Catalytic properties of MGAT3, a putative triacylglycerol synthase[S]

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Abstract **Acyl-coenzyme A:monoacylglycerol acyltransferase 3 (MGAT3) is a member of the MGAT family of enzymes that catalyze the synthesis of diacylglycerol (DAG) from monoacylglycerol (MAG), a committed step in dietary fat absorption. Although named after the initial identification of its MGAT activity, MGAT3 shares higher sequence homology with acyl-coenzyme A:diacylglycerol acyltransferase 2 (DGAT2) than with other MGAT enzymes, suggesting that MGAT3 may also possess significant DGAT activity. This study compared the catalytic properties of MGAT3 with those of MGAT1 and MGAT2 enzymes using both MAG and DAG as substrates. Our results showed that in addition to the expected MGAT activity, the recombinant MGAT3 enzyme expressed in Sf-9 insect cells displayed a strong DGAT activity relative to that of MGAT1 and MGAT2 enzymes in the order MGAT3 > MGAT1 > MGAT2. In contrast, none of the three MGAT enzymes recognized biotinylated acyl-CoA or MAG as a substrate. Although MGAT3 possesses full DGAT activity, it differs from DGAT1 in catalytic properties and subcellular localization. The MGAT3 activity was sensitive to inhibition by the presence of 1% CHAPS, whereas DGAT1 activity was stimulated by the detergent. Consistent with high sequence homology with DGAT2, the MGAT3 enzyme demonstrated a similar subcellular distribution pattern to that of DGAT2, but not DGAT1, when expressed in COS-7 cells. Our data suggest that MGAT3 functions as a novel triacylglycerol (TAG) synthase that catalyzes efficiently the two consecutive acylation steps in TAG synthesis.**—Cao, J., L. Cheng, and Y. Shi. **Catalytic properties of MGAT3, a putative triacylglycerol synthase.** ***J. Lipid Res.*** **2007.** 48: **583–591.**

Supplementary key words acyl-coenzyme A:monoacylglycerol acyltransferase • acyltransferase • monoacylglycerol

In mammals, the synthesis of triacylglycerol (TAG) serves a critical function in multiple important physiological processes, including intestinal nutrition absorption, surplus energy storage in cells, lactation, attenuation of lipotoxicity, lipid transportation, and signal transduction (1–4). There are two main biochemical pathways for TAG synthesis. The monoacylglycerol (MAG) pathway begins with the acylation of MAG with fatty acyl-CoA catalyzed by acyl-coenzyme A:monoacylglycerol acyltransferase (MGAT), producing diacylglycerol (DAG). This dominates the synthesis of TAG inside enterocytes after feeding because of the large amount of 2-MAG and fatty acids released from dietary lipids (5). The MAG pathway is also active in adipose tissue that stores excess energy in the form of TAG (6). The other is the glycerol-3-phosphate (G3P) pathway, a de novo pathway involved in TAG synthesis in most tissues, including the small intestine. The G3P pathway begins with the acylation of G3P with fatty acyl-CoA, producing lysophosphatidic acid, followed sequentially by further acylation and dephosphorylation to yield DAG (1). The two pathways share the final step in converting DAG to TAG, which is catalyzed by acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) (1, 7).

In the past few years, great strides have been made in the identification and characterization of the enzymes of the two pathways as well as in the elucidation of underlying mechanisms that regulate TAG synthesis, as a result of rapid progress in genomics, bioinformatics, and transgenics. The use of bioinformatics approaches bypassed the difficulties in the purification of these enzymes from primary tissues because most of the enzymes are intrinsic membrane proteins. The identification of the first DGAT, DGAT1, was achieved by a sequence homology search to ACAT1 (7). The recent purification and cloning of a DGAT2 from the oleaginous fungus *Mortierella remmaniana* sparked the molecular identification of a family of mammalian MGAT/DGAT enzymes, including DGAT2 and three isoforms of

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Abbreviations: DAG, diacylglycerol; DGAT, acyl-coenzyme A:diacylglycerol acyltransferase; ER, endoplasmic reticulum; G3P, glycerol-3-phosphate; MAG, monoacylglycerol; MGAT, acyl-coenzyme A:monoacylglycerol acyltransferase; TAG, triacylglycerol.

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[S] The online version of this article (available at http://www.jlr.org) contains additional two figures.

MGAT (MGAT1, MGAT2, and MGAT3) (8–11). Intriguingly, DGAT2 enzyme shares very high sequence homology with the MGAT enzymes but not with DGAT1, suggesting that *DGAT2* and *MGAT* genes likely share a common genetic origin. This is supported in part by the colocalization of *DGAT2* gene with *MGAT2* gene on the same region of human chromosome 11.

Although multiple isoforms are involved in catalyzing the same step in TAG synthesis, they may play distinct functional roles, as suggested by differential tissue distribution and subcellular localization of the DGAT/MGAT family of enzymes. The MGAT1 mRNAs are detected mainly from stomach, kidney, and adipose tissue, whereas MGAT2 and MGAT3 exhibit highest expression in the small intestine (10–12). Similarly, DGAT1 is ubiquitously expressed in many tissues, with the highest level in small intestine, whereas DGAT2 expression is most abundant in liver (7, 9). The functional distinction of these isoforms can also be achieved by different subcellular localization within the cells (13, 14). In yeast cells, there are two distinct pools of DGAT enzymes that exhibit different subcellular localization. Although such studies have not been carried out with mammalian DGAT enzymes, this notion is partly supported by distinct phenotypes of mice deficient in DGAT enzymes. For example, DGAT1 knockout mice developed resistance to diet-induced obesity, whereas mice with targeted deletion of the DGAT2 gene developed lipopenia and neonatal lethality, which were not compensated for by the presence of the other isoform in the same tissue (15).

The recent cloning and characterization of the three MGAT isoforms has laid a foundation to investigate their functional differences. Among the three MGAT isoforms identified to date, MGAT3 exhibits some unique features. Compared with *MGAT1* and *MGAT2* genes, the *MGAT3* gene only exists in higher mammals and humans, and not in rodents, based on bioinformatic analysis. Although the *MGAT3* gene was named according to its initially discovered MGAT activity, the MGAT3 enzyme actually shares higher sequence homology with DGAT2 than with MGAT1 and MGAT2, suggesting that the enzyme may possess significant DGAT activity. In this report, we investigated the catalytic properties of MGAT3 and compared them with those of MGAT1 and MGAT2. Our results demonstrate that MGAT3 exhibited significantly higher DGAT activity than MGAT1 and MGAT2 when either MAGs or DAGs were used as substrates, suggesting that MGAT3 functions as a putative TAG synthase. Furthermore, subcellular localization analysis indicated that MGAT3 was localized exclusively in the endoplasmic reticulum (ER) in a pattern similar to that of DGAT2 but quite distinct from that of DGAT1.

MATERIALS AND METHODS

Materials

rac-1-Monolauroylglycerol, *rac*-1-monooleoylglycerol, *sn*-2-monooleoylglycerol, *sn*-1,2-dioleoylglycerol, *rac*-1,2-dioleoylglycerol, *sn*-1,3-dioleoylglycerol, 1,2,3-trioleoylglycerol, oleic acid, lauroyl-CoA, and oleoyl-CoA were purchased from Sigma-Aldrich (St. Louis, MO). 1-[^{14}C]monooleoylglycerol, 2-[^{14}C]monooleoylglycerol, [^{14}C]lauroyl-CoA, and [^{14}C]oleoyl-CoA (50 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Biotinylated monolauroylglycerol, dilauroylglycerol, and lauroyl-CoA were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL).

Cloning and expression of MGATs and DGATs

The full-length coding sequences of mouse MGAT1, human MGAT2, human MGAT3, and human DGAT1 were cloned by PCR amplification with Marathon-ready cDNA prepared from tissues as suggested previously as templates (7, 9–11, 16). PCR amplification was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The resulting PCR products were cloned into the pPCR-script Amp SK(+) vector (Stratagene) and verified by sequencing analysis. The cDNA insert of each gene was then subcloned into the pFastBac vector for expression in *Spodoptera frugiperda* 9 (Sf-9) insect cells using a Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA). The pFastBac vector containing a cDNA fragment of the relevant gene was transformed into DH10Bac™ *Escherichia coli* cells to generate a recombinant bacmid. High-titer recombinant baculovirus was generated by transfecting the bacmid DNA into Sf-9 cells followed by several rounds of amplification to increase viral titer. After infection with recombinant baculoviruses for an optimized time, Sf-9 cells were harvested in ice-cold PBS, pelleted by centrifugation, lysed, and assayed immediately for enzyme activity or frozen in liquid N_2 for later use. Cell pellets were lysed by homogenizing in 20 mM NaCl with 20 up-and-down strokes in a motor-driven Dounce homogenizer (Heidolph, Germany) followed by 3 passages through a 27 gauge needle. The protein concentration in homogenate was determined with the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's instructions. Protein expression levels were checked by Western blot analysis for each enzyme, and similar amount of proteins were used in each of the enzyme assays.

In vitro assays for MGAT and DGAT activities

MGAT and DGAT activities were determined as described previously (9). Briefly, MGAT activity was determined by measuring the incorporation of the acyl moiety into diacylglycerol with [^{14}C]acyl-CoA and monoacylglycerols. DGAT activity was measured by analyzing the incorporation of the [^{14}C]oleoyl moiety into trioleoylglycerol with [^{14}C]oleoyl-CoA and *sn*-1,2-dioleoylglycerol or *sn*-1,3-dioleoylglycerol. The acyl acceptors were introduced into the reaction mixture by dissolving in ethanol (<1% in volume). Unless indicated otherwise, 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 cold oleoyl-CoA or 20 μM [^{14}C]oleoyl-CoA, 20 μM [^{14}C]monooleoylglycerols (50 Ci/mmol) or 200 μM MAGs, and 100 μg of cell homogenate protein from Sf-9 cells as a source of enzyme, as indicated above. The cell homogenate from Sf-9 cells infected with the wild-type control virus was used as a negative control for the enzyme assays. The effect of detergent on enzyme activity was determined by the addition of 1% CHAPS to the enzymatic reactions. After 10 min of incubation at room temperature, lipids were extracted with chloroform-methanol (2:1, v/v) and centrifuged to remove debris. The organic phase containing lipids was dried under a speed vacuum and separated by the Linear-K Preadsorbent TLC Plate (Whatman, Inc., Clifton, NJ) with hexane-ethyl ether-acetic acid (80:20:1, v/v/v), a solvent that resulted in good separation of *sn*-1,2(2,3)-DAGs from *sn*-1,3-DAGs. For resolution of biotinylated DAG and TAG, TLC plates were resolved by toluene-chloroform-methanol (85:15:5, v/v/v). Individual lipid moieties were identified

by standards with exposure to I_2 vapor. The TLC plates were exposed to a Phosphor Screen to assess the incorporation of ^{14}C-labeled acyl moieties into respective lipid products. Phosphoimaging signals were visualized and quantified using a Storm 860 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant software.

Immunocytohistochemistry

To facilitate subcellular colocalization analysis of MGAT and DGAT enzymes, mammalian expression vectors were engineered by PCR amplification using anchored primers that carry the sequence for the FLAG (DYKDDDDK) or myc epitope. A set of expression plasmids was engineered by this method, which resulted in the attachment of the FLAG sequence to the C terminus of MGAT2 and MGAT3 or to the N terminus of DGAT1. Similarly, a myc epitope was attached to the N terminus of DGAT2 using this method. The tagged cDNA fragments were subcloned into the pcDNA3.1(+) vector for transient expression in COS-7 cells. For immunohistochemical analysis, cells were grown and transfected on a coverslip (BD Biosciences, Bedford, MA). 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), monoclonal anti-myc antibody (5.0 μg/ml; Sigma), or rabbit anti-calnexin N-terminal polyclonal antibody (1.0 μg/ml; StressGen Biotechnologies Corp., Victoria, Canada). 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).

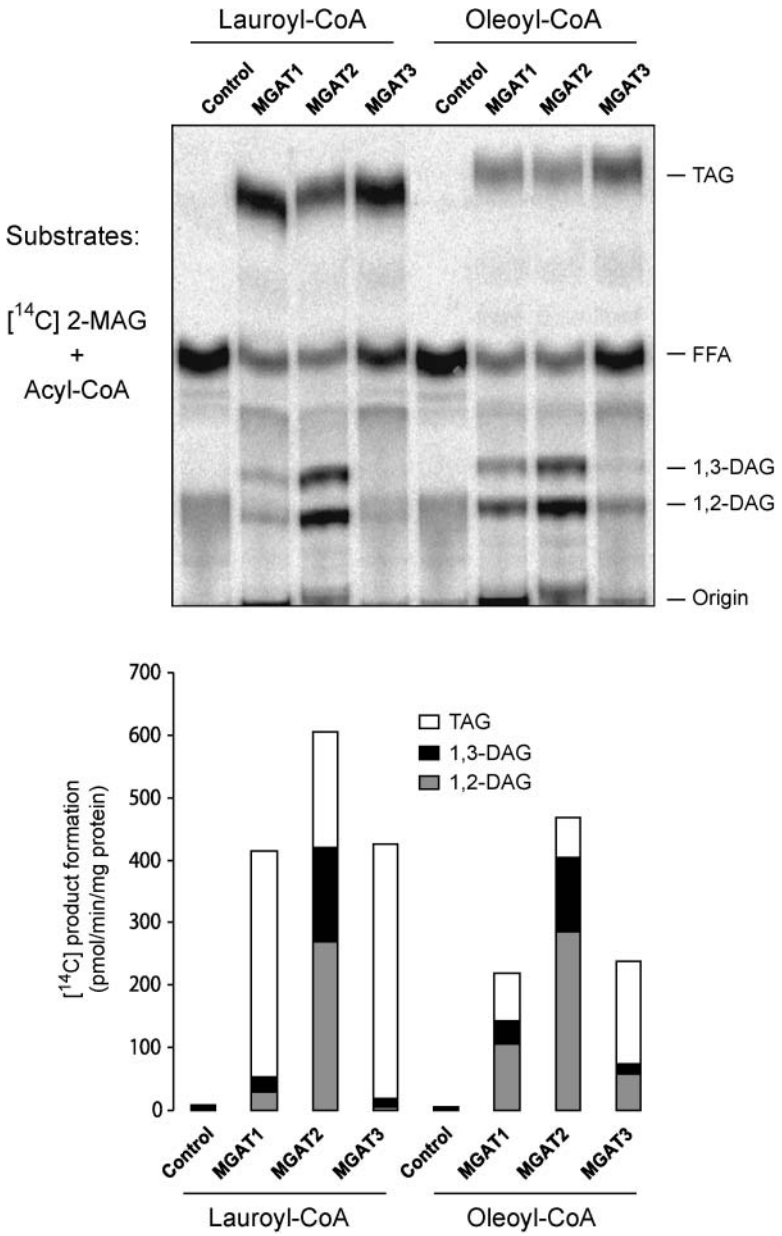

Fig. 1. Acylation activities of acyl-coenzyme A:monoacylglycerol acyltransferase 1 (MGAT1), MGAT2, and MGAT3 toward 2-[^{14}C]monooleoylglycerol and two different acyl-CoAs, lauroyl-CoA or oleoyl-CoA. The acyltransferase assays were conducted by incubating 40 μM acyl-CoA with 20 μM 2-[^{14}C]monooleoylglycerol in the presence of 100 μg of cell lysates from Sf-9 cells infected with either the wild-type baculovirus (control) or recombinant baculovirus expressing MGATs, as described in Materials and Methods. Representative TLC analyses are shown in the upper panel, and quantitative specific activity data are shown in the lower bar graph. Note that the formation of radiolabeled triacylglycerol (TAG) represents acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity, whereas the total formation of radiolabeled diacylglycerol (DAG) and TAG represents MGAT activity, because radiolabeled monooleoylglycerols were used as substrate. MAG, monoacylglycerol.

RESULTS

Comparative analyses of DGAT activity associated with MGAT enzymes using MAGs as substrates

MGAT3 is a member of the MGAT family of enzymes but shares higher sequence homology with DGAT2 than with other MGAT enzymes (data not shown). To test the hypothesis that MGAT3 functions as a TAG synthase that catalyzes two consecutive steps in TAG synthesis, we compared both MGAT and DGAT activities of MGAT3 with those of MGAT1 and MGAT2, using MAG as a substrate. We first analyzed the three MGAT enzymes for their DGAT activities using 2-[^{14}C]monooleoylglycerol substrate and lauroyl-CoA or oleoyl-CoA as acyl donor. As shown in **Fig. 1**, all three MGAT enzymes demonstrated significant DGAT enzyme activities, as indicated by the presence of high levels of radiolabeled TAG. Additionally, all three isoforms exhibited a preference for lauroyl-CoA over oleoyl-CoA as an acyl donor for their DGAT activities, as shown by the relative abundance of radiolabeled TAG over DAG. Consistent with the high sequence homology with DGAT2 enzyme, the MGAT3 enzyme demonstrated the highest DGAT activity, as shown by the near absence of radiolabeled DAG.

We next compared the DGAT activities of the three MGAT enzymes toward different MAG isomers using [^{14}C]oleoyl-CoA as an acyl donor. As expected, the major MGAT enzymatic products for *sn*-1-MAG and *sn*-2-MAG were 1,3-DAG and 1,2- or 2,3-DAG, respectively (**Fig. 2**). In contrast to MGAT1 and MGAT2, MGAT3 displayed much stronger DGAT activity, which is supported by the high level of [^{14}C]TAG produced when different *sn*-1-MAGs were used as substrates (Fig. 2). Again, MGAT2 displayed strong MGAT activity, but its DGAT activity was relatively low.

Profiling of DGAT activity of MGAT enzymes using DAGs as substrates

We next directly examined the DGAT activity of the three MGAT enzymes with 1,2-DAG or 1,3-DAG as acyl acceptor and [^{14}C]oleoyl-CoA as acyl donor, using DGAT1 as a positive control. As shown in **Fig. 3**, all three MGAT enzymes possessed DGAT activity in the order MGAT3 > MGAT1 > MGAT2, consistent with the data from previous experiments using [^{14}C]-MAG as an acyl acceptor.

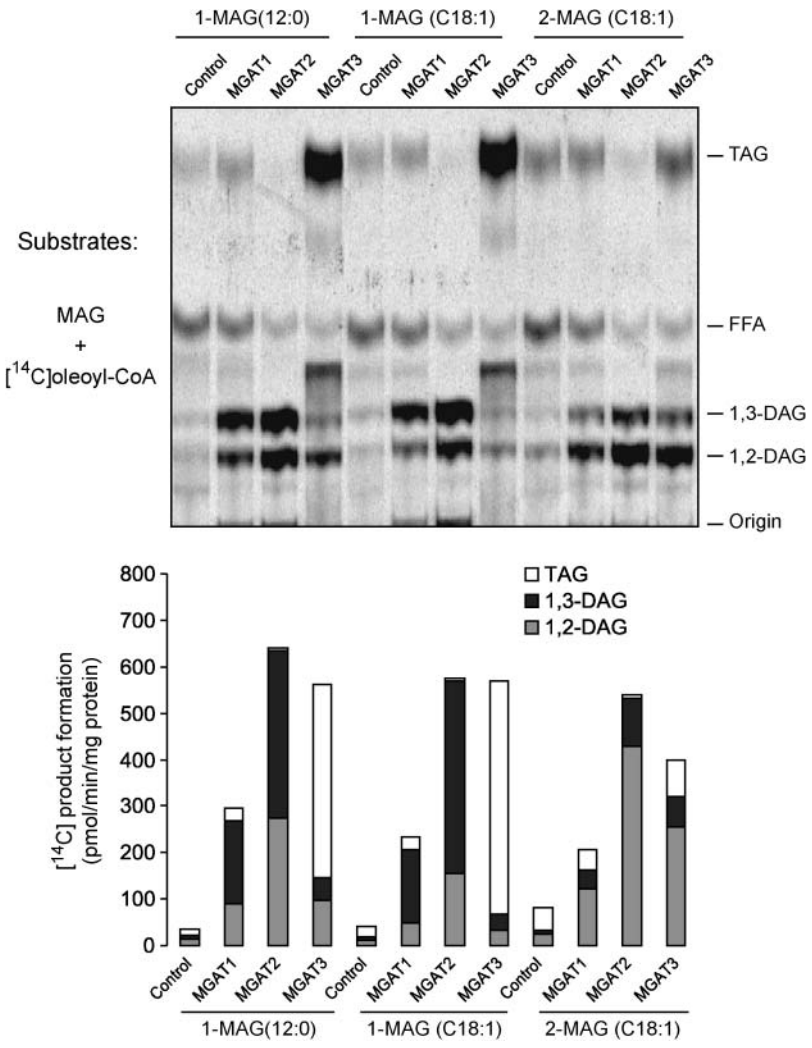

Fig. 2. Acylation activities of MGAT1, MGAT2, and MGAT3 toward [^{14}C]oleoyl-CoA and different MAGs. The experiment was conducted as described for Fig. 1 with the exception of different application of substrates. In this experiment, 20 μM [^{14}C]oleoyl-CoA and 200 μM of various MAGs were used. Representative TLC analyses are shown in the upper panel, and quantitative specific activity data are shown in the lower bar graph.

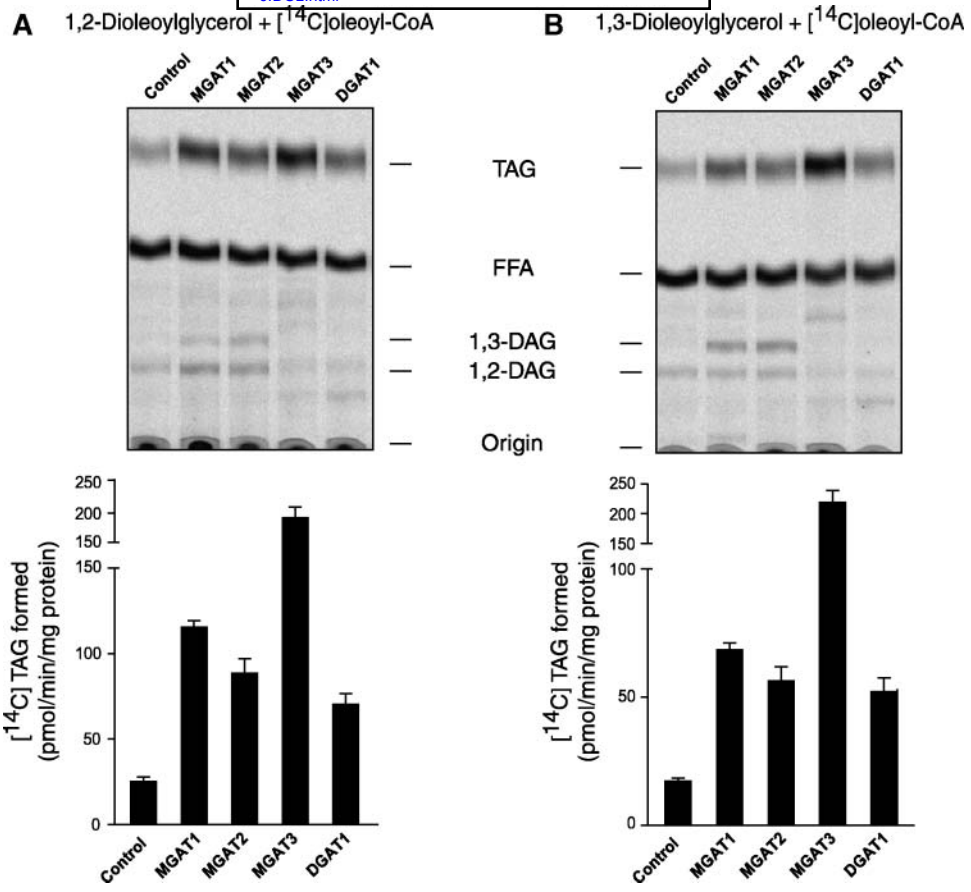

Fig. 3. Analysis of DGAT activities of MGAT1, MGAT2, and MGAT3 and DGAT1 toward 1,2-DAG (A) or 1,3-DAG (B). DGAT enzyme assays were conducted by incubating 20 μM [^{14}C]oleoyl-CoA with 200 μM 1,2-dioleoylglycerol or 1,3-dioleoylglycerol in the presence of 100 μg of cell lysates from Sf-9 cells infected with either the wild-type baculovirus (control) or recombinant baculovirus expressing MGATs or DGAT1, as described in Materials and Methods. Representative TLC analyses are shown in the upper panels, and quantitative DGAT specific activity data are shown in the lower bar graphs. Error bars represent SD.

Differential effects of the zwitterionic detergent CHAPS on the activity of the MGAT and DGAT enzymes

Detergents have been used widely in solubilizing membrane-associated proteins and protein complexes, including MGAT and DGAT from various primary tissues. The use of detergent often led to substantial loss of enzyme activities (17, 18). Although both MGAT3 and DGAT1 enzymes recognize DAG as substrate, the two enzymes share no sequence homology in protein sequence. To differentiate the two enzymes in catalytic features, we next analyzed the responses of MGAT and DGAT enzymes to treatment with zwitterionic detergent. The results showed that the activity of all three MGAT enzymes was severely inhibited by 1% CHAPS, as indicated by a dramatic decrease in the formation of DAG and TAG in the presence of detergent (**Fig. 4A**). In contrast, both MGAT and DGAT activities of DGAT1 enzyme were largely retained in the presence of 1% CHAPS (Fig. 4A). Although the DGAT activity of the MGAT enzymes was also inhibited by the detergent, it is not clear whether this was caused by the reduction of DAG that was used as substrate for the DGAT activity of the MGAT enzymes. To address the issue, we next analyzed the effect of detergent on the DGAT activity of MGAT enzymes by directly using DAG as substrate. As shown in Fig. 4B, the DGAT activity of all three MGAT enzymes was inactivated by the presence of 1% CHAPS. In contrast to a slight reduction in DGAT activity when MAG was used as a substrate, DGAT1 activity was significantly stimulated by the presence of detergent when DAG was used as a substrate (Fig. 4B). These results suggest that DGAT and MGAT activities of the MGAT enzymes are inseparable, whereas the MGAT and DGAT activities of the DGAT1 enzyme are dispensable.

The effect of substrate biotinylation on MGAT enzyme activities

Biotin is routinely used for assay development because of its high affinity for streptavidin. Attachment of biotin to the acyl chain of MAG or acyl-CoA would facilitate the separation of biotinylated DAG from its substrates by means of scintillation proximity assay. To evaluate the effect of biotinylation of fatty acyl chains on MGAT activity, we analyzed the MGAT activity of MGAT2 and MGAT3 using either biotinylated lauroyl-CoA or biotinylated

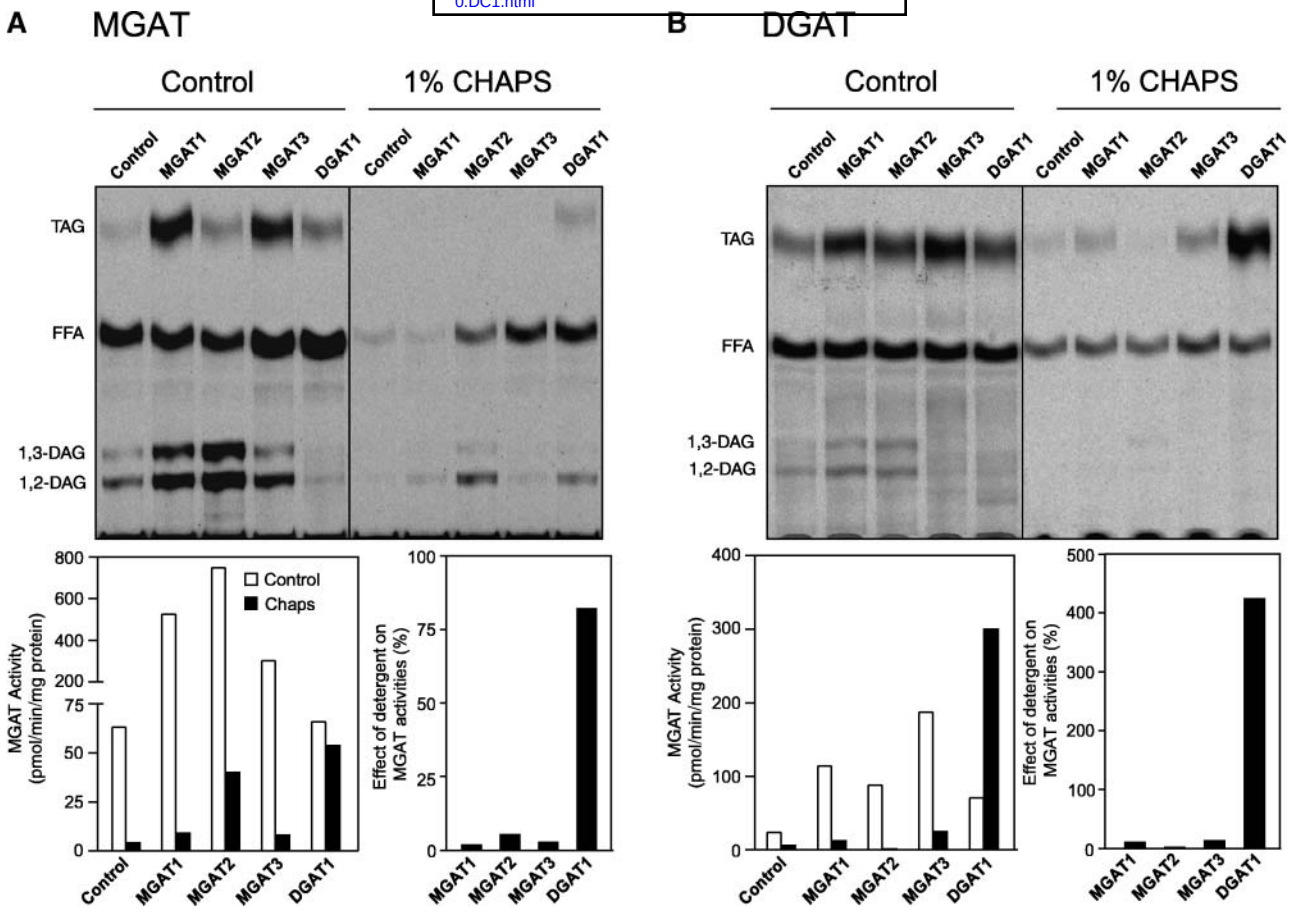

Fig. 4. Effects of CHAPS on MGAT activity (A) and DGAT activity (B) of MGAT1, MGAT2, and MGAT3 and DGAT1. The reaction was conducted by incubating 200 μM *sn*-2-monooleoylglycerol (A) or *sn*-1,2-dioleoylglycerol (B) and 20 μM [^{14}C]oleoyl-CoA for 10 min at room temperature with 100 μg of protein in the absence or presence of 1% CHAPS, followed by lipid extraction and TLC analysis. Specific MGAT and DGAT activities are shown by representative TLC analyses (upper panels) and quantified as shown in the lower bar graphs. The effects of CHAPS on the enzyme activities are also expressed as percentage of enzyme activity obtained in the absence of the detergent.

monolauroylglycerol as substrate. As shown in **Fig. 5**, the recombinant MGAT2 and MGAT3 showed no catalytic activities toward either biotinylated monolauroylglycerol (Fig. 5A, lanes 4–6) or biotinylated lauroyl-CoA (Fig. 5B, lanes 4–6). In contrast, both enzymes demonstrated high acyltransferase activities toward unconjugated lauroyl-CoA and monolauroylglycerol (Fig. 5A, B, lanes 1–3), as indicated by the increased production of radiolabeled 1,2-DAG by MGAT2 and TAG by MGAT3 compared with the wild-type control. MGAT1 also did not show catalytic activity toward biotinylated substrates (data not shown). These results suggest that biotinylated MAG and acyl-CoA are no longer recognized as substrates by the MGAT enzymes.

Subcellular localization of MGAT3 and DGAT enzymes in COS-7 cells

To examine differences in the subcellular localization of MGAT3 and DGAT1, we next performed immunocytohistochemical analyses of recombinant MGAT3 and DGAT1 tagged with the FLAG epitope as well as myc-tagged DGAT2 in transiently transfected COS-7 cells. Forty-eight hours after transfection, cells were processed for indirect immunofluorescence staining with antibodies specific for the FLAG epitope (green) and calnexin (red), an ER-resident protein used as a positive marker. Cells were also counterstained with 4′,6-diamino-phenylindole to visualize nuclei (blue). The FLAG-MGAT3 protein expressed in COS-7 cells displayed a perinuclear and punctate pattern (**Fig. 6A**) that colocalized with the ER marker calnexin (Fig. 6B), as demonstrated by the yellow color in the merged image (Fig. 6C). These results demonstrated conclusively that MGAT3 was an ER-associated protein. Likewise, myc-DGAT2 also localized in the ER, as suggested by colocalization with the ER marker calnexin (Fig. 6D–F). In contrast, the staining pattern of FLAG-DGAT1 was quite different, and not restricted to the ER, as indicated by the lack of complete overlap between FLAG-DGAT1 and calnexin (Fig. 6G–I). This difference is further supported by the lack of completed colocalization between DGAT1 and DGAT2 when coexpressed in the same cell (Fig. 6J–L). Furthermore, coexpression of DGAT1 and DGAT2 resulted in an enlarged ER structure (Fig. 6J–L), which is independent of the amount of DNA used in the transfection. As a negative control of the immunostaining process, no significant

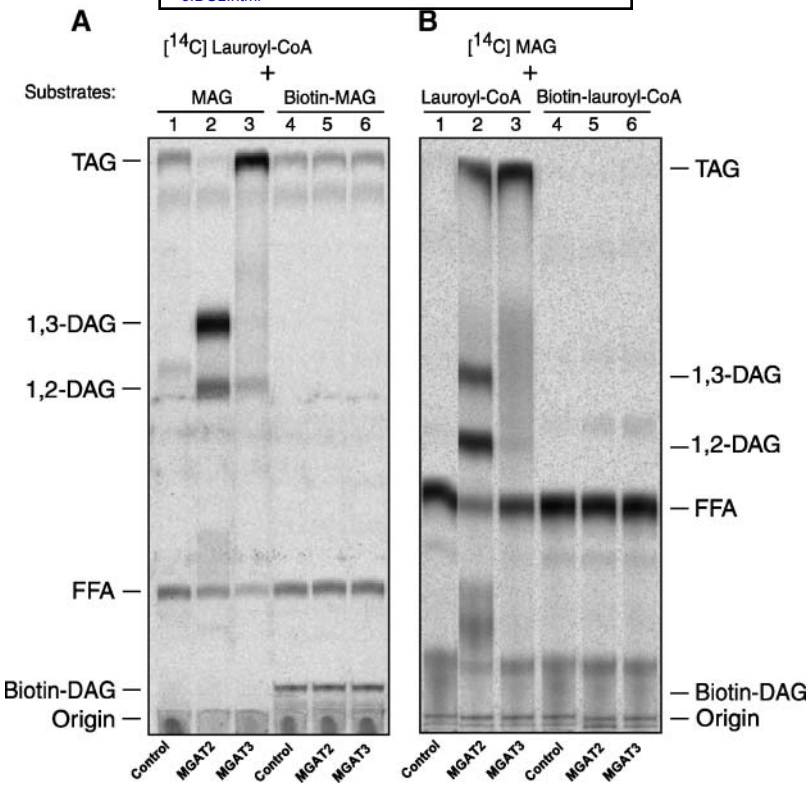

Fig. 5. Loss of recognition of biotinylated substrates by MGAT2 and MGAT3. The reaction was conducted by incubating 100 μg of cell lysates with 20 μM ^{14}C-labeled and 40 μM biotin-labeled substrates for 10 min at room temperature. Although a significant acylation activity in MGAT2- and MGAT3-containing cell lysates toward unconjugated substrates was detected (lanes 1–3 in both panels), such an activity was not observed toward their biotinylated MAG (A, lanes 4–6) or lauroyl-CoA (B, lanes 4–6).

staining was observed in mock-transfected cells stained with anti-FLAG or anti-myc antibody or with normal mouse IgG (data not shown). These data demonstrated that DGAT1 and DGAT2 exhibited some differences in subcellular distribution.

DISCUSSION

In eukaryotes, TAG synthetic pathways play important roles in energy storage, synthesis of phospholipids, lipoprotein trafficking, and detoxification of free fatty acids. TAG also plays pivotal roles in the maintenance of skin integrity, as shown by the phenotype of DGAT2 knockout mice (15). An efficient process of TAG synthesis and breakdown is required to maintain energy homeostasis under different physiological conditions. Thus, excessive storage of TAG in fat tissues causes obesity, whereas depletion of fat tissues results in lipodystrophy that is associated with metabolic diseases such as insulin resistance and diabetes (19). Hence, modulation of TAG synthesis by targeting appropriate enzymes involved in the process may offer treatment options for obesity (20, 21), an ongoing epidemic of the developed nations.

The importance of TAG synthesis is also reflected by the complexity of TAG synthesis pathways. For example, each step of the TAG synthesis pathways is catalyzed by multiple isoforms of enzymes that differ in tissue distribution and/or subcellular localization. Among the three MGAT isoforms identified to date, MGAT3 possesses some unique features. The *MGAT3* gene is found only in higher mammals and humans, but not in rodents. Although named after its enzyme activity, MGAT3 actually shares higher sequence homology with DGAT2 than with other MGAT isoforms. In this report, we investigated catalytic properties and subcellular localization of human MGAT3 and compared these features with those of MGAT isoforms and DGAT enzymes. Our results show that the recombinant MGAT3 enzyme demonstrated significantly higher DGAT activity than did MGAT1 and MGAT2 in the order MGAT3 > MGAT1 > MGAT2 when either MAG or DAG was used as substrate, suggesting that MGAT3 functions as a TAG synthase. These results also support the notion that MGAT and DGAT2 enzymes evolved from a common ancestral gene and may provide guidance for the identification of residues responsible for the DGAT activity of MGAT enzymes.

Although the MGAT3 enzyme exhibited strong DGAT activity, its catalytic properties are quite different from those of DGAT1. MGAT3 was very sensitive to treatment with 1% CHAPS, whereas DGAT1 activity was stimulated by the treatment, indicating a different catalytic mechanism. Interestingly, MGAT3 activities were sensitive to detergent

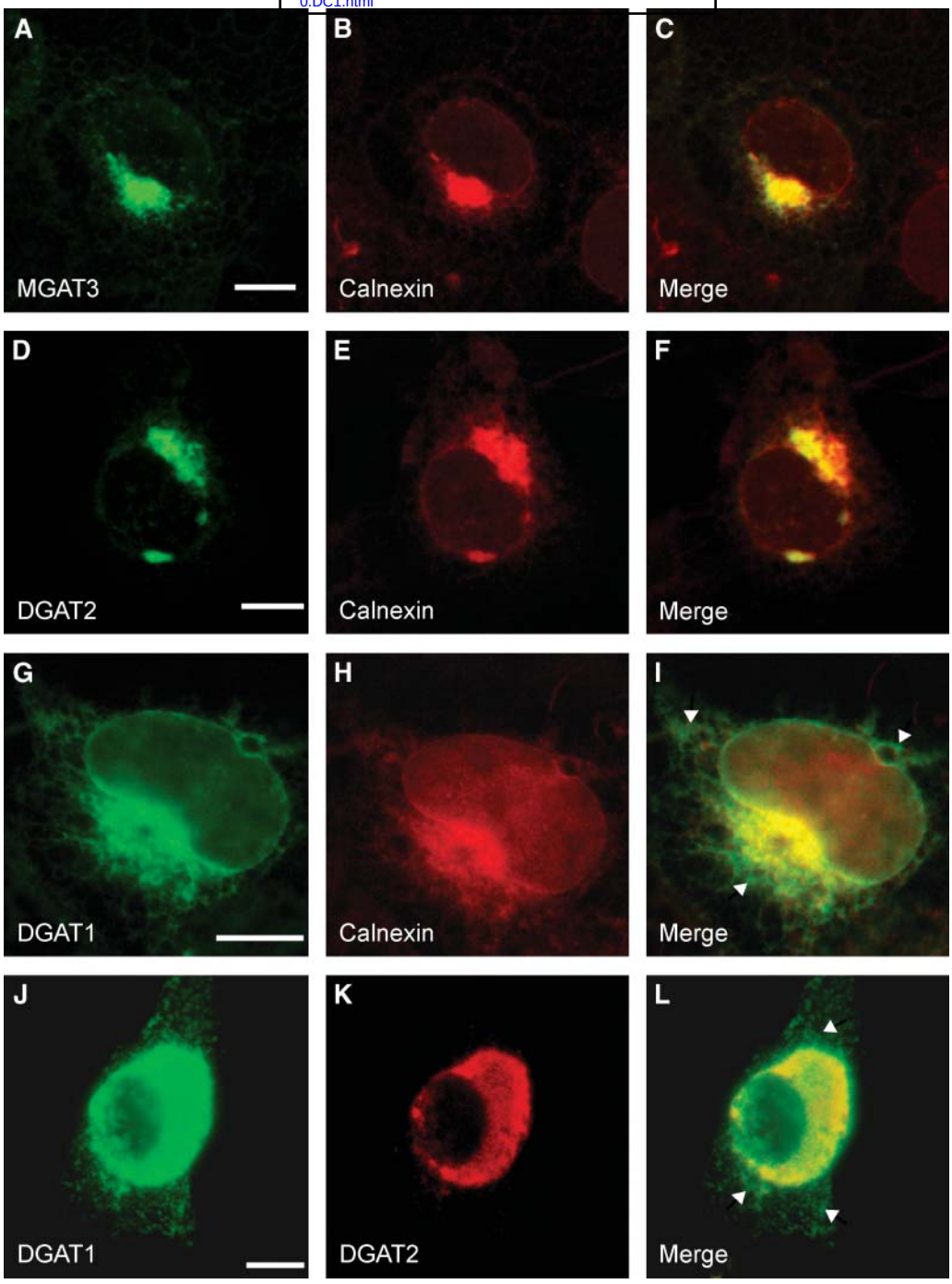

Fig. 6. Subcellular localization of MGAT and DGAT enzymes expressed in COS-7 cells. COS-7 cells were transiently transfected with FLAG-tagged MGAT3 (A), FLAG-tagged DGAT1 (G), and myc-tagged DGAT2 (D), as described in Materials and Methods. Forty-eight hours after transfection, cells were processed for indirect immunofluorescence staining with monoclonal antibodies specific for the tag peptide or calnexin (B,E,H,K), a resident endoplasmic reticulum transmembrane protein. The merged images are shown at right (C,F,I,L). The arrowheads in I and J highlight the staining pattern of DGAT1 that did not colocalize with that of calnexin and DGAT2, respectively. Yellow indicates the colocalization of the two proteins. Bars = 20 μm.

inactivation when either MAG or DAG was used as substrate, suggesting that the DGAT and MGAT activities of the MGAT enzymes are inseparable.

In mammals, there are two isoforms of DGAT enzymes that catalyze the final step in TAG synthesis. The two DGAT enzymes share little sequence homology and exhibit different phenotypes when inactivated in mice. Mice deficient in DGAT1 enzymes were viable and resistant to diet-induced obesity, whereas DGAT2 knockout mice developed lethal lipopenia (2, 15). These pathophysiological conditions were not compensated for by DGAT1 that was ubiquitously expressed in all tissues, suggesting that the

two enzymes are found in different subcellular localizations. To provide direct evidence for how the MGAT3 enzyme differs from the DGAT enzymes in subcellular localization, we compared the subcellular distribution of MGAT3 with that of DGAT1 and DGAT2 enzymes by immunohistochemistry. Consistent with high sequence homology with DGAT2, both MGAT3 and DGAT2 proteins exhibited typical staining patterns of ER, as indicated by colocalization with the ER-resident protein calnexin. Similar results were also obtained with MGAT1 and MGAT2 enzymes (data not shown). Although DGAT1 is also localized in the ER, the protein appears to have a wider distribution, so that its localization goes beyond the site of the ER, which is supported by a lack of complete colocalization of DGAT1 with calnexin or DGAT2 enzyme when coexpressed in the same cells. In support of this notion, overexpression of DGAT1 results in the accumulation of small lipid droplets around the cell periphery, whereas overexpression of DGAT2 leads to increases in large cytosolic lipid droplets (15). This difference is also supported by previous reports on rat liver and yeast DGAT enzymes. Two types of DGAT activities were detected from liver microsomes: one is overt and regulates the cytosolic TAG pools, and the other is latent and plays a role in TAG secretion (22, 23). In yeast cells, inactivation of the yeast *DGAT1* gene resulted in the reduction of DGAT activity on lipid particles, but without significant effect on DGAT activity in the ER (24). Because the MGAT enzymes share similar subcellular localization with DGAT2, it can be envisaged that they may share common functional roles, which remain to be elucidated in future studies of mice with targeted deletion of MGAT enzymes.

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