

# Mitochondrial Glycerol Phosphate Acyltransferase Directs the Incorporation of Exogenous Fatty Acids into Triacylglycerol\*

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The mitochondrial isoform of glycerol-3-phosphate acyltransferase (GPAT), the first step in glycerolipid synthesis, is up-regulated by insulin and by high carbohydrate feeding via SREBP-1c, suggesting that it plays a role in triacylglycerol synthesis. To test this hypothesis, we overexpressed mitochondrial GPAT in Chinese hamster ovary (CHO) cells. When GPAT was overexpressed 3.8-fold, triacylglycerol mass was 2.7-fold higher than in control cells. After incubation with trace [<sup>14</sup>C]oleate (~3 μM), control cells incorporated 4.7-fold more label into phospholipid than triacylglycerol, but GPAT-overexpressing cells incorporated equal amounts of label into phospholipid and triacylglycerol. In GPAT-overexpressing cells, the incorporation of label into phospholipid, particularly phosphatidylcholine, decreased 30%, despite normal growth rate and phospholipid content, suggesting that exogenous oleate was directed primarily toward triacylglycerol synthesis. Transiently transfected HEK293 cells that expressed a 4.4-fold increase in GPAT activity incorporated 9.7-fold more [<sup>14</sup>C]oleate into triacylglycerol compared with control cells, showing that the effect of GPAT overexpression was similar in two different cell types that had been transfected by different methods. When the stable, GPAT-overexpressing CHO cells were incubated with 100 μM oleate to stimulate triacylglycerol synthesis, they incorporated 1.9-fold more fatty acid into triacylglycerol than did the control cells. Confocal microscopy of CHO and HEK293 cells transfected with the GPAT-FLAG construct showed that GPAT was located correctly in mitochondria and was not present elsewhere in the cell. These studies indicate that overexpressed mitochondrial GPAT directs incorporation of exogenous fatty acid into triacylglycerol rather than phospholipid and imply that (a) mitochondrial GPAT and lysophosphatidic acid acyltransferase produce a separate pool of lysophosphatidic acid and phosphatidic acid that must be transported to the endoplasmic reticulum where the terminal enzymes of triacylglycerol synthesis are located, and (b) this pool remains relatively separate from the pool of lysophosphatidic acid and phosphatidic acid that contributes to the synthesis of the major phospholipid species.

Excess accumulation of triacylglycerol in adipocytes is a ma-

yor factor contributing to the current epidemic of obesity-related disorders, including type 2 diabetes, and excess accumulation of triacylglycerol in non-adipocytes has been linked to lipid-mediated apoptosis (1, 2). Although obesity research has focused on the storage of calories that are ingested in excess of energy needs, the observation that mice null for diacylglycerol acyltransferase are resistant to diet-induced obesity suggests the possibility that, independent of energy intake or expenditure, the amount of triacylglycerol stored can be regulated biochemically (3). This remarkable observation further suggests that the synthesis of triacylglycerol can be specifically regulated and that regulation of the synthetic pathway could determine how much triacylglycerol is stored in cells. If this were true, enzymes in the biosynthetic pathway of triacylglycerol synthesis could provide targets for therapy.

The pathway for triacylglycerol synthesis begins with the acylation of glycerol 3-phosphate at the *sn*-1 position by glycerol 3-phosphate acyltransferase (GPAT)<sup>1</sup> (EC 2.3.1.15), the committed step for the synthesis of all glycerolipids. After acylation by GPAT to form lysophosphatidic acid, LPA acyltransferase acylates the *sn*-2 position to form phosphatidic acid, which is then hydrolyzed to form diacylglycerol. Phosphatidic acid is the precursor for the anionic phospholipids, phosphatidylinositol, phosphatidylglycerol, and cardiolipin, whereas diacylglycerol is the precursor for the quantitatively most prominent phospholipids, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, as well as for triacylglycerol. Most of the enzymes required for these synthetic steps are located in the endoplasmic reticulum, but LPA acyltransferase activity and a specific GPAT isoform are also located on the mitochondrial outer membrane (4).

The rate-limiting step of phosphatidylcholine synthesis, CTP:phosphocholine cytidyltransferase, undergoes complex post-translational regulation by membrane lipid composition, subcellular location, and phosphorylation state (5). It has been generally assumed that phospholipid synthesis is tightly regulated in accordance with requirements for membrane turnover and cell proliferation (6), but that the synthesis of triacylglycerol is controlled primarily by the amount of fatty acid available. Thus, triacylglycerol synthesis is enhanced only when fatty acid synthesis or uptake exceeds the cellular need for phospholipid synthesis. This view is supported by cell culture

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<sup>1</sup> The abbreviations used are: GPAT, glycerol-3-phosphate acyltransferase; ACAT, acyl-CoA, cholesterol acyltransferase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DGAT, diacylglycerol acyltransferase; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LPA, lysophosphatidic acid; MEM, minimal essential medium; PPAR, peroxisome proliferator activation receptor; PBS, phosphate-buffered saline; SREBP, sterol regulatory element-binding protein; TLC, thin layer chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.

studies showing that when the concentration of media fatty acid is low, it is primarily incorporated into phospholipid, and when fatty acid concentration is high, the formation of triacylglycerol is greatly favored (7, 8). In addition, overexpression of putative fatty acid transporters can increase triacylglycerol stores (9).

On the other hand, the view that triacylglycerol storage is unregulated is inconsistent with the observation that DGAT knockout mice remain slim on a high fat diet even though they are able to synthesize triacylglycerol by a DGAT-independent mechanism (3). Indirect evidence that triacylglycerol synthesis is regulated also comes from studies of the mitochondrial isoform of GPAT, which is transcriptionally controlled by diet changes that promote or depress the synthesis of triacylglycerol. For example, carbohydrate feeding or high insulin concentrations increase liver GPAT mRNA, whereas fasting or lack of insulin down-regulates GPAT mRNA (10), probably via insulin-mediated regulation of SREBP-1c (11, 12). Mitochondrial GPAT mRNA is also elevated 12-fold in pancreatic  $\beta$ -cells from obese diabetic Zucker *fa/fa* rats, implying a role for triacylglycerol synthesis in the observed  $\beta$ -cell steatosis (13). Acute GPAT regulation has also been reported in mitochondria; consistent with the need to decrease triacylglycerol synthesis and increase energy-producing oxidation in the face of limited nutrients, 5-amino-4-imidazolecarboxamide riboside, a synthetic activator of AMP-activated kinase, the proposed sensor of cellular fuel deprivation, acutely decreases GPAT activity (14).

Despite these data suggesting that mitochondrial GPAT regulates triacylglycerol synthesis in cells, this hypothesis has not been examined directly. To determine whether mitochondrial GPAT plays a specific role in triacylglycerol synthesis, we created a stable cell line in which the expression of mitochondrial GPAT could be regulated.

#### EXPERIMENTAL PROCEDURES

**Materials**—Silica gel G plates were from Whatman. [ $^3\text{H}$ ]Glycerol and [ $^{14}\text{C}$ ]oleic acid were from PerkinElmer Life Sciences. Essentially fatty acid-free BSA, anti-FLAG M2 monoclonal antibody, and digitonin were from Sigma. Lipid standards were from Doosan Serdary Research Laboratories. Tissue culture supplies were from Corning, and culture medium and ultrafiltered FBS were obtained from Life Technologies, Inc.

**Construction of pBI-EGFP/GPAT-FLAG and Establishment of Double-stable Transfected CHO Cell Line**—GPAT with a FLAG epitope (DYKDDDDK) at the C terminus was constructed by adding the FLAG coding sequence by polymerase chain reaction amplification in a two-step procedure. The plasmid pcDNA3.1 containing the entire GPAT open reading frame flanked by *Bam*HI and *Xho*I sites was used as a template for the amplification. In the first step, the oligonucleotide (5' *Bam*GPAT) ATCGCGGATCCACCATGGAGGAGT was used as the 5' primer, and the oligonucleotide **CATCATCATCCTTGTAGTCGAGCACGACGAAGCTGAGAATGTA** (3' GPAT-F1), which adds 19 nucleotides encoding the FLAG epitope (shown in bold), was used as the 3' primer. The amplification product was used as a template for the second polymerase chain reaction step, using the same 5' primer and the 3' primer oligonucleotide CGCC **TCTAGACTACTTGTATCATCATCCTTGTAGTCGAGCA** (3' *Xba* FL2), designed to add the last 5 nucleotides corresponding to the FLAG epitope, a stop codon, and a *Xba*I restriction site. The resulting second amplification product was digested with *Bam*HI and *Xba*I and inserted in the multicloning site of the plasmid pcDNA3.1 to generate pcDNA3.1-GPAT-FLAG. *Mlu*I and *Nhe*I sites flanking GPAT-FLAG were added by polymerase chain reaction, and the amplified GPAT-FLAG product was subcloned into the mammalian expression vector pBI-EGFP-tet (CLONTECH), a bidirectional response plasmid that allows simultaneous expression of both green fluorescent protein and GPAT-FLAG under the control of a single tetracycline (doxycycline)-responsive element. CHO cells were cotransfected with the pBI-EGFP/GPAT-FLAG construct and a pTet-Off plasmid (CLONTECH), which expresses a hybrid protein and activates transcription in the absence of doxycycline. When doxycycline is present, neither GPAT nor EGFP are expressed. To establish the double-stable transfected cell line, CHO cells in 35-mm dishes were cotrans-

fectected with pBI-EGFP/GPAT-FLAG (1  $\mu\text{g}$ ) and pTet-Off (2  $\mu\text{g}$ ) using Transfast transfection reagent (Promega). Twenty-four h after transfection, cells were selected with 600  $\mu\text{g}/\text{ml}$  G418 for 2 weeks. Positive GFP clones were identified under UV light microscopy and diluted into a 96-well plate. After continued selection with G418 for 6–8 weeks, lines were selected that expressed green fluorescent protein in 100% of the cells. Cells were routinely cultured in MEM with 10% heat-inactivated FBS plus 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (normal medium) at 34 °C, 5%  $\text{CO}_2$ , and 100% humidity, in the presence or absence of doxycycline (2  $\mu\text{g}/\text{ml}$ ) to repress or induce, respectively, GPAT overexpression. The medium was changed every other day.

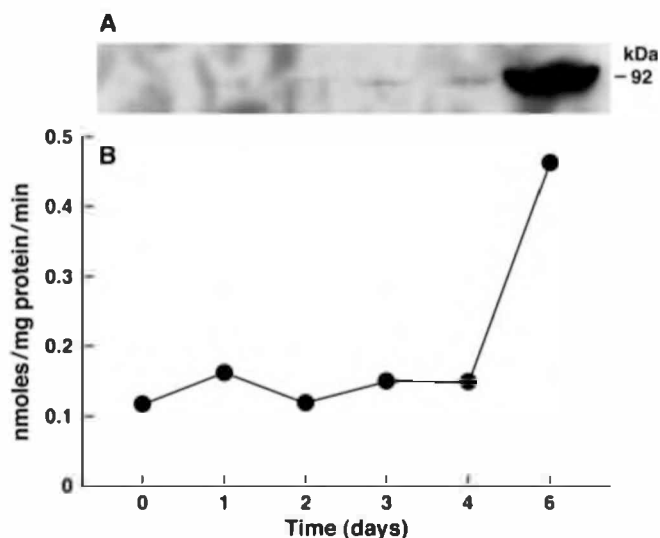
Transient transfection and immunocytochemical analysis of GPAT expression were performed as described previously (15) with modifications. CHO K1 cells were trypsinized and seeded in 100-mm tissue culture dishes containing six to eight glass coverslips. The cells were incubated at 37 °C until 70% confluence and then transfected with cationic liposomes (LipofectAMINE, Life Technologies, Inc.) with pcDNA 3.1 vector alone or with pcDNA3.1-GPAT-FLAG. After 27 h cells were washed with PBS and incubated for 15 min with 0.1  $\mu\text{M}$  Texas Red, a mitochondrial marker (Mito-tracker, Molecular Probes) in culture medium, rinsed with PBS, and fixed with 2% formaldehyde at room temperature for 10 min. After washing with PBS, cells were permeabilized with 5  $\mu\text{g}/\text{ml}$  digitonin in buffer A (0.3 M sucrose, 25 mM  $\text{MgCl}_2$ , 0.1 M KCl, 1 mM EDTA, 10 mM PIPES buffer, pH 6.8) for 5 min on ice, incubated with a 1/1000 dilution of anti-FLAG M2 monoclonal antibody (Sigma) for 60 min, washed with PBS, and incubated for 45 min with a 1/200 dilution of fluorescein isothiocyanate (FITC)-conjugated secondary antibody: goat anti-mouse IgG (Santa Cruz Biotechnology). Cells were then washed in PBS, and the coverslips were mounted. Confocal microscopy was performed on a Zeiss LSH 210 fluorescence microscope equipped with excitation argon laser 488/514 to visualize cells stained with the Texas Red and FITC antibodies. Images were saved and analyzed using Photoshop v.5.5 and Image Processing Tool Kit to overlay multiband fluorescence images.

**Total Particulate Preparations and Enzyme Assays**—CHO cells that had been grown continuously in either the presence or absence of doxycycline were washed with PBS, scraped into Medium 1 (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol), homogenized with 10 up and down strokes in a Teflon-glass homogenizing vessel, and centrifuged at 100,000  $\times g$  for 1 h to obtain total particulate (membrane) fractions. GPAT activity was measured with 10–40  $\mu\text{g}$  of protein after incubation for 15 min on ice in the absence (total GPAT) or presence (mitochondrial GPAT) of 2 mM *N*-ethylmaleimide. The 10-min assay at room temperature contained 2 mM [ $^3\text{H}$ ]glycerol 3-phosphate and 0.75 mM palmitoyl-CoA (16). Microsomal GPAT activity was calculated as total activity minus *N*-ethylmaleimide-resistant activity. Acyl-CoA synthetase activity was assayed using 1–5  $\mu\text{g}$  of protein, 50  $\mu\text{mol}$  of [ $^{14}\text{C}$ ]palmitic acid, 10 mM ATP, 5 mM dithiothreitol, and 0.25 mM CoA for 10 min at 37 °C (17). DGAT activity was assayed using 5–20  $\mu\text{g}$  of protein, 0.2 mM *sn*-1,2-dioleoylglycerol, and 30  $\mu\text{M}$  [ $^3\text{H}$ ]palmitoyl-CoA for 10 min at room temperature (18).

**GPAT Protein Expression**—Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, which was probed with anti-FLAG M2 antibody (Sigma), washed extensively, and then probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). GPAT-FLAG protein was visualized by a reaction with Supersignal chemiluminescent substrate (Pierce) and exposed to x-ray film.

**Cell Radiolabeling and Lipid Analysis**—CHO cells (stably overexpressing GPAT) were seeded in 60-mm dishes, grown to near confluence in the continuous presence or absence of 2  $\mu\text{g}/\text{ml}$  doxycycline, and incubated with either trace ( $\sim 3$   $\mu\text{M}$ ) or 100  $\mu\text{M}$  [ $^{14}\text{C}$ ]oleic acid (0.25  $\mu\text{Ci}/\text{dish}$ ) in medium supplemented with 0.5% BSA. HEK293 (human embryonic kidney) cells in 60-mm dishes were transfected with pcDNA3.1-GPAT-FLAG or pcDNA3.1 (control) for 18 h, then incubated with trace ( $\sim 3$   $\mu\text{M}$ ) [ $^{14}\text{C}$ ]oleic acid (0.25  $\mu\text{Ci}/\text{dish}$ ) in medium supplemented with 0.5% BSA. At various time points, the medium was removed, and the cells were washed with 0.1% BSA in ice-cold PBS scraped in ice-cold methanol and  $\text{H}_2\text{O}$ . Total lipids were extracted (19) and concentrated in a Savant SpeedVac concentrator. Neutral lipids were resolved by TLC using a solvent system consisting of hexane: ethylether:acetic acid (80:20:1; v/v). Phospholipid species were separated by TLC in a one-dimensional solvent system of chloroform:methanol:acetic acid:water (50:37.5:3.5:1.5; v/v). All samples were chromatographed in parallel with pure lipid standards. The [ $^{14}\text{C}$ ]labeled lipids were detected using a Bioscan Image System.

**Determination of SREBP-1 and PPAR $\gamma$  Levels**—Cell monolayers at 80% confluence were scraped from the dishes, washed twice with ice-



**FIG. 1. Time course of mitochondrial GPAT expression and activity in Tet-Off cells transfected with pBI-EGFP/GPAT-FLAG.** After growing routinely in the presence of 2  $\mu\text{g/ml}$  doxycycline, cells in 100-mm dishes were placed in media without doxycycline. At the intervals shown, cells from each dish were scraped, homogenized, and centrifuged to obtain total particulate preparations for measurement of mitochondrial GPAT expression (with M2 anti-Flag antibody) (A) and specific activity (B).

cold PBS, resuspended in 0.25 M sucrose, 1 mM EDTA, 1 mg/ml protease inhibitor mixture (Sigma), and 10 mM Tris buffer, pH 7.4, and disrupted by several passages through a 22-gauge syringe needle. Proteins from total membranes from GPAT overexpressing CHO cells and controls were separated in SDS-PAGE and blotted onto nitrocellulose membranes. Immunoblots were performed using the corresponding polyclonal antibody against full-length (128 kDa) and mature (68 kDa) SREBP-1 and PPAR $\gamma$  (Santa Cruz Biotechnologies). Anti-rabbit horseradish peroxidase-conjugated antibody was used as secondary antibody. Immunoreactive bands were detected using a chemiluminescence detection kit (Pierce).

**Other Measurements**—Protein was measured using serum bovine albumin as the standard (20). Triacylglycerol was measured using a TGO-Trinder kit (Sigma), and phospholipid was measured by phosphate analysis (21). Distribution of [ $^{14}\text{C}$ ]oleate in the *sn*-1 and *sn*-2 positions of phospholipids was examined by phospholipase A $_2$  (*Naja naja*) (Sigma) hydrolysis of phospholipids labeled for 48 h with [ $^{14}\text{C}$ ]oleate as described previously (22). Lipids extracts were chromatographed with hexane/ethyl ether/acetic acid (80:20:1; v/v) to separate unesterified fatty acids from total polar lipids and counted in the Bioscan Image System. Data are presented as means  $\pm$  S.D. Differences between the transfected cell lines and the controls were analyzed by Student's *t* test.

## RESULTS

A stable Tet-Off CHO cell line was constructed that expressed both EGFP and mitochondrial GPAT with a FLAG-epitope at the C terminus. EGFP was used to ensure that every cell expressed the construct. When cells were grown in the absence of doxycycline, fluorescence microscopy showed expression of both EGFP and the GPAT-FLAG epitope in every cell; no fluorescence was observed when 2  $\mu\text{g/ml}$  doxycycline was present (data not shown). Withdrawing doxycycline from the medium resulted in expression of GPAT protein at 6 days and a 3.3-fold increase in GPAT specific activity over the endogenous basal activity (Fig. 1). Adding doxycycline to the medium repressed GPAT activity and protein expression within 24 h, suggesting that GPAT protein is rapidly degraded (data not shown). A dose response showed that plasmid-mediated GPAT expression (ascertained by Western blot analysis of the FLAG epitope) was nearly completely blocked when as little as 0.05  $\mu\text{g/ml}$  doxycycline was present in the medium for 24 h.

To determine whether increased mitochondrial GPAT expression resulted in secondary effects on related enzymes of glycerolipid synthesis, we measured activities of microsomal GPAT, acyl-CoA synthetase, and diacylglycerol acyltransferase in total particulate preparations from the Tet-Off CHO cells grown with (control) or without (GPAT overexpressing) doxycycline (Table I). Mitochondrial GPAT specific activity was 3.8-fold higher in the overexpressing cells compared with controls, but no changes were observed in the specific activities of the other enzymes.

**GPAT-overexpressing CHO Cells Contain More Triacylglycerol**—GPAT-overexpressing CHO cells in medium without added fatty acid contained 2.7-fold more triacylglycerol mass than control cells (Fig. 2). When the cells were incubated with 100  $\mu\text{M}$  oleate to stimulate triacylglycerol accumulation, both groups of cells increased their neutral lipid content at similar rates throughout the 24-h incubation time, but the mass of triacylglycerol was always greater in the GPAT overexpressing cells (3.1-, 2.2-, and 1.6-fold higher at 3, 6, and 24 h, respectively).

**GPAT Expressed in CHO Cells Is Targeted to Mitochondria**—Because the final steps in the enzyme pathway that synthesizes triacylglycerol are located in the endoplasmic reticulum, it was surprising to find that overexpression of a mitochondrial enzyme resulted in excess triacylglycerol content. Thus, we wondered whether GPAT might be overexpressed in an aberrant location that could provide LPA directly for triacylglycerol biosynthesis. Because the EGFP expressed by GPAT overexpressing CHO cells would interfere with FITC excitation at 488 nm, we studied CHO cells that had been transiently transfected with an identical GPAT-FLAG construct. Mitochondrial GPAT activity at 28 h after transfection increased from 0.14 to 0.86 nmol/min/mg of protein. Anti-FLAG indirect antibody staining recognized the expressed GPAT (Fig. 3b) and matched the specific mitochondrial marker signal (Fig. 3, c and d). Both the cells transfected with the empty vector and those that had not been exposed to the first antibody emitted fluorescence in the FITC band only at background levels. Thus, FITC staining was not due to nonspecific binding of the anti-FLAG antibody to mitochondria. When the overlapping image (Fig. 3d) was subtracted from the GPAT localization image (Fig. 3b) using Boolean operator transformation, the resulting image was completely dark, indicating that GPAT was present only in mitochondria and not in other locations.

**GPAT-overexpressing CHO Cells Synthesize Excess [ $^{14}\text{C}$ ]Triacylglycerol**—Because GPAT-overexpressing CHO cells accumulated more triacylglycerol mass, we measured the ongoing rate of triacylglycerol formation in the absence of added medium fatty acid. Cells were incubated with tracer amounts ( $\sim 3 \mu\text{M}$ ) of [ $^{14}\text{C}$ ]oleic acid for up to 24 h. Cells that overexpressed GPAT showed a significantly higher incorporation of [ $^{14}\text{C}$ ]oleate into triacylglycerol (2.7-, 2.5-, and 3.4-fold higher at 3, 6, and 24 h, respectively) compared with control cells (Fig. 4A). Overexpression of GPAT did not affect [ $^{14}\text{C}$ ]oleic acid incorporation into cholesterol esters (Fig. 4B).

**Synthesis of [ $^{14}\text{C}$ ]Phospholipid in GPAT-overexpressing CHO Cells**—Not only is GPAT the committed step for triacylglycerol synthesis, but it is also required for the synthesis of all the glycerophospholipids. Therefore we examined ongoing incorporation of low amounts of [ $^{14}\text{C}$ ]oleate ( $\sim 3 \mu\text{M}$ ) into the different phospholipid species in the GPAT-overexpressing CHO cells and in controls (Fig. 5). At each time point up to 24 h, GPAT overexpressing cells showed 30% decreased incorporation of [ $^{14}\text{C}$ ]oleate into total phospholipids. [ $^{14}\text{C}$ ]Oleate-labeled phosphatidylcholine decreased 30–35% and [ $^{14}\text{C}$ ]PI plus [ $^{14}\text{C}$ ]phosphatidylserine decreased 40–50%, but [ $^{14}\text{C}$ ]phosphatidyleth-

TABLE I  
Glycerolipid synthetic activities in control cells and cells that over-express mitochondrial GPAT

Mitochondrial GPAT, microsomal GPAT, acyl-CoA synthetase, and diacylglycerol acyltransferase were assayed as described under "Experimental Procedures" in three independent total particulate preparations from cells that were grown continuously with (control) or without (GPAT) doxycycline. Data are expressed as mean  $\pm$  S.D. Numbers in parentheses represent the percent of total GPAT specific activity.

	Mitochondrial GPAT	Microsomal GPAT	Acyl-CoA synthetase	Diacylglycerol acyltransferase
	<i>nmol/min/mg protein</i>			
Control (+doxycycline)	0.11 $\pm$ 0.01	0.85 $\pm$ 0.22	11.0 $\pm$ 2.5	0.37 $\pm$ 0.03
GPAT (-doxycycline)	0.42 $\pm$ 0.09 <sup>a</sup>	0.62 $\pm$ 0.26	10.7 $\pm$ 3.1	0.36 $\pm$ 0.01
	(11%)	(89%)		
	(40%)	(60%)		

<sup>a</sup>  $p < 0.01$  compared to control.

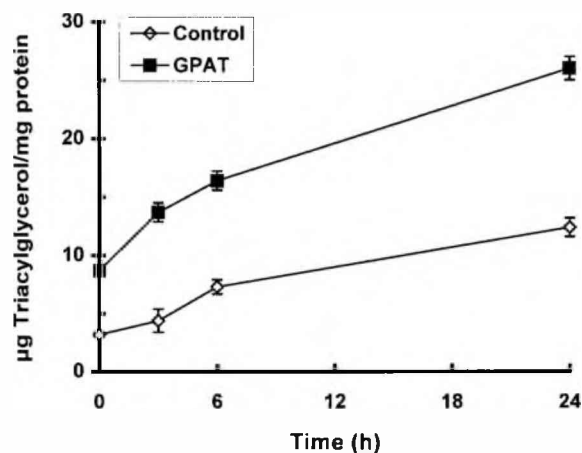


FIG. 2. GPAT overexpression promotes triacylglycerol accumulation in CHO cells. Preconfluent CHO cells were grown in MEM 10% FBS in the presence (Control) or absence (GPAT) of 2  $\mu$ g/ml doxycycline. At time 0 they were supplemented with 100  $\mu$ M sodium oleate for up to 24 h. Cells were collected, homogenized, and extracted as described under "Experimental Procedures." Total cell triacylglycerol was measured at the intervals shown. Values represents the means  $\pm$  S.D. for three 100-mm dishes. Some error bars are hidden by the symbols.

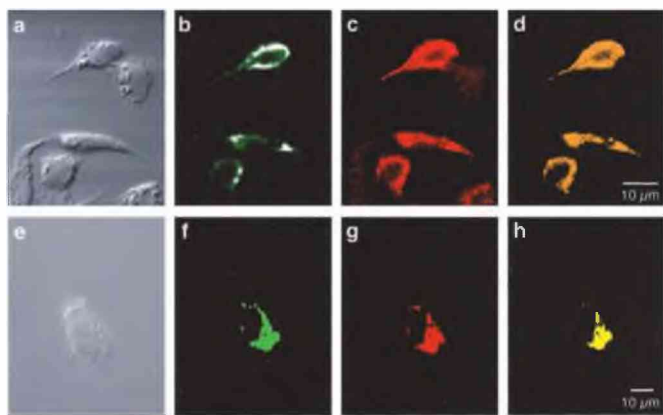


FIG. 3. GPAT colocalizes with mitochondria. CHO cells (a–d) or HEK293 cells (e–h) were transiently transfected with GPAT-FLAG for 27 h and stained as described under "Experimental Procedures." a and e, phase-contrast microscopy; b and f, anti-FLAG primary antibody and FITC-conjugated secondary antibody; c and g, Texas Red Mito-Tracker; d and h, overlapped image of b and c or f and g. The scale bars represent 10  $\mu$ m. The figure is representative of three to four independent transfections for each cell line.

anolamine decreased relatively little (15%). Viewed as a whole, the control cells incorporated 4.7 times more label into phospholipid than triacylglycerol, but the GPAT overexpressing cells incorporated the <sup>14</sup>C label equally into the two lipid species. With trace [<sup>14</sup>C]oleate plus 10% FBS (which contains 21–28  $\mu$ M fatty acid (7)), the cells are exposed to medium that

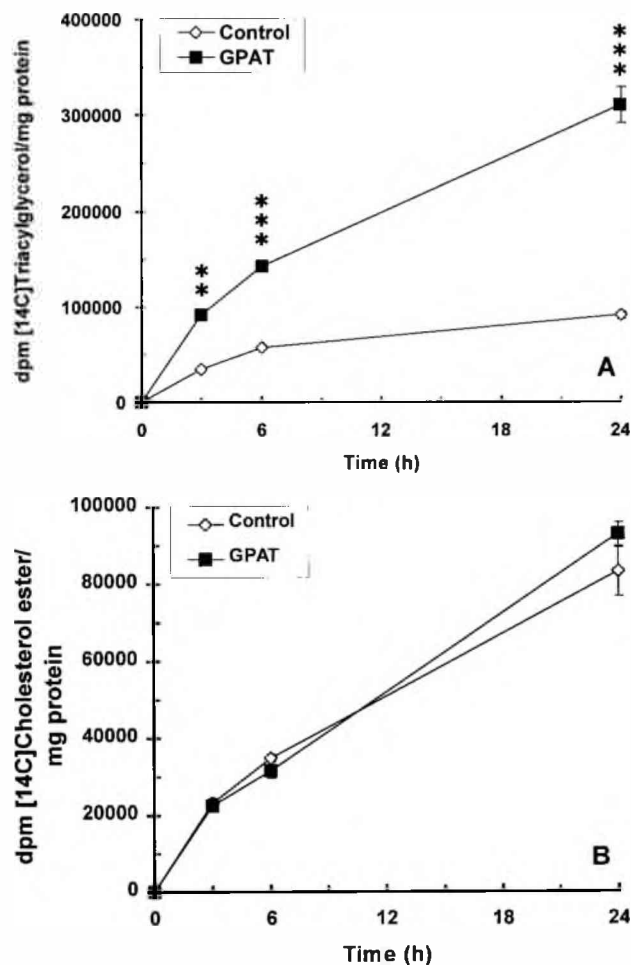


FIG. 4. GPAT overexpression stimulates incorporation of [<sup>14</sup>C]oleate into triacylglycerol but not cholesterol ester. Nearly confluent CHO cells, maintained in 10% FBS, MEM in the presence (Control) or absence (GPAT) of 2  $\mu$ g/ml doxycycline, were incubated with 0.25  $\mu$ Ci of [<sup>14</sup>C]oleic acid/dish in the same media plus 0.5% BSA for up to 24 h. Lipids were extracted and separated as described under "Experimental Procedures." Data points represent the means  $\pm$  S.D. of an experiment performed in triplicate. Some error bars are hidden by the symbols. A, [<sup>14</sup>C]triacylglycerol; B, [<sup>14</sup>C]cholesterol ester. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$  compared with the control.

contains  $\sim$ 30  $\mu$ M fatty acid. Thus, the incorporation data are surprising, because normally with concentrations of media oleate of less than 30  $\mu$ M, four to five times more label is incorporated into phospholipid than triacylglycerol, as occurred in the control cells. To enable radiolabel to be distributed equally in the two lipid fractions in fibroblasts, total media oleate concentrations must be greater than 60  $\mu$ M (8).

**Phospholipid Synthesis Was Not Impaired in GPAT-overexpressing CHO Cells**—Diminished incorporation of [<sup>14</sup>C]oleate into phospholipid could result from a decrease in the rate of

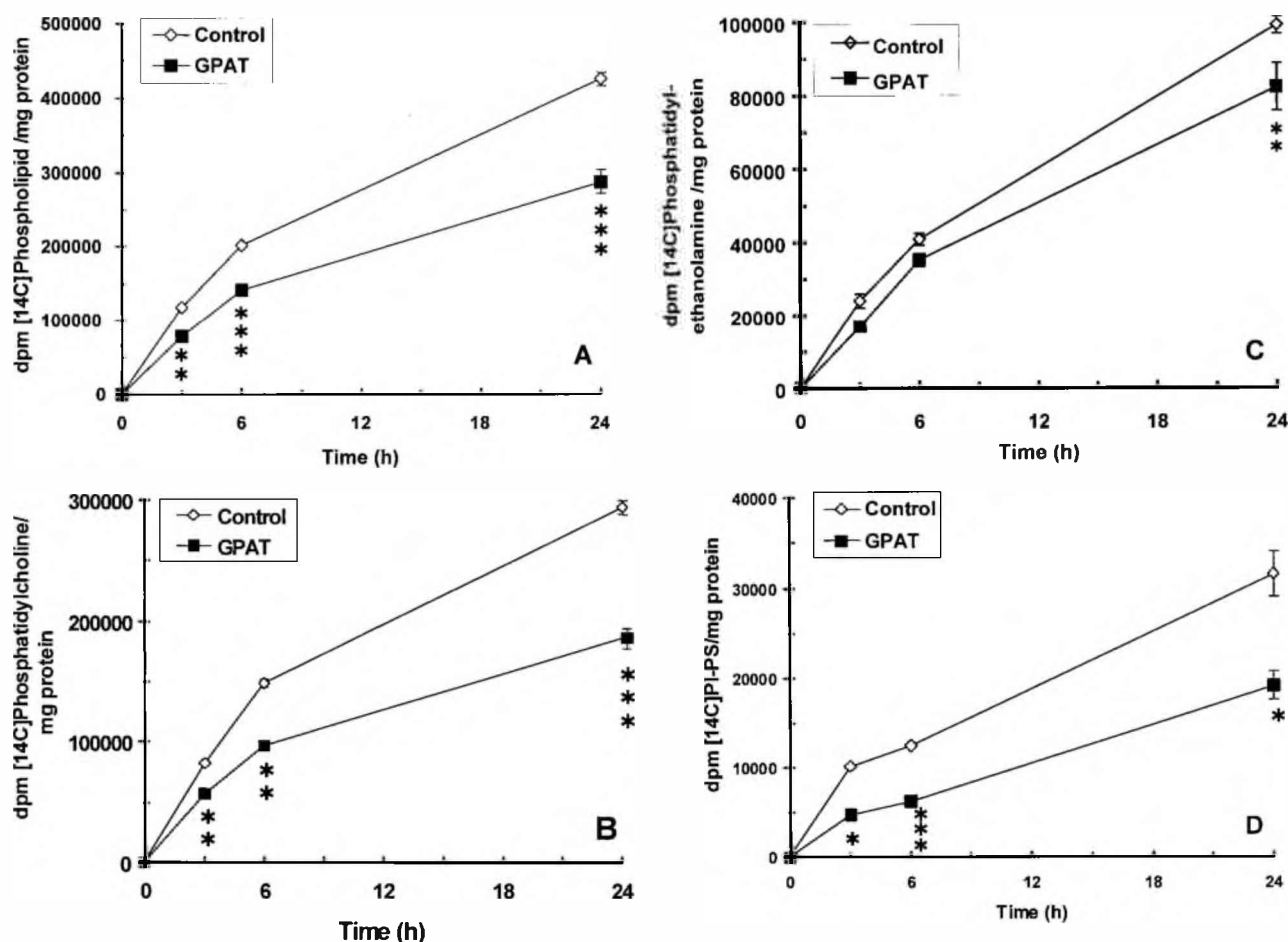


FIG. 5. **GPAT overexpression inhibits incorporation of [<sup>14</sup>C]oleate into phospholipid.** Nearly confluent CHO cells grown in 10% FBS, MEM plus (Control) or minus (GPAT) 2  $\mu$ g/ml doxycycline were labeled with 0.25  $\mu$ Ci of [<sup>14</sup>C]oleic acid/dish in the same media for up to 24 h. At each time point, cell lipids were extracted, and polar lipid species were separated by TLC. Each value corresponds to the mean  $\pm$  S.D. of an experiment performed in triplicate. Some error bars are smaller than the symbols. A, [<sup>14</sup>C]-labeled total phospholipids; B, [<sup>14</sup>C]phosphatidylcholine; C, [<sup>14</sup>C]phosphatidylethanolamine; and D, [<sup>14</sup>C]phosphatidylinositol (PI) plus phosphatidylserine (PS). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$  compared with the control.

phospholipid synthesis or because exogenously added fatty acids are incorporated preferentially into triacylglycerol rather than phospholipid. We examined these possibilities by comparing the rate of cell division of control CHO cells and CHO cells that overexpressed GPAT. The rate of growth was identical for the two cell types (Fig. 6), phospholipid content was 0.42, 0.34, and 0.28  $\mu$ g/ $\mu$ g of protein in GPAT, control, and vector-transfected cells, respectively, and these differences were not statistically significant. Thus, phospholipid synthesis and accretion appeared to be unaffected despite the diminished incorporation of [<sup>14</sup>C]oleate. Because mitochondrial GPAT prefers saturated over unsaturated fatty acids, it has been hypothesized that one of the enzyme's functions might be to direct the incorporation of saturated fatty acids into the *sn*-1 position of phospholipids (4). To test this idea, GPAT overexpressing and control cells were incubated with [<sup>14</sup>C]oleate ( $\sim 3 \mu$ M). After 48 h total lipids were extracted and treated with phospholipase A<sub>2</sub>, which hydrolyzes the fatty acid at the *sn*-2 position of phospholipids. The [<sup>14</sup>C]oleate content of hydrolyzed (*sn*-2 fatty acids) and nonhydrolyzed (*sn*-1 acyl-lysophospholipid) was quantified. In both GPAT-overexpressing and control cells, 70% of the label was released, showing that there had been equivalent incorporation of oleate (30%) at the *sn*-1 position in control and GPAT overexpressing cells. This study indicates that a 3.8-fold increase in GPAT specific activity did not affect the positional distribution of a monounsaturated fatty acid.

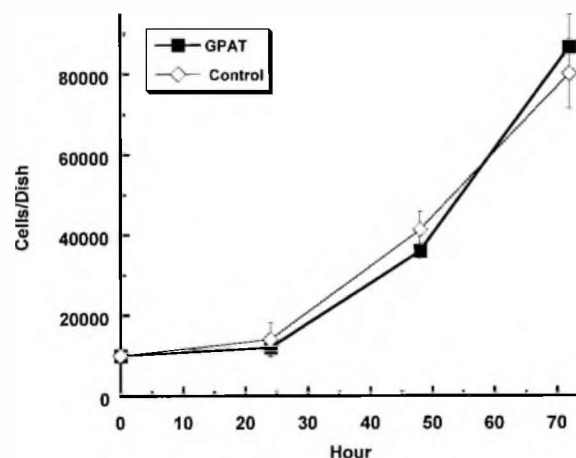
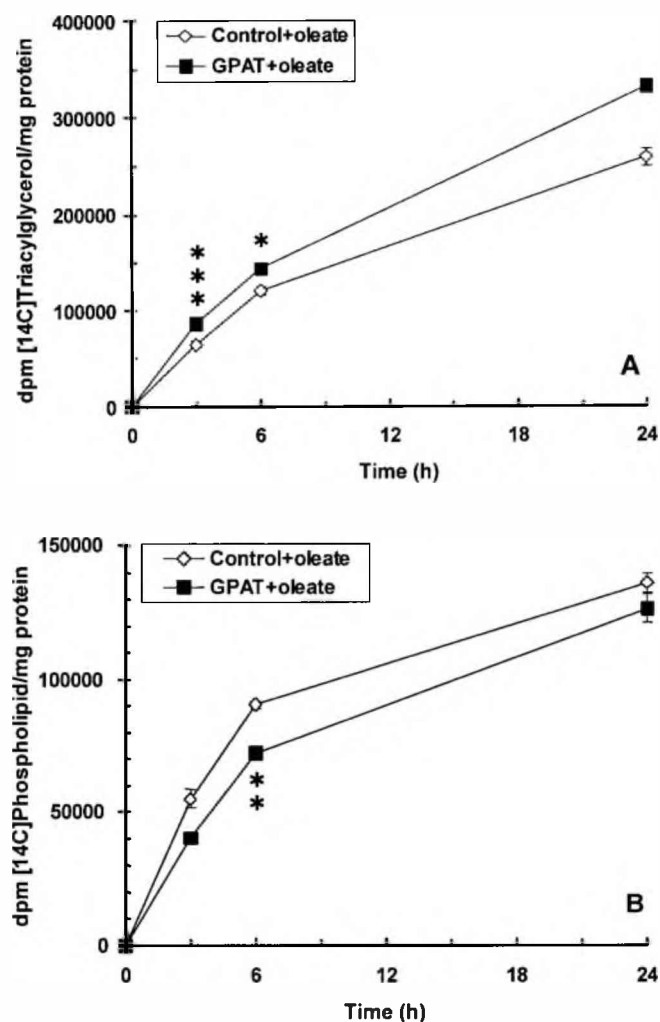


FIG. 6. **Growth rate was identical in control and GPAT-overexpressing cells.** pBI-EGFP/GPAT-FLAG CHO cells were grown in the presence or absence of 2  $\mu$ g/ml doxycycline. At time 0, cells growing in the presence or absence of doxycycline were trypsinized and plated in 35-mm plates (10,000 cells/dish) in the continued presence (Control) or absence (GPAT) of doxycycline. Cells were trypsinized and counted at 24, 48, and 72 h. Data are expressed as means  $\pm$  S.D. of three dishes for each time point.

*Incorporation of [<sup>14</sup>C]Oleic Acid into Triacylglycerol and Phospholipids in the Presence of 100  $\mu$ M Oleate*—As shown in Figs. 4 and 5, when a low concentration of fatty acid was



**FIG. 7. Fatty acid enhances incorporation of [<sup>14</sup>C]oleic acid into triacylglycerol by overexpressed GPAT.** Cells were incubated with 0.25  $\mu$ Ci of [<sup>14</sup>C]oleic acid/dish in 0.5% BSA, 10% FBS, MEM plus 100  $\mu$ M sodium oleate for up to 24 h. At each time point total lipids were extracted from the cells and separated by TLC. Values represent the means  $\pm$  S.D. of three experiments. Some error bars are hidden by the symbols. A, [<sup>14</sup>C]triacylglycerol; B, [<sup>14</sup>C]phospholipid. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.005$  compared with the control.

present in the media, GPAT overexpression in CHO cells greatly enhanced the incorporation of [<sup>14</sup>C]oleate into triacylglycerol while concomitantly decreasing incorporation into phospholipid. We next asked whether this pattern of lipid synthesis in CHO cells overexpressing GPAT would be altered if the media contained the high concentration of fatty acid commonly used in experiments designed to promote triacylglycerol synthesis and accumulation in cultured cells. When the formation of newly synthesized [<sup>14</sup>C]triacylglycerol was measured in the presence of 100  $\mu$ M [<sup>14</sup>C]oleate, the incorporation of [<sup>14</sup>C]oleate into triacylglycerol was only 1.3-fold higher in the GPAT-overexpressing cells compared with control cells (Fig. 7A), and similar amounts of label were incorporated into phospholipid in the control and overexpressing cells (Fig. 7B). When 100  $\mu$ M oleate was present, the ratio of triacylglycerol to phospholipid was 2.6 to 1 in GPAT-overexpressing cells compared with 1.9 to 1 in control cells (Table II). Thus, whether oleate availability was high or low, GPAT overexpression promoted incorporation of exogenous oleate into triacylglycerol.

**Incorporation of [<sup>14</sup>C]Oleate by Transiently Transfected HEK293 Cells**—HEK293 cells transiently transfected with pcDNA3.1-GPAT-FLAG showed maximum mitochondrial GPAT

protein expression and activity between 18 and 36 h after transfection with a 30% decline by 48 h. At 24 h after transfection, mitochondrial GPAT specific activity was 0.33 nmol/min/mg of protein, a 4.4-fold increase compared with the control cells (0.074 nmol/min/mg of protein), and the GPAT-overexpressing cells and the control cells contained similar amounts of triacylglycerol and phospholipid (data not shown). Confocal microscopy showed that mitochondrial GPAT was present only in mitochondria (Fig. 3, e–h). From 18 to 42 h after transfection, incorporation of trace [<sup>14</sup>C]oleate ( $\sim 3 \mu$ M) into TAG was 9.7-fold higher in the GPAT-overexpressing cells than the controls (Fig. 8A). Unlike the stably overexpressing CHO cells, the transiently transfected line also incorporated more label into phospholipid ( $19.6 \times 10^5$  dpm/mg of protein in GPAT overexpressers versus  $6.8 \times 10^5$  dpm/mg of protein in control cells) (Fig. 8B) and more in cholesterol esters ( $7.6 \times 10^4$  dpm/mg of protein in GPAT overexpressers versus  $3.4 \times 10^4$  dpm/mg of protein in control cells). It is likely that the high rate of label incorporation into phospholipid in the transiently transfected HEK293 cells occurred, because only a small number of the HEK293 cells actually expressed the GPAT construct. Nevertheless, the ratios of label incorporated into triacylglycerol versus phospholipid were 1:12.4 in the control cells and only 1:3.7 in the GPAT-overexpressing cells, again showing the decisive effect of mitochondrial GPAT expression on the synthesis of triacylglycerol relative to phospholipid.

**The Transcription Factors SREBP-1 and PPAR $\gamma$  Are Unaffected in GPAT Overexpressing CHO Cells**—SREBP-1 and PPAR $\gamma$  are well known transcriptional factors that stimulate lipogenesis by increasing the levels of lipid synthetic enzymes. Since the effects of GPAT overexpression on lipid metabolism could be secondary to up-regulation of these lipogenic factors, or since the transcription factors might be down-regulated by the excess triacylglycerol mass in GPAT-overexpressing cells, we determined the levels of both SREBP-1 and PPAR $\gamma$  in control and GPAT-overexpressing CHO cells. No changes in the amount of full-length or mature SREBP-1 or in PPAR $\gamma$  levels were detected in GPAT overexpressers when compared with controls (data not shown).

#### DISCUSSION

The regulation of triacylglycerol synthesis remains poorly understood, in large part, because molecular tools have only recently become available for several of the enzymes in the triacylglycerol synthetic pathway (4). Enzyme isoforms have been identified for most of the steps in the pathway, but the separate role of each isoform has not yet been delineated for any glycerolipid synthetic enzyme. For GPAT, only the mitochondrial isoform has been cloned (23, 24), but the microsomal activity can be distinguished by its sensitivity to sulfhydryl reagents like *N*-ethylmaleimide (4). High carbohydrate feeding and insulin increase the mitochondrial GPAT activity and mRNA in liver, but have little effect on the microsomal activity (4, 10). Mitochondrial GPAT is up-regulated transcriptionally during adipocyte differentiation (11, 25) and probably during neonatal liver development (26). The mitochondrial GPAT promoter contains carbohydrate-responsive sequences and sterol regulatory element sites (11, 27), and SREBP-1c mRNA is up-regulated by insulin (12). Purified recombinant AMP-activated kinase also acutely decreases mitochondrial, but not microsomal GPAT activity in isolated membrane fractions from hepatocytes, suggesting that phosphorylation acutely decreases GPAT activity when cell ATP levels are low, thereby diminishing glycerolipid synthesis (14).

In most tissues and in cultured cells, mitochondrial GPAT comprises only 10% of total GPAT activity, and its role has remained obscure. It has been proposed that mitochondrial

TABLE II  
Relative incorporation of oleate into phospholipid and triacylglycerol

Data are derived from the 24-h experiments shown in Figs. 4, 5, and 7 and expressed as means  $\pm$  S.D. TAG, triacylglycerol; PL, phospholipid.

Added oleate $\mu\text{M}$	Cell line	Triacylglycerol dpm/mg protein	Phospholipid dpm/mg protein	Ratio TAG:PL
3	Control (+doxycycline)	91,378 $\pm$ 3,633	425,548 $\pm$ 8,772	1:4.7
3	GPAT (-doxycycline)	310,534 $\pm$ 18,516	287,773 $\pm$ 16,068	1:1
100	Control (+doxycycline)	258,601 $\pm$ 8,913	136,068 $\pm$ 3,402	1.9:1
100	GPAT (-doxycycline)	332,278 $\pm$ 4,246	126,534 $\pm$ 5,519	2.6:1

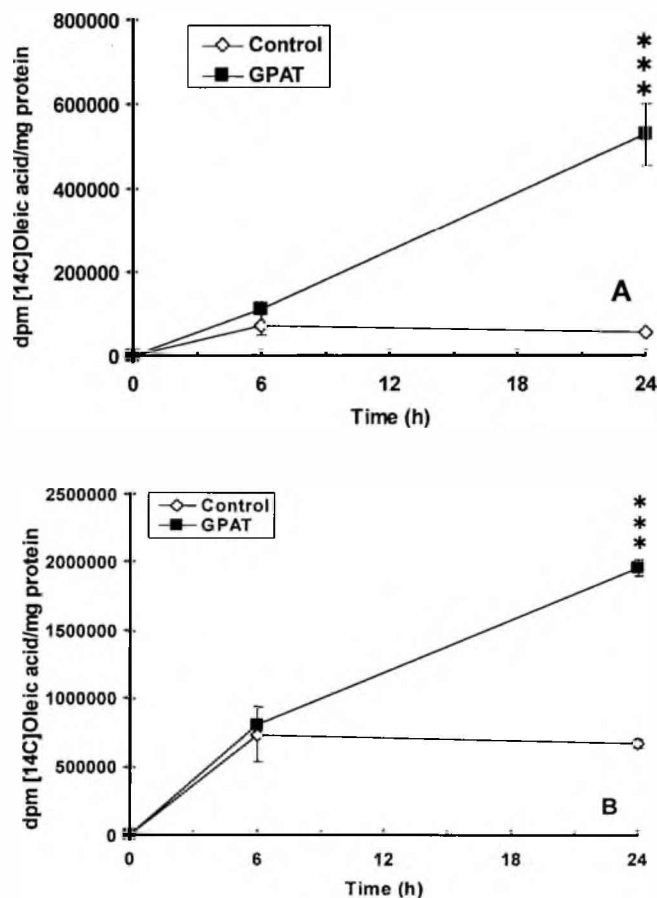


FIG. 8. GPAT overexpression in HEK293 cells stimulates incorporation of [ $^{14}\text{C}$ ]oleate into triacylglycerol and phospholipid. HEK293 cells, maintained in 10% FBS, MEM, were transfected with pcDNA3.1-GPAT-FLAG (GPAT) or pcDNA3.1 (Control) for 18 h, then incubated with 0.25  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]oleic acid/dish in the same media plus 0.5% BSA for 6 or 24 h. Lipids were extracted and separated as described under "Experimental Procedures." Data points represent the means  $\pm$  S.D. of an experiment performed in triplicate that was repeated three times. Some error bars are hidden by the symbols. \*\*\*,  $p < 0.005$  compared with the control.

GPAT provides precursors for phosphatidylglycerol and cardiolipin and that it increases the saturated fatty acid content at the *sn*-1 position of phospholipids (4). In liver the mitochondrial isoform comprises 50% of total activity, and its mRNA is markedly up-regulated by insulin, suggesting a strong relationship with lipogenesis and triacylglycerol synthesis. Although up-regulation of GPAT activity might be expected for a cell faced with marked increases in its fatty acid content, one might predict that regulation of triacylglycerol synthesis would involve the microsomal rather than the mitochondrial isoform, because the specific activity of the microsomal isoform increases 70-fold during both adipocyte differentiation (16) and postnatally when the rat pup's primary energy source changes from glucose to lipid (26). Furthermore, the microsomal isoform

is located on the endoplasmic reticulum where the terminal steps in triacylglycerol synthesis take place (4). Our studies, however, support the view that mitochondrial GPAT controls the entry of exogenously derived fatty acids into triacylglycerol stores. CHO cells, which normally contain little mitochondrial GPAT, accumulate triacylglycerol when mitochondrial GPAT expression is increased 3.8-fold, from 11 to 40% of total GPAT specific activity.

Thus, our finding that overexpressing mitochondrial GPAT directs incorporation of fatty acids into triacylglycerol in preference to phospholipid is surprising, because GPAT is the first step in a complex pathway that has redundant isoenzymes and two major branch points. With low concentrations of fatty acid substrate in the media, CHO and HEK293 cells that overexpress GPAT incorporated 3-fold and 9.7-fold more labeled oleate into triacylglycerol, respectively, than did control CHO cells in which doxycycline suppressed GPAT expression or HEK293 cells in which only the pcDNA3.1 vector was present. Furthermore, CHO cells that stably overexpressed GPAT incorporated equal amounts of [ $^{14}\text{C}$ ]oleate into triacylglycerol and phospholipid, even though the amount of fatty acid provided in the media was present at a concentration that usually promotes the incorporation of fatty acid primarily into phospholipid (7, 8, 28), and, as expected, the control CHO cells incubated with low concentrations of [ $^{14}\text{C}$ ]oleate incorporated 4.7-fold more label into phospholipid. Control HEK293 cells incubated with a low concentration of [ $^{14}\text{C}$ ]oleate incorporated 12.4-fold more label into phospholipid than triacylglycerol, but transient transfection of the HEK293 cells with GPAT lowered this ratio to 1:3.4. At 100  $\mu\text{M}$  oleate ( $\sim 120 \mu\text{M}$  fatty acid, including the contribution from FBS), the effect of overexpressed GPAT was still evident in CHO cells, and more oleate was incorporated into triacylglycerol than was seen with control cells, thereby confirming that triacylglycerol synthesis is not driven solely by the amount of available fatty acid. These data indicate that GPAT does not merely promote fatty acid uptake and entry into the pathway of glycerolipid synthesis. If increased flux were GPAT's sole contribution, one would expect equally enhanced [ $^{14}\text{C}$ ]oleate incorporation into phospholipid and triacylglycerol in the GPAT overexpressing cells. Instead, cells incorporated label preferentially in triacylglycerol, both at low and high media fatty acid concentrations. We hypothesize that because only a fraction of the HEK293 cells were transfected, the nontransfected cells incorporated oleate into phospholipid at the high rate that is usually observed in the presence of low amounts of exogenous fatty acid (7, 8). Even so, the presence of GPAT promoted an increase in oleate incorporation into triacylglycerol that was more than three times greater than into phospholipid.

Triacylglycerol is stored in cells as cytosolic lipid droplets, which also contain cholesterol esters. It is not known whether the synthetic pathways for both of these neutral lipids are co-regulated. Our results clearly show, however, that when triacylglycerol mass and the rate of incorporation of labeled fatty acid into triacylglycerol increase severalfold in CHO cells, the incorporation of label into cholesterol ester is unaffected

(Fig. 4, A and B), and cholesterol ester mass is unchanged (data not shown), but that incorporation into cholesterol ester increases in HEK293 cells. Thus, triacylglycerol and cholesterol ester pathways may not share the same pool of activated fatty acid in CHO cells. Similarly, when CHO cells are treated with phospholipase C, their pool of cytosolic triacylglycerol expands, but the cellular content of cholesterol ester does not change (29). Results from the HEK293 cells, however, suggest the possibility that access to activated fatty acid pools may differ in different cell types, perhaps related to the presence of ACAT1 versus ACAT2.

Overexpression of GPAT decreases the incorporation of externally provided fatty acid into phosphatidylcholine and phosphatidylinositol plus phosphatidylserine while having little effect on phosphatidylethanolamine. Preferential use of oleate for phosphatidylethanolamine rather than phosphatidylcholine synthesis is also observed in fetal human fibroblasts when oleate is scarce (7).

The present study extends the concept that GPAT controls glycerolipid synthesis by raising additional questions related to the independent functions of the two GPAT isoforms and to the trafficking of lipid intermediates within cells. Because mitochondrial GPAT and LPA acyltransferase are both present on the mitochondrial outer membrane, their LPA and/or phosphatidic acid products must be transported to the endoplasmic reticulum where the final steps occur in the synthesis of both triacylglycerol and most of the major phospholipids. Although the liver isoform of fatty acid-binding protein is reported to transport LPA (30), LPA and phosphatidic acid should diffuse through the cytosol as readily as lysophosphatidylcholine, because the energy of the membrane off-rate of the acyl group is the major barrier (31). Alternatively, there may be membrane contact regions between the mitochondrial outer membrane and the endoplasmic reticulum that could facilitate the transport of pathway intermediates.

More difficult to understand is the mechanism by which mitochondrial synthesis of LPA could direct this intermediate toward LPA acyltransferase and phosphatidic acid phosphohydrolase that link primarily with DGAT and triacylglycerol synthesis while providing little phosphatidic acid or diacylglycerol substrate that can be used by enzymes of phospholipid synthesis. Use of oleate rather than other fatty acids should not present a problem, because when triacylglycerol synthesis is promoted by incubating cells with 100  $\mu\text{M}$  [ $^{14}\text{C}$ ]oleate, incorporation of radiolabel into phospholipid remains stable (7, 8). Thus, oleate-containing LPA and phosphatidic acid are excellent substrates for phospholipid synthesis. Instead, the current data suggest the possibility that mechanisms exist within the endoplasmic reticulum to channel lipid intermediates toward independent pathways. Studies of fibroblasts from a patient with neutral lipid storage disease indicated that *de novo* and recycling pools of diacylglycerol did not mix (32), and strong evidence was presented in fibroblasts and hepatocytes for func-

tionally separate pools of acyl-CoA (22, 33).

The apparent reciprocal regulation of carnitine palmitoyltransferase 1 and mitochondrial GPAT by AMP-activated kinase has suggested that GPAT present on the mitochondrial outer membrane may compete for acyl-CoAs with carnitine palmitoyltransferase 1 (14). Thus, an increase in mitochondrial GPAT activity might divert acyl-CoAs away from oxidation and toward glycerolipid synthesis. To test this hypothesis, further studies are needed in cells that perform a significant amount of  $\beta$ -oxidation.

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**Mitochondrial Glycerol Phosphate Acyltransferase Directs the Incorporation of Exogenous Fatty Acids into Triacylglycerol**

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