties of MGAT2 and DGAT1 enzymes.

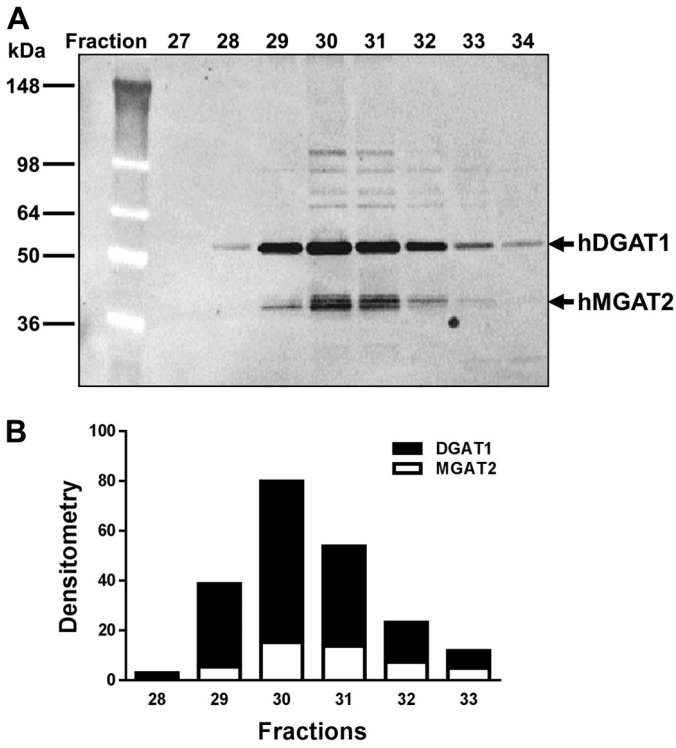

FIGURE 3. **Co-purification of MGAT2 with DGAT1 transiently expressed in COS-7 cells by gel filtration chromatography.** *A*, COS-7 cells were transiently co-transfected with expression vector for FLAG-tagged MGAT2 with FLAG-tagged DGAT1. After 48 h of transfection, protein lysates were loaded onto a gel filtration column for purification. Column fractions were resolved on SDS-PAGE and analyzed by Western blot analysis using anti-FLAG antibodies. *B*, quantitative analysis of MGAT2 and DGAT2 in different elution fractions from *A* by densitometry.

FLAG-tagged MGAT2 and DGAT1, the two prominent enzymes responsible for dietary fat absorption, in COS-7 cells and performed Western analysis without reducing reagents and heating. As shown in Fig. 2*A*, each enzyme formed a homodimer in intact cells without any treatment with cross-linking reagent, which is consistent with previous reports that the DGAT1 formed dimeric and tetrameric proteins (26). Moreover, co-expression of both enzymes in COS-7 cells resulted in heterodimerization of the two enzymes. The results were further corroborated by the findings that the heterodimerization was stimulated dose-dependently with the increased expression level of DGAT1 (Fig. 2*B*).

To provide further evidence for the heterodimerization of MGAT2 with DGAT1, we next analyzed the elution profile of two enzymes co-expressed in COS-7 cells by gel filtration chromatography. As shown in Fig. 3*A* (quantified in Fig. 3*B*), the two enzymes co-eluted from a gel filtration column, further confirming that the two enzymes form a protein complex in intact cells.

Two distinct family members of DGAT enzymes, known as DGAT1 and DGAT2, have been characterized in different organisms, including mammals, fungi, and plants. Although both DGAT1 and DGAT2 catalyze the same step in TAG synthesis, the two enzymes do not share any sequence homology. Furthermore, they exhibit distinct functional features, including tissue distribution and metabolic phenotypes, in knock-out mice. In contrast to DGAT1, DGAT2, the founding member of the DGAT2/MGAT family, shares high sequence homology with MGAT1–3. We next investigated whether DGAT2 could dimerize with MGAT2 or DGAT1 by co-expression in COS-7 cells. Again, we observed the homodimerization of MGAT2 or DGAT1 and heterodimerization of MGAT2 and DGAT1 (Fig. 4, *lanes 2–4*). Surprisingly, MGAT2 did not dimerize with DGAT2. In addition, DGAT2 failed to dimerize with DGAT1 (Fig. 4).

To investigate the underlying mechanisms by which DGAT2 failed to dimerize with DGAT1, we co-expressed DGAT1 and DGAT2 in COS-7 cells and investigated their subcellular localization by confocal imaging analysis (Fig. 5). Consistent with previous reports that both DGAT1 and DGAT2 are localized in the ER (20, 27, 28), the two enzymes completely overlapped with each other in subcellular localizations, as highlighted by *arrows* (Fig. 5*D*), excluding the possibility that the two enzymes do not co-localize within cells.

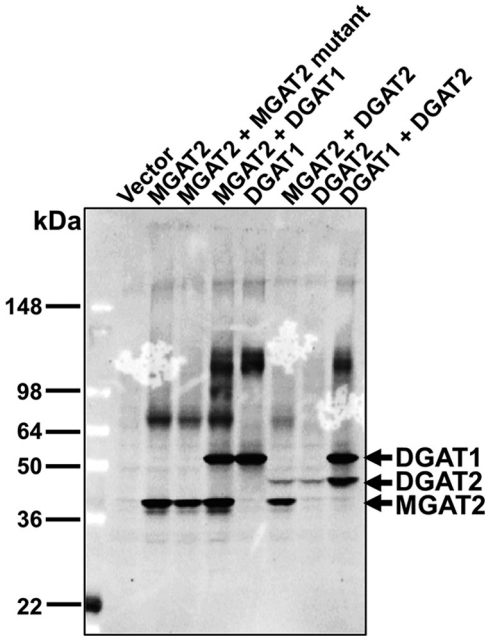

FIGURE 4. **MGAT2 heterodimerizes with DGAT1 but not DGAT2 in intact COS-7 cells.** COS-7 cells were transiently transfected with expression vector for FLAG-tagged MGAT2, DGAT1, DGAT2, or the indicated combinations. At 48 h post-transfection, cell lysates were dissolved in a sample buffer without reducing reagents and boiled, resolved on SDS-PAGE, and analyzed by Western blot analysis using anti-FLAG antibodies. The *arrows* highlight different moieties of MGAT2, DGAT1, DGAT2, and various dimers and heterodimers of the enzymes.

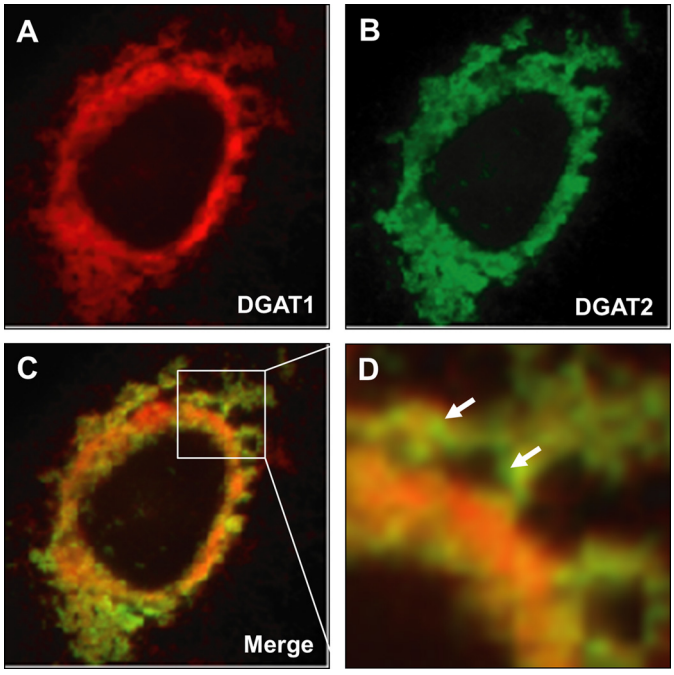

FIGURE 5. **Subcellular localization analysis of DGAT1 and DGAT2 in COS-7 cells.** COS-7 cells were transiently co-transfected with FLAG-tagged DGAT1 (*A*) and Myc-tagged DGAT2 (*B*). Forty-eight hours after transfection, cells were processed for indirect immunofluorescence staining with monoclonal antibodies specific for FLAG (*green*) or Myc (*red*). The *merged images* are shown in *C* and *enlarged* in *D*. *Yellow*, complete overlap; *arrowheads* in *D*, staining pattern of DGAT2 that did not co-localize with that of DGAT1.

An N-terminal Signal Peptide Is Required for MGAT2 Enzyme Activity but Not Its Dimerization or Subcellular Localization—DGAT1 was reported to form a homotetramer, which requires the N terminus (26, 29). Like DGAT1, MGAT2 also possesses a signal peptide at the N terminus (Fig. 6*A*, highlighted in *red*). To determine a role of the signal peptide in regulating dimerization and enzyme activity, we generated a deletion mutant that lacks the N-terminal signal peptide (Fig. 6, *A* and *B*). When transiently expressed in COS-7 cells, the deletion mutant exhibited both monomers and dimers, suggesting that the signal peptide is not required for dimerization. Likewise, the deletion mutant also heterodimerized with DGAT1 (Fig. 6*C*), further confirming that the signal peptide does not play an essential role in dimerization.

We next determined a role of the signal peptide in regulating subcellular localization and enzyme activity. As shown in Fig. 7*A*, ablation of the signal peptide did not affect its localization in ER, as evidenced by co-localization with calnexin, an ER marker protein. Surprisingly, the signal peptide is required for MGAT enzyme activity. Deletion of the signal peptide completely abolished its enzyme activity (Fig. 7*B*, quantified in Fig. 7*C*).

Amino Acids 39–90 of Human DGAT1 Mediates Both Homo- and Heterodimerization—DGAT1 was reported to form a homotetramer, which requires the N terminus (26, 29). To identify domains potentially required for the heterodimerization with MGAT2, we generated a set of deletion mutants deficient in the potential dimerization domains (Δ2–35 and Δ2–80), interspaced between two transmembrane domains (Δ152–172) and the predicated catalytic domain (Δ406–425), respectively (Fig. 8*A*). The deletion mutants were transiently expressed in 293E cells and analyzed for their propensity to homodimerize or heterodimerize. As shown in Fig. 8*B*, deletion of amino acids 2–35 did not significantly affect DGAT1 homodimerization or heterodimerization with MGAT2. Likewise, neither the intertransmembrane space between amino acids 152 and 172 nor the catalytic domain at amino acids 406–425 was required for the dimerizations. In contrast, deletion of amino acids 2–80 totally abolished the ability of DGAT1 to form homodimers and to form heterodimers with MGAT2. The deletion mutant overlapped with the Δ2–35 mutant for the first 33 amino acids, suggesting that amino acids 35–80 are essential for the dimerizations, as highlighted in Fig. 8*A*.

Co-expression of MGAT2 with DGAT1 Significantly Promoted Lipogenesis in Vivo—The findings that MGAT2 formed a complex with DGAT1 prompted us to investigate whether the interaction will promote lipogenesis by ORO staining. The results show that transient expression of MGAT2 or DGAT1 alone significantly stimulated lipogenesis in COS-7 cells, as evidenced by increased staining of ORO. Strikingly, co-expression of MGAT2 with DGAT1 significantly promoted lipogenesis, which is supported by an increased number of cells stained positive for ORO and a significant increase in the intensity of the staining (Fig. 9*A*, quantified in Fig. 9*B*), implicating a synergy of the two enzymes in catalyzing the synthesis of triglycerides *in vivo*.

DISCUSSION

Enzymes involved TAG synthesis play an important role in dietary fat absorption in intestinal enterocytes and fat deposition in white adipose tissues. Among the two TAG synthesis pathways, the MGAT pathway plays an important role in dietary absorption in intestinal enterocytes, whereas the glycerol

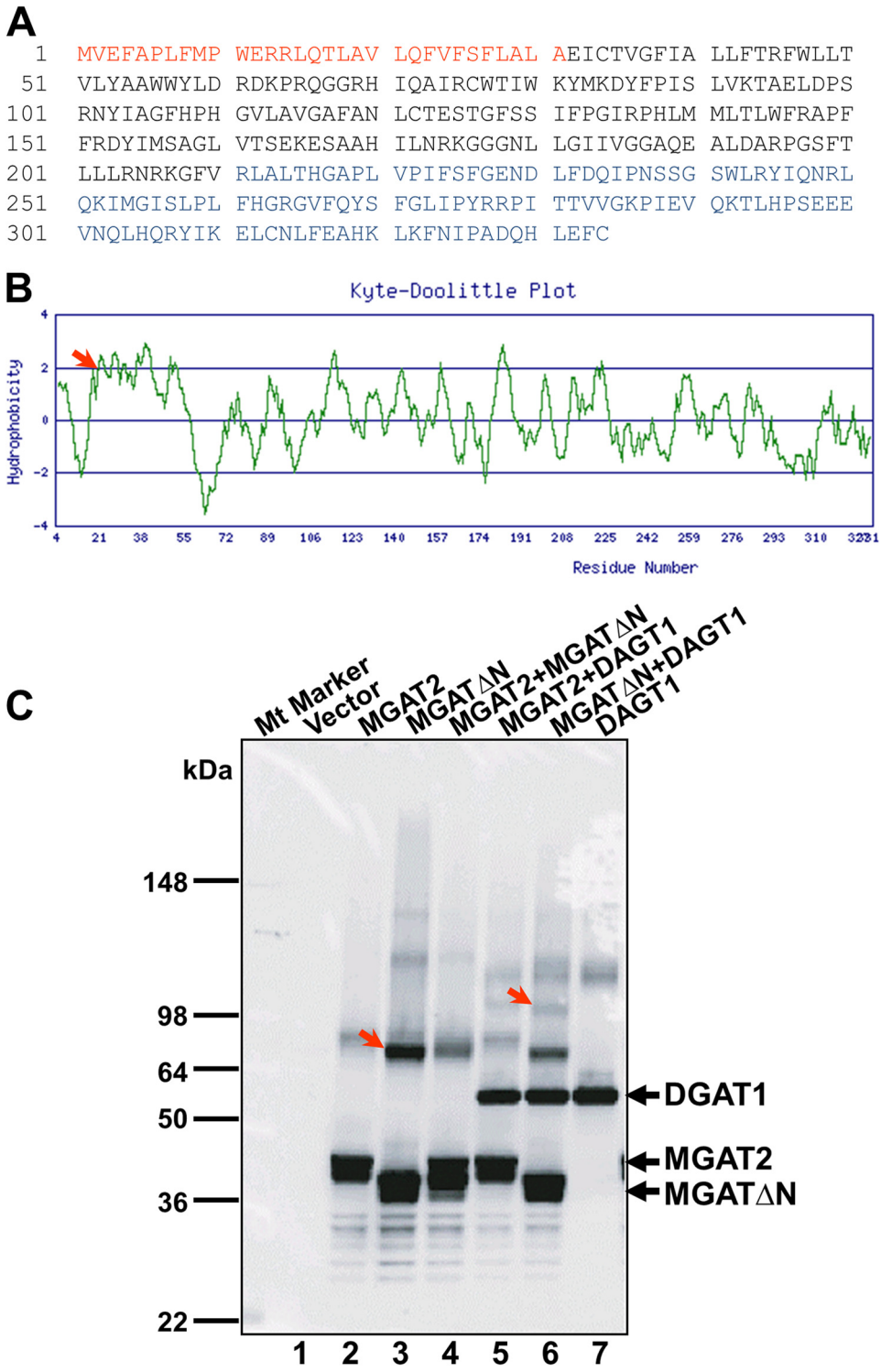

FIGURE 6. **The N-terminal signal peptide of MGAT2 is not required for dimerization.** *A* and *B*, highlight of signal peptide sequence (*red type*) and hydrophobicity profile of MGAT2. *C*, COS-7 cells were transiently transfected with expression vector for FLAG-tagged MGAT2, a deletion mutant lacking the N-terminal signal peptide (MGATΔN), DGAT1, or the indicated combinations. At 48 h post-transfection, cell lysates were dissolved in a sample buffer without reducing reagents and boiled, resolved on SDS-PAGE, and analyzed by Western blot analysis using anti-FLAG antibodies. *Red arrows*, homodimerization of MGATΔN and heterodimerization of MGATΔN with DGAT1.

3-phosphate pathway is mainly involved in *de novo* synthesis of TAG in lipogenic tissues. The two pathways merge at the final step for TAG synthesis catalyzed by DGAT. Thus, DGAT enzymes are involved in both dietary fat absorption and *de novo* lipogenesis. Consistent with a role of MGAT enzymes in dietary fat absorption, all three MGAT enzymes identified recently are predominantly expressed in gastrointestinal tissues. MGAT1 is most abundantly expressed in stomach and kidney, whereas MGAT2 and MGAT3 are predominantly expressed in small intestine. The MGAT2 expression forms a pattern that corre-

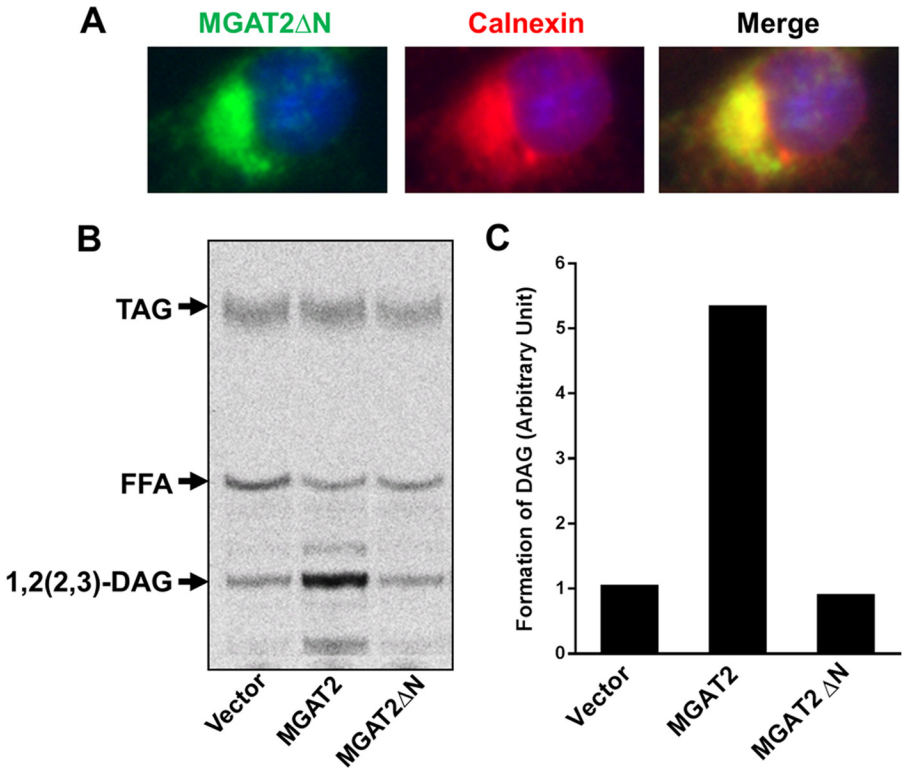

FIGURE 7. **Deletion of the N-terminal signal peptide of MGAT2 abolishes its enzyme activity but not its ER localization.** COS-7 cells were transiently co-transfected with FLAG-tagged MGATΔN or vector controls. *A*, 48 h after transfection, cells were processed for indirect immunofluorescence staining with monoclonal antibodies specific for FLAG (*green*) or calnexin (*red*), an ER-resident protein, and examined by confocal image analysis. *Yellow*, complete overlap between MGATΔN and calnexin. *B*, analysis of MGAT activity of MGATΔN or vector controls transiently expressed in COS-7 cells by TLC. *C*, quantitative analysis of diacylglycerol (*DAG*) formation from enzymatic reactions shown in *B*.

lates well with that of dietary absorption and is induced by a high fat diet (18). In contrast, the two DGAT enzymes identified thus far are widely expressed, which is consistent with their involvement in both TAG synthetic pathways.

Enzymes involved in TAG are believed to form a large synthase complex that consists of MGAT, DGAT, fatty acid-activating enzymes, acyl-CoA ligase, and carrier proteins. The formation of the complex is believed to facilitate the channeling of intermediates in a sequential ordered ping-pong acyl transfer along the metabolic pathway (30). However, investigation of the components and assembly process of the TAG synthase complex has been hindered by the absence of the purified enzymes and identification of encoding genes. There have been many attempts previously to purify the TAG synthase complex. In one of the studies, Manganaro and Kuksis (31) purified a large enzyme complex of 350–375 kDa that co-purified with TAG synthesis activity. However, the purification was carried out in the absence of detergent; thus, it may contain aggregated proteins. Further efforts in purifying the TAG synthase complexes have encountered problems of protein instability of purified enzyme due to the use of detergent in the purification process that disrupts the tight lipid-protein interaction necessary for optimal activity. The recent identification and cloning of several metabolic enzymes involved in TAG synthesis, such as MGAT and DGAT enzymes, has made it possible to decipher components and the assembly process of the TAG synthase complex.

The present studies provide direct evidence that the human MGAT2 is an oligomeric protein that forms dimers and tetramers under semidenatured conditions as well as inside intact cells. The formation is quite strong, as evidenced by the presence of dimmers in SDS-PAGE without treatment with cross-linking reagent, and such a dimer can only be completely eliminated by boiling the sample in reducing agent before the electrophoresis. Treatment of live cells with cross-linking reagent not only enhanced the formation of dimers but also promoted the formation of tetramers, suggesting that MGAT2 forms a homotetrameric protein inside the cells.

The recombinant MGAT2 formed heterodimers with DGAT1 but not with DGAT2. This is unexpected because the MGAT enzymes share more than 40% sequence homology with DGAT2 but not with DGAT1. Although the two DGAT enzymes catalyze the same reaction, the two DGAT enzymes share no sequence homology. DGAT1 enzyme is a member of the ACAT/MBOAT enzyme family, members of which also include a number of acyl-CoA-dependent acyltransferases responsible for the modification of phospholipids, cholesterol, and signaling peptides, such as grehlin and Wnt (32). In addition, DGAT1 has been characterized as a multifunctional enzyme, catalyzing the synthesis of triacylglycerols, waxes, and retinyl esters (33). Findings from knock-out mice also revealed that DGAT1 and DGAT2 function differently *in vivo*. Mice with targeted deletion of DGAT1 are viable and can still synthesize TAG. The DGAT1-deficient mice exhibit increased metabolic rate and activity and are resistant to diet-induced obesity (25). In contrast, inactivation of DGAT2 resulted in mice that displayed neonatal lethality due to defective TAG synthesis (34). Both genetic and pharmacological studies

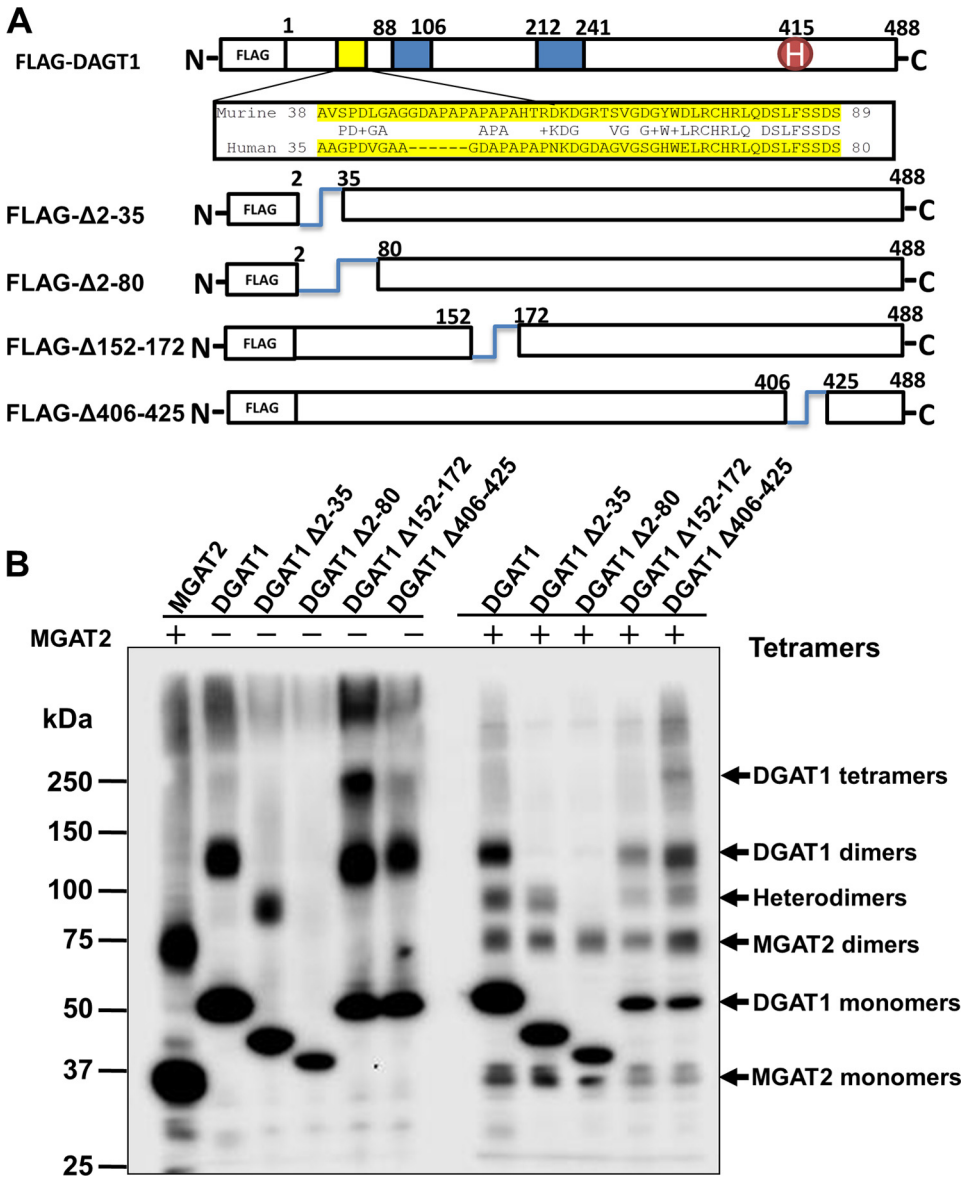

FIGURE 8. **Systematic deletion analysis of DGAT1 domains required for dimerization.** *A*, diagram depicting DGAT1 deletion mutants, including the predicted transmembrane domains of human DGAT1 (*filled boxes*), the putative active site histidine at position 415 (*H*), and detailed sequence information for amino acids 35–80 and their corresponding murine sequence. *B*, Western blot analysis of dimerization of MGAT2 with DGAT1 deletion mutants by SDS-PAGE. NIH 293E cells were transiently transfected with expression vector for FLAG-tagged MGAT2 or FLAG-tagged DGAT1 or co-transfected with FLAG-tagged MGAT2 and the indicated FLAG-tagged DGAT1 deletion mutants. At 48 h post-transfection, cell lysates were dissolved in a sample buffer without reducing reagents and boiling, resolved on SDS-PAGE, and analyzed by Western blot analysis using anti-FLAG antibodies. *Arrows*, different moieties of MGAT2, DGAT1, DGAT2, and various dimers and heterodimers of the enzymes.

revealed an important role for DGAT1 in mediating dietary fat absorption. In an acute lipid challenge model, inactivation of DGAT1 by either genetic deletion or small molecule inhibitors resulted in marked reduction in serum triglyceride after an oral administration of a bolus of corn oil (25, 35). Similarly, a body of evidence supports a predominant role of MGAT2 in dietary fat absorption (18, 24). Therefore, our findings that MGAT2 and DGAT1 form heterodimers are also revealing and provide molecular evidence lending support to the notion of a TAG synthase complex with protein components including MGAT, DGAT, and acyl-CoA synthase along the ER membrane. The formation of MGAT2/DGAT1 heterodimers is expected to bring the intermediate substrate (*i.e.* 1,2-DAG) to the proximity of the next step catalytic enzyme (*i.e.* DGAT1) and to largely increase the efficiency of TAG synthesis. This will also help meet the body's need to absorb the large amount of fatty acids and monoacylglycerol after a meal. In contrast, we found that MGAT2 did not interact with DGAT2, suggesting that DGAT2 may play a less important role in dietary fat absorption (22–24). Additionally, our deletion analysis identified a 45-amino acid region in the N terminus of DGAT1 that is required for both homodimerization and heterodimerization with MGAT2, lending further support to the specificity of the interaction. In contrast, an N-terminal signal peptide of MGAT2 is required for enzyme activity but not for heterodimerization with DGAT1 or subcellular localization in the ER. Furthermore, in support of the functional importance of the interaction, co-expression of MGAT2 with DGAT1 significantly enhanced lipo-

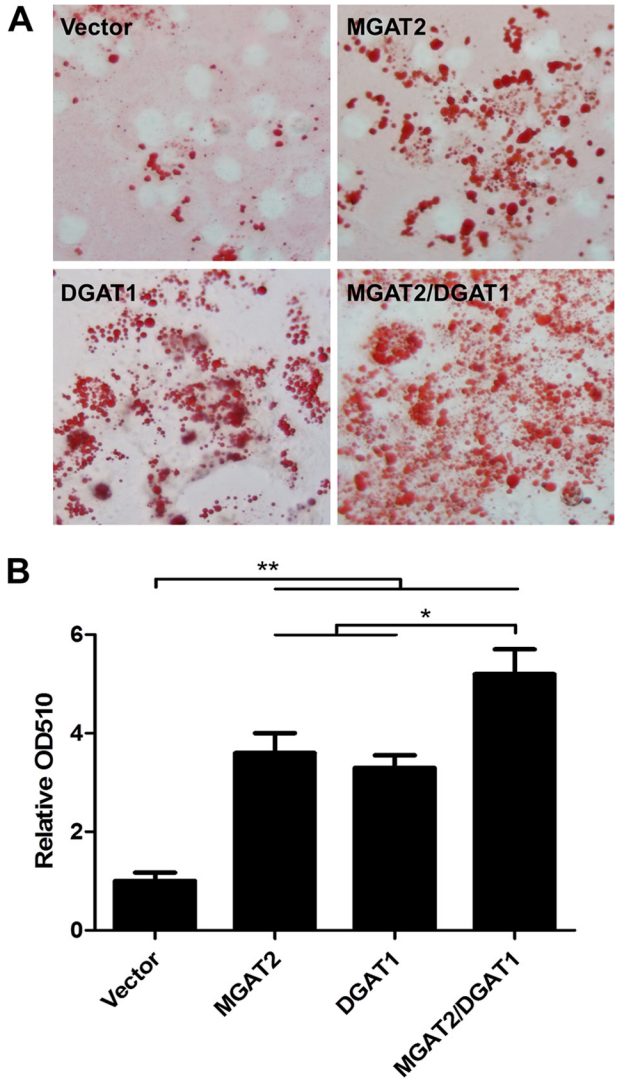

FIGURE 9. **Co-expression of MGAT2 with DGAT1 promotes lipogenesis *in vivo*.** *A*, COS-7 cells were transiently transfected with MGAT2, DGAT1, or a combination. After 36 h of transfection, the transfected cells were incubated with 0.5 mM sodium oleate complexed to 0.67% fatty acid-free bovine serum albumin for 12 h, fixed with 4% paraformaldehyde, and subjected to ORO staining. Cell images were captured by using an Olympus IX71 inverted microscope. *B*, quantification of ORO. Cells from *A* were directly dissolved in 100% isopropyl alcohol to extract ORO, measured for absorbance at 510 nm, and quantified. Error bars, S.E.

genesis in COS-7 cells, supporting a synergy of the two enzymes in catalyzing the synthesis of TAG *in vivo*.

In summary, our findings in this study provide evidence at the molecular level showing that MGAT2 functions as a dimeric or tetrameric protein and selectively heterodimerizes with DGAT1 in mammalian cells. Our data also shed light on the role of a short signal peptide at the N terminus of MGAT2 in catalytic activity, localization, and interaction with partnering proteins. Additional studies on the relation between dimer/tetramer or heterodimer formation and TAG-biosynthesizing efficiency may be warranted to elucidate the functional importance of such a process.

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