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Overexpression of Diacylglycerol Acyltransferase-1 Reduces Phospholipid Synthesis, Proliferation, and Invasiveness in Simian Virus 40-transformed Human Lung Fibroblasts*

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Diacylglycerol (DAG) is a versatile molecule that participates as substrate in the synthesis of structural and energetic lipids, and acts as the physiological signal that activates protein kinase C. Diacylglycerol acyltransferase (DGAT), the last committed enzyme in triacylglycerol synthesis, could potentially regulate the content and use of both signaling and glycerolipid substrate DAG by converting it into triacylglycerol. To test this hypothesis, we stably overexpressed the DGAT1 mouse gene in human lung SV40-transformed fibroblasts (DGAT cells), which contains high levels of DAG. DGAT cells exhibited a 3.9-fold higher DGAT activity and a 3.2-fold increase in triacylglycerol content, whereas DAG and phosphatidylcholine decreased by 70 and 20%, respectively, compared with empty vector-transfected SV40 cells (Control cells). Both acylation and *de novo* synthesis of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin were reduced by 30–40% in DGAT cells compared with controls, suggesting that DGAT used substrates for triacylglycerol synthesis that had originally been destined to produce phospholipids. The incorporation of [^{14}C]DAG and [^{14}C]fatty acids released from plasma membrane by additions of either phospholipase C or phospholipase A_2 into triacylglycerol was increased by 6.2- and 2.8-fold, respectively, in DGAT cells compared with control cells, indicating that DGAT can attenuate signaling lipids. Finally, DGAT overexpression reversed the neoplastic phenotype because it dramatically reduced the cell growth rate and suppressed the anchorage-independent growth of the SV40 cells. These results strongly support the view that DGAT participates in the regulation of membrane lipid synthesis and lipid signaling, thereby playing an important role in modulating cell growth properties.

Amphipatic lipids, such as diacylglycerols (DAG)[1] and acyl-CoAs exhibit dual actions, as substrates for membrane and energy storage glycerolipids, as well as second messengers for signaling transduction events. As a substrate for *de novo* glycerolipid synthesis, DAG is a common intermediate for both triacylglycerol and phospholipid synthesis. Studies performed with permeabilized cells indicate that the utilization of *de novo* synthesized DAG for either neutral or polar lipid synthesis is controlled by DGAT and CDP-choline (ethanolamine) phosphotransferase activities, suggesting the presence of a common DAG pool that is shared for both lipid synthetic routes (1). Nevertheless, at least one other DAG pool is available for glycerolipid synthesis, because DAG that is released from triacylglycerol stores in human fibroblasts can be converted to phospholipids (2). Moreover, hepatic cells might contain different DAG pools that are used to synthesize triacylglycerol for storage or for lipoproteins (3, 4). Although no definitive evidence exists for specific DAG pools for polar and neutral glycerolipid synthetic pathways, segregation of DAG toward different metabolic routes seems to occur according to the need of the cell. For instance, when phospholipid synthesis is inhibited, DAG originally destined to form phospholipids is re-directed toward triacylglycerol (5).

Growth factors and hormones activate phospholipases C and D to promote a bi-phasic accumulation of DAG that triggers signaling events (6). As a signaling effector, DAG regulates cell growth and differentiation by activating several isoforms of protein kinase C (PKC). A short-term release of DAG is caused by the hydrolysis of phosphatidylinositol by several isoforms of phosphoinositide-specific PLC (7). A second wave of DAG produced by cytokine-activated PC hydrolysis is needed to fully develop mitogenesis. In this regard, quiescent fibroblasts treated with platelet-derived growth factor or bacterial PC-PLC increase DAG levels and a concomitant strong mitogenic response (8). Persistent accumulation of intracellular DAG has also been linked to oncogenic transformation. Thus, sustained high levels of DAG produced by overactivation of PC-PLC induce a transformed phenotype in NIH 3T3 cells (9). Moreover, neoplastic transformation by *simian virus 40* (*sv40*), and by *ras*-, *src*-, and *fps* oncogenes is accompanied by an excess content of intracellular DAG (10–12).

Not only are appropriate intracellular levels of signaling DAG necessary for mitogenesis, but a proper supply of DAG substrate for glycerolipid synthesis is also required by proliferating cells. To prepare for mitosis, cells must synthesize new membrane phospholipids; hence precursors for phospholipid synthesis must be available. Thus, mitogenic signals stimulate the formation of new PC by activating the expression of CTP:

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[1] The abbreviations used are: DAG, diacylglycerol; BSA, bovine serum albumin; DGAT, diacylglycerol acyltransferase; FBS, fetal bovine serum; MEM, minimal essential medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PBS, phosphate-buffered saline; SM, sphingomyelin.

phosphocholine cytidylyltransferase (13) and by diverting DAG toward PC synthesis, rather than triacylglycerol formation (5). Moreover, in neurite outgrowth induced by nerve growth factor, PC synthesis is enhanced by an increase in DAG levels together with an activation of the CDP-choline DAG phosphotransferase (14). Membrane lipid synthesis is also up-regulated in neoplastic cells, hence a constant supply of lipid precursors for new membranes is required to sustain the unrestricted proliferation of tumor cells. In this regard, increased synthesis and turnover of phospholipids has been observed in neoplastic cells (10, 11, 15).

Cells can regulate the content and destiny of DAG by lipolysis (16), phosphorylation by DAG kinases (17–20), and synthesis of PC (21). DAG released from plasma membrane may also be directly incorporated into triacylglycerol, suggesting the presence of a novel mechanism for terminating DAG signals based on the synthesis of a storage lipid (22).

A key enzyme involved in DAG and triacylglycerol metabolism is acyl-CoA-diacylglycerol acyltransferase 1 (DGAT1) (23). DGAT1, and the recently discovered DGAT2 (24), catalyze the last committed step in mammalian triacylglycerol synthesis by esterifying DAG with a fatty acid. The DAG used as a DGAT substrate is primarily derived from the *de novo* glycerol 3-phosphate pathway. Other sources of DAG for triacylglycerol synthesis include that produced from monoacylglycerol in intestinal cells (25), from the hydrolysis of triacylglycerol (2, 3), and from DAG released from plasma membrane phospholipids (22). Because both DGAT genes are widely expressed in mouse and human tissues (23, 24), the presence of two DGAT enzymes in non-adipose tissues suggests that the synthesis of triacylglycerol might be linked to cell functions other than energy storage. For instance, adenoviral expression of DGAT1 in pancreatic islets increased the formation of triacylglycerol by 100% but impaired the secretion of insulin after glucose stimulation (26).

To understand the role of DGAT in terminating DAG signals, we tested the hypothesis that DGAT overexpression channels DAG and/or fatty acids toward triacylglycerol, thereby sequestering either proliferative lipid signals and/or lipid substrates for membrane biogenesis with a consequent decrease in phospholipid synthesis, normalization of cell proliferation, and a reversion in the abnormal cytological phenotype.

EXPERIMENTAL PROCEDURES

Materials—Normal human lung fibroblasts (WI38) and the derived SV40-transformed strain were obtained from the American Type Culture Collection (Manassas, VA). LipofectAMINE™, cell culture media, G418 (Geneticin™) antibiotic, and other culture reagents were from Invitrogen. Ultrafiltered fetal bovine serum (FBS) was from Gensa (Buenos Aires, Argentina). Cell culture supplies were from Greiner BioOne (Frickenhausen, Germany). Restriction enzymes and other molecular biology reagents were purchased from Promega (Madison, WI). [^{14}C]Oleic acid and [γ-^{32}P]ATP were from Amersham Biosciences, [*methyl*-^{14}C]choline and [^{3}H]glycerol were purchased from PerkinElmer Life Sciences. Fatty acid-free bovine serum albumin (BSA), phospholipase A_2 (PLA_2, from *Naja naja*), PC-specific phospholipase C (PC-PLC, from *Bacillus cereus*), and anti-FLAG M2 monoclonal antibody were from Sigma. Pure lipid standards were from Doosan Serdary (Yongin, Korea). 1,6-Diphenyl-1,3,5-hexatriene was purchased from Aldrich. Silica Gel 60 chromatography plates were from Merck (Darmstadt, Germany). Analytical-grade solvents were from Carlo Erba (Milano, Italy). DAG kinase membrane suspension was from Calbiochem.

Cell Culture—Cells were routinely cultured in 100-mm Petri dishes in minimum essential medium with Earle's salts (MEM) with 10% heat-inactivated FBS, 1% penicillin (100 units/ml), streptomycin (10 μg/ml), 1% nonessential amino acids, and 1% MEM vitamins (growing medium), at 37 °C, 5% CO_2, and 100% humidity. Normal human lung fibroblasts were used for the experiments before reaching the 20th passage.

Generation of Stable DGAT1 Overexpressing SV40-transformed Cells—Mouse DGAT1 cDNA with an N-terminal FLAG epitope (kind gift of Dr. Robert V. Farese Jr., Gladstone Institute of Cardiovascular Disease, University of California) was cloned into the restriction sites EcoRV and XbaI of a pCDNA3 mammalian expression vector (Invitrogen). SV40-transformed cells, grown in 100-mm dishes up to 50% confluence, were washed twice with serum-free Opti-MEM and then transfected with 12 μg of either DGAT1-pCDNA3 plasmid or empty vector plus 35 μl of LipofectAMINE™ (2 mg/ml) in 0.5 ml of Opti-MEM plus 20% FBS. After 5 h incubation, medium was replaced by 10% FBS MEM and transfected cells were grown for an additional 24 h. Then, positive transfectants were selected by culturing the cells in 10% FBS, MEM containing geneticin (600 μg/ml) for 15 days.

Ten DGAT1-transfected clones were isolated by using cloning cylinders and were grown in 100-mm Petri dishes with 10% FBS, MEM plus G418 antibiotic (300 μg/ml) for 15 days. Triacylglycerol synthetic rate and content were analyzed in each DGAT-transfected clone, as well as in empty vector-transfected SV40 cells (Control cells), and two DGAT clones (DGAT-A and DGAT-V cells) that showed the highest synthesis of triacylglycerol (measured by the incorporation of [^{14}C]oleate into lipids during a 24-h period) were selected for the experiments.

Metabolic Labeling—Preconfluent control and DGAT cells, cultured in 60-mm dishes, were incubated for up to 48 h either with [^{14}C]oleic acid (0.25 μCi/dish), [^{3}H]glycerol (5 μCi/dish) or [*methyl*-^{14}C]choline (0.5μCi/dish), in the presence or absence of 100 μM oleate, in 10% FBS, MEM supplemented with 0.5% BSA. At the end of each labeling period, the radioactive medium was discarded and cell monolayers were washed twice with 0.1% BSA in ice-cold phosphate-buffered saline (PBS), to eliminate residual label on the cell surface. Next, cells were scraped from the dishes with two additions of 1 ml of ice-cold methanol and 0.5 ml of H_2O. Total cellular lipids were obtained according to the procedure of Bligh and Dyer (27) and aqueous and lipid fractions were concentrated in a Savant SpeedVac concentrator.

Analysis and Quantitation of Lipids and Choline-derived Metabolites—For mass lipid determinations, cells were grown until near confluence in 100-mm dishes and total lipid extracts were obtained as described above. Neutral and polar lipids species were separated on Silica Gel 60 thin-layer chromatography (TLC) plates using one-dimensional single development procedures. Routinely, neutral lipid separation was carried out with the following solvent system:hexane:ethyl ether:acetic acid, 80:20:2 (by volume). Polar lipid species were resolved in a solvent system consisting of chloroform:methanol:ammonium hydroxide:water, 50:37.5:3.5:2 (by volume). Known amounts of pure lipid standards were seeded and run on the TLC plates in parallel to samples. To both solvent systems 100 μM 1,6-diphenyl-1,3,5-hexatriene was added to visualize the lipid spots on the plate under UV light (22). Fluorescent lipid spots from samples and standards were photographed and lipid mass was quantified using a DS120 Kodak Image system. For the [^{14}C] and [^{3}H] labeling experiments, individual fluorescent lipid spots were scraped into plastic vials and radioactivity levels were determined in a liquid scintillation counter.

To separate water-soluble choline metabolites, aqueous phases were dried and dissolved in H_2O, spotted on silica gel chromatoplates, and resolved with 0.6% sodium chloride, methanol, 30% ammonium hydroxide; 50:50:5 (by volume). Pure standards were used as carriers and were added to the samples before chromatography. Radioactive spots were detected in a Berthold II radiometric scanner, scraped into vials, and counted.

Total 1,2-DAG was quantitated by the DAG kinase assay according to the procedure of Preiss *et al.* (28), using [γ-^{32}P]ATP as substrate. The results of lipid determinations were expressed in nanomoles of lipid per mg of protein.

Cell Proliferation—To evaluate cell growth, 1.5×10^4 cells were seeded in duplicate 60-mm dishes. Twenty-four hours later, medium was removed and replaced with fresh growing medium. At this time and up to 144 h cells were then trypsinized, and counted in an hemocytometer. Cell viability was determined by trypan blue exclusion.

[^{3}H]Thymidine Incorporation into Total DNA—To measure the rate of DNA synthesis, cells grown in triplicate 60-mm dishes were pulsed with [^{3}H]thymidine (1 μCi/dish) in 2 ml of growing media for 3 h at 37 °C. Media was removed and cell monolayers were washed twice with ice-cold PBS, and then precipitated with 5% trichloroacetic acid for 10 min at 4 °C. The acid-insoluble material was solubilized with 0.2% SDS in 0.1 N NaOH and an aliquot was counted in a liquid scintillation counter.

Cloning in Soft Agar—Control and DGAT cells were plated in quadruplicates at 1×10^4 cells per 60-mm dishes in MEM containing 10% FBS and 0.3% (w/v) agar onto a bottom layer of 0.6 (w/v) agar in MEM. After 3 and 5 weeks, the presence or absence of multicellular colonies (more than eight cells) in both cell groups was verified under the

microscope. For macroscopic analysis, cell colonies were stained with ethidium bromide in PBS for 30 min, visualized, and photographed under UV light.

Cell Radiolabeling and Phospholipase Treatments—Near confluent control and DGAT cells grown in 60-mm dishes were incubated with trace amounts of [^{14}C]oleic acid (0.25 μCi/dish) in growing medium supplemented with 0.5% BSA for 48 h. After labeling, medium was removed and monolayers were washed twice with 0.1% BSA in warm PBS. Next, cells were treated with 10 units/dish of either PC-PLC or PLA_2, or 100 μM oleic acid, for 6 h. After these treatments, the medium was discarded, and cell monolayers were washed with ice-cold PBS and scraped with two additions of ice-cold methanol. Lipid extraction and separation, as well as the quantitation of radioactivity levels of lipid species, were performed as stated above.

DGAT Enzyme Assay—Cells were grown in 100-mm Petri dishes until 80–90% confluence. Cells were then trypsinized, washed twice with ice-cold PBS, and resuspended in 10 mM Tris-HCl buffer, pH 7.4, with protease inhibitor mixture (Sigma). Cell homogenates were obtained by sonication on an ice-water bath for 10 s at 50% output and stored at −70 °C until use. DGAT activity was determined using 100 and 200 μg of cell homogenate protein, 200 μM diacylglycerol in acetone, and 30 μM [^{3}H]palmitoyl-CoA following the procedure of Coleman (29).

Western Blot Analysis of FLAG-DGAT—Preconfluent control and DGAT cells were homogenized in 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS plus 1% protease inhibitor mixture by sonication in an ice-water bath. Proteins from total cell homogenates (150 μg) were separated by SDS-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel and then transferred onto nitrocellulose membranes. After blocking, membranes were incubated with mouse anti-FLAG as primary antibody and donkey anti-mouse IgG-horseradish peroxidase conjugate as secondary antibody. Visualization of FLAG-DGAT protein was performed using a SuperSignal West Pico detection kit (Pierce).

Other Methods—Protein content was measured as described by Lowry *et al*. (30). [^{3}H]Palmitoyl-CoA was synthesized enzymatically (31).

RESULTS

Stable DGAT Overexpression Results in Massive Accumulation of Triacylglycerol and a Decrease in the Levels of DAG and PC—From a pool of SV40-transformed cells transfected with DGAT1 cDNA and selected by resistance to G418 antibiotic resistance, several clones of DGAT overexpressing cells were isolated. Two clones, DGAT-A and DGAT-V, which had the highest rate of TAG synthesis and TAG content were selected for the experiments. DGAT-A cells exhibited a high level of FLAG-DGAT protein expression (Fig. 1*A*), which correlated with a 3.9-fold increase in the *in vitro* DGAT activity (Fig. 1*B*), when compared with control cells. In addition, the DGAT-V cells exhibited a 10-fold increase in DGAT activity over the controls. However, because this DGAT-overexpressing clone showed an extremely low cell proliferation rate (see below), because of feasibility, most of the experiments were performed using the DGAT-A clone.

To determine whether FLAG-DGAT protein was functional in the whole cells, the content of triacylglycerol mass in control and DGAT-A cells was analyzed by TLC and densitometric scanning. Accordingly, DGAT-A cells showed a 3.2-fold increase in triacylglycerol levels, with respect to control cells (Fig. 1, *C* and *D*). These cells also exhibited a higher level of cholesteryl esters, but this finding was not further investigated. We also observed that triacylglycerol accumulated as numerous small cytosolic droplets (Oil Red O sensitive) surrounding the nucleus (data not shown). This pattern of triacylglycerol depot was similar to that observed in other non-adipose cells (32), indicating that no aberrant localization of triacylglycerol droplets occurred in the DGAT-overexpressing cells. Taken as a whole, this group of observations indicate that the DGAT-transfected clones contained high amounts of functional DGAT enzyme that promoted the accumulation of its product triacylglycerol.

Additionally, the mass of the main phospholipids, PC and PE, as well as 1,2-DAG was determined in control cells and DGAT overexpressors (Table I). PC content decreased ~20% in DGAT-A cells compared with empty vector-transfected SV40 cells, whereas the levels of PE did not show significant changes between the cell groups. Interestingly, the content of total 1,2-DAG in DGAT cells was reduced by 70% with respect to control values, strongly suggesting that the overexpressed DGAT activity is depleting the DAG sources that can be used for phospholipid production by preferentially channeling this lipid intermediate into triacylglycerol stores.

Synthesis of Neutral Lipids Versus Polar Lipids in DGAT Overexpressing Cells: Regulation of Lipid Partitioning by DGAT—Because it has been hypothesized that PC and triacylglycerol biosynthetic pathways share the same pool of lipid substrates, we analyzed both the synthesis of neutral and polar lipids in control and DGAT-overexpressing cells incubated with traces of [^{14}C]oleic acid. Labeling of total lipids was slightly decreased (~20%) in DGAT-A cells compared with controls. When the synthesis of the [^{14}C]lipid species was analyzed, it was observed that the formation of triacylglycerol was 2.3- and 5.5-fold higher in DGAT cells than in control cells at 24 and 48 h, respectively (Fig. 2*A*). On the other hand, synthesis of total polar lipids was decreased by 35% in DGAT overexpressors, both at 24 and 48 h (Fig. 2*B*). Decreases in both PC and PE, the only polar lipid species labeled with [^{14}C]oleate in both cell groups, accounted for the lower phospholipid labeling (data not shown). Additionally, the levels of cellular [^{14}C]1,2-DAG, measured after a 24-h incubation with trace amounts of [^{14}C]oleate, was ~30% decreased in DGAT cells compared with empty vector-transfected cells (6694 ± 836 *versus* 4602 ± 608 dpm/mg of protein, respectively). This observation further confirms that DGAT overexpression preferentially redirects DAG molecules toward the synthesis of triacylglycerol, decreasing the DAG pool available for the formation of phospholipids.

Oleic acid can act as a substrate for both acylation of preexisting phospholipids as well as for the *de novo* synthesis of polar lipids, whereas labeled glycerol is a substrate exclusively for the *de novo* synthetic pathway, therefore labeling of lipids with [^{3}H]glycerol would help to determine whether this route is specifically affected by DGAT overexpression. Thus, preconfluent control and DGAT-A cells were labeled with [^{3}H]glycerol for 24 h in the presence of 100 μM oleate to enhance lipid synthesis, and labeled neutral and total phospholipids were analyzed. The incorporation of [^{3}H]glycerol into total cell lipids was significantly depressed in DGAT cells compared with the mock-transfected group (Fig. 3), suggesting a specific down-regulation of the *de novo* pathway for glycerolipid formation in the DGAT overexpressors. However, despite a decreased *de novo* lipid formation, most of the [^{3}H]glycerol was incorporated into triacylglycerol in DGAT cells at the expense of both PC and PE labeling (Fig. 3, *inset*), indicating that *de novo* synthesized lipid substrates were re-directed toward the synthesis of triacylglycerol in the DGAT-SV40 cells.

Decreased de Novo Synthesis of PC and SM in DGAT Overexpressors—Because both the [^{14}C]oleic acid and [^{3}H]glycerol experiments pointed to a deficit in the synthesis of the lipid backbone of phospholipids, we examined the synthesis of the polar head for PC using [^{14}C]choline as tracer. After incubating the monolayers with [^{14}C]choline for 24 h, labeled lipids and choline aqueous metabolites were analyzed. As shown in Fig. 4*A*, synthesis of PC and SM was 38 and 30% lower, respectively, in DGAT cells than in controls, in agreement with the [^{14}C]oleate and [^{3}H]glycerol experiments. More than 90% of the water-soluble choline metabolites was phosphorylcholine (Fig. 4*C*) and DGAT cells had 30% less phosphorylcholine than mock-transfected controls. DGAT overexpressors showed a ~9-fold increase in [^{14}C]CDP-choline compared with the control cells (Fig. 4*D*). This unusual accumulation of CDP-choline,

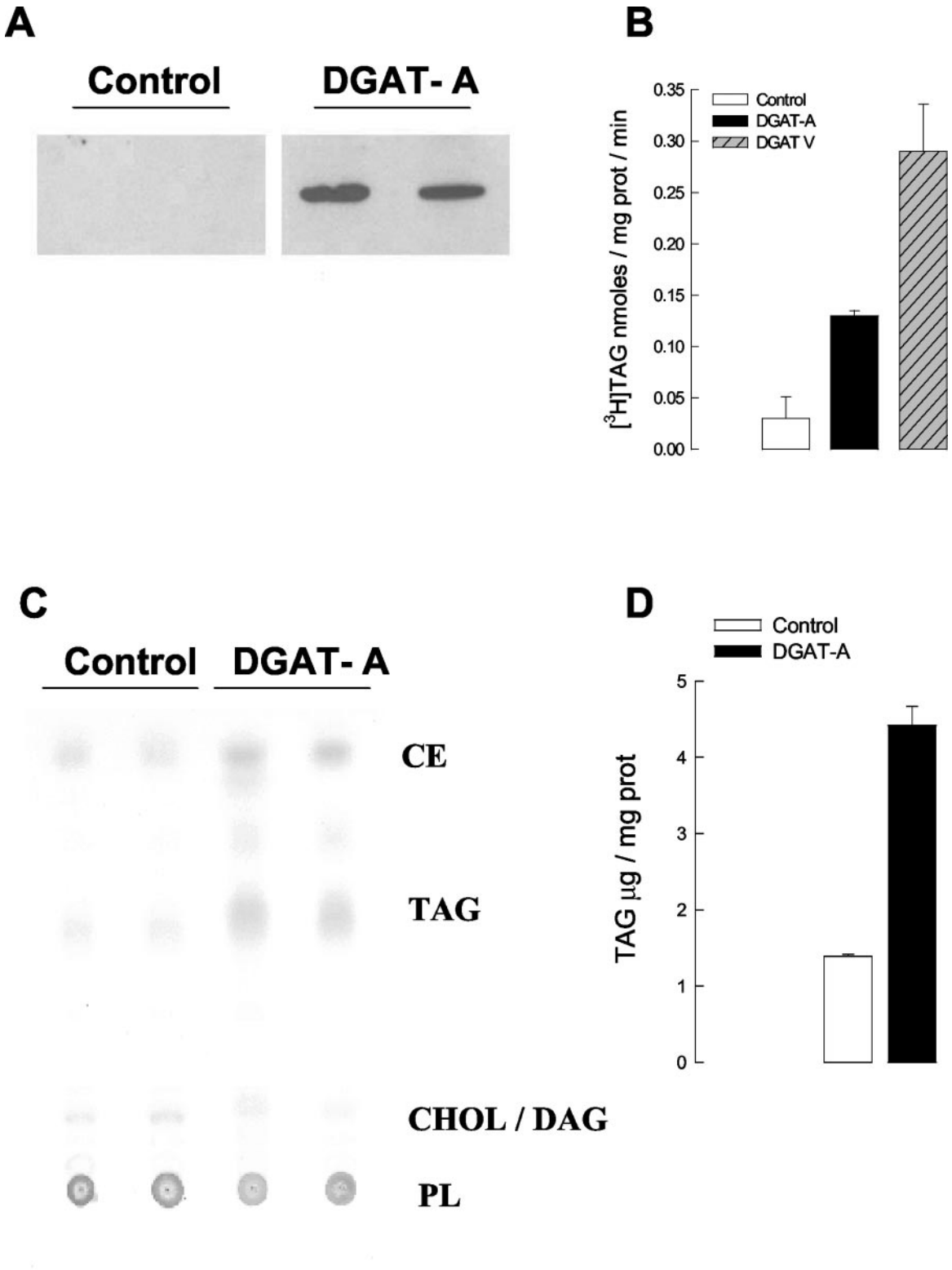


FIG. 1. **High levels of DGAT expression and activity induce massive accumulation of triacylglycerol in SV40-transformed fibroblasts stably transfected with FLAG-DGAT cDNA.** Near confluent empty vector-transfected (*Control*) and DGAT cells were scraped and homogenized by sonication. FLAG-DGAT protein expression levels (*A*) were analyzed by Western blot in duplicate samples of DGAT-A cells using anti-FLAG antibody. DGAT activity (*B*) was measured in triplicate or quadruplicate samples of DGAT-A and DGAT-V cells using [^{14}C]palmitoyl-CoA and diacylglycerol as substrates. Cell triacylglycerol (*TAG*) content (*C* and *D*) were determined in quadruplicate samples of the DGAT-A cell clone by TLC followed by scanning densitometry. *CHOL*, cholesterol.

together with the decreased formation of [^{14}C]choline-labeled PC, strongly indicates a deficient level of the co-substrate DAG available for the CDP-choline diacylglycerol phosphotransferase reaction.

Formation of Triacylglycerol from DAG and Fatty Acids Released from Plasma Membrane Is Enhanced by DGAT Overexpression—We have previously shown that DAG derived from turnover of plasma membrane phospholipids is converted to

TABLE I
Levels of PC, PE, and 1,2-DAG in control and DGAT-A cells

SV40-transformed cells stably transfected with either empty vector (Control) or DGAT1 cDNA (DGAT-A) were grown until near confluence in MEM, 10% FBS in the presence of 300 μg/ml geneticin. Cells were harvested and the content of lipid species was determined as described under "Experimental Procedures." Values represent mean ± S.D. of three separate determinations (PC and PE) or the average of duplicate determinations for two independent experiments, with a difference between determinations of less than 10% (1,2-DAG).

Nanomole of lipid/mg protein	Control	DGAT-A
PC	156 ± 14	121 ± 16[a]
PE	58 ± 12	67 ± 11
1,2-DAG	0.409	0.116

[a] $p < 0.05$, Student's t test.

triacylglycerol (22), suggesting a role for DGAT in the metabolism of signaling lipids. To determine whether overexpression of DGAT would enhance the channeling of plasma membrane-released lipids toward triacylglycerol formation, membrane phospholipids of DGAT-A and control cells were prelabeled with trace amounts of [^{14}C]oleic acid for 48 h and, after cell monolayers were treated with either PC-PLC or PLA_2 for 6 h, the formation of [^{14}C]triacylglycerol was estimated. As shown in Fig. 5, in cells exposed to bacterial PC-PLC, the synthesis of new triacylglycerol was increased 6.2-fold in DGAT cells compared with the control group, confirming that DGAT can use membrane-released signaling DAG to produce triacylglycerol. Additionally, DGAT cells incubated with PLA_2 exhibited a 2.8-fold increase in [^{14}C]triacylglycerol labeling, indicating that fatty acids originating from the plasma membrane are also substrates for DGAT activity.

Effects of DGAT Stable Overexpression on Cell Proliferation and Colony Forming Ability—Given the fact that overexpressed DGAT could reduce phospholipid synthesis and sequester membrane-released lipids with potential signaling functions into triacylglycerol, we addressed the question of whether cell growth was affected by these alterations in lipid metabolism. Fig. 6 shows the growth curve for the normal human lung fibroblast WI38 strain, non-transfected SV40-transformed WI38 cells, and SV40 cells stably transfected with either DGAT cDNA or empty vector. As expected, the neoplastic SV40 cells, both non-transfected and transfected with vector only, displayed a much faster growth rate than the normal fibroblasts. Remarkably, DGAT-A and DGAT-V cell clones grew at a rate even slower than normal WI38 fibroblasts. Moreover, the growth rate of the DGAT clones was inversely correlated to their DGAT activities (Fig. 1*A*), having the DGAT-V cells with the highest DGAT activity and the lowest cell replication rate. Additionally, the DNA synthesis rate was compared between mock and DGAT-transfected SV40 cells (Fig. 7). The incorporation of [^{3}H]thymidine into cellular DNA was 50% lower in DGAT overexpressors with respect to empty vector-transfected controls, confirming the profound effect of DGAT overexpression on cell growth properties of the neoplastic SV40 cells.

To further analyze whether the decreased growth rates in DGAT-overexpressing neoplastic cells was accompanied by other modifications in the transformation phenotype, control and DGAT-A cells were tested for their abilities to form colonies on a soft agar surface. Cells were grown on agar for 21 days and up to 5 weeks, to allow the formation of colonies of more than eight cells. Whereas empty vector-transfected cells produced multiple colonies after 21 days incubation, DGAT overexpression completely eliminated the anchorage-independent growth of the neoplastic SV40 cells (Fig. 8). Moreover, the DGAT cells were not able to produce colonies of more than 3–5 cells even after 5 weeks of incubation (data not shown).

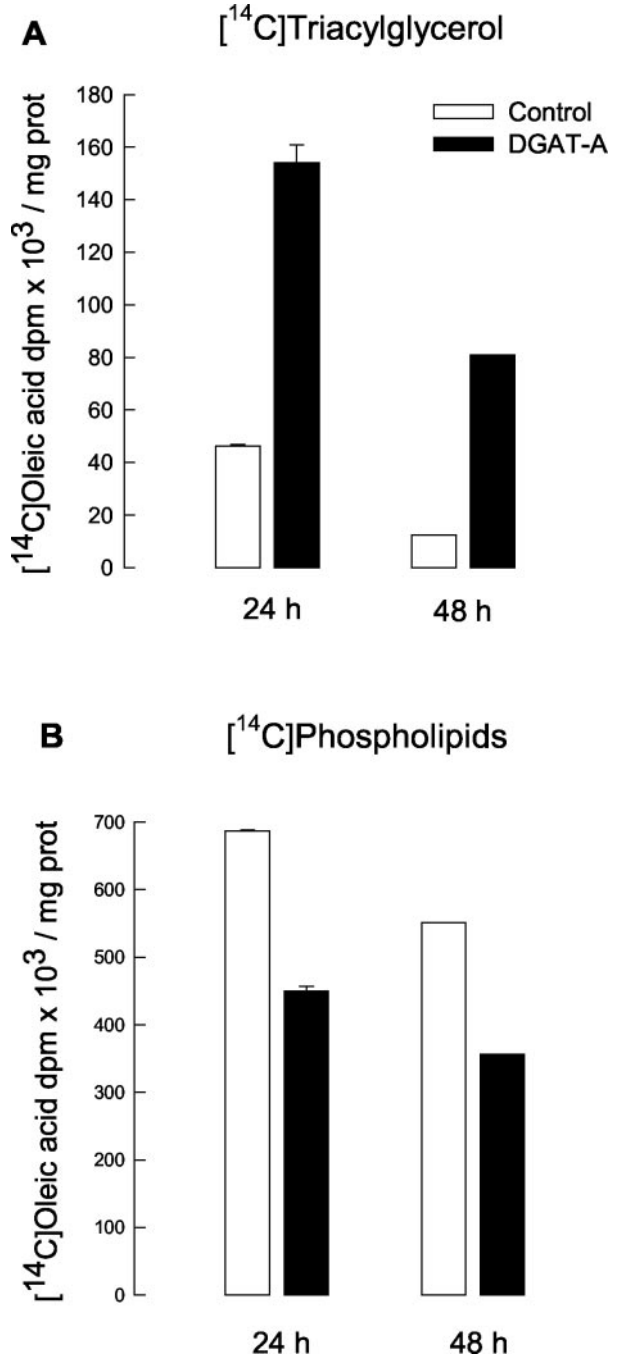


FIG. 2. **Incorporation of [^{14}C]oleic acid into triacylglycerol (*A*) and total phospholipids (*B*) in control and DGAT-A cells.** Cells were incubated with 0.25 μCi of [^{14}C]oleic acid in 0.5% BSA, 10% FBS, MEM for 24 and 48 h. Lipids were extracted and chromatographed as described under "Experimental Procedures." Data points represent the mean ± S.D. of an experiment performed in triplicate (24 h) and duplicate (48 h). Variation between duplicates was less than 5%.

DISCUSSION

Much of the interest in the regulation of triacylglycerol synthesis, and specially DGAT activity, relates to its role in storing energy. Excessive depots of triacylglycerols in adipose cells is strongly related to the development of lipid dysmetabolism-related disorders such as obesity and type 2 diabetes, diseases currently considered epidemic in Western countries. However, in cells in which neutral lipids are not primarily used as energy store, new functions for both triacylglycerols and DGAT have been identified. In human skin fibroblasts, triacylglycerol can act as a donor of lipid precursors, mostly DAG and fatty

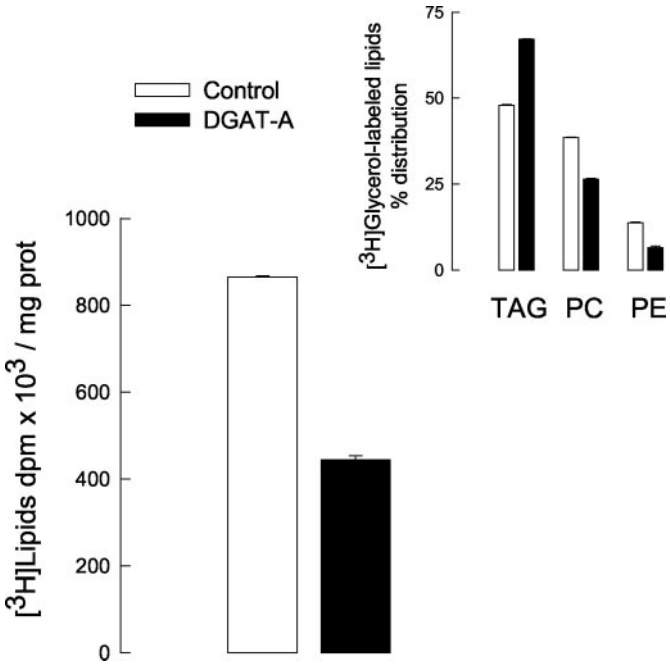


FIG. 3. **DGAT overexpression inhibits the incorporation of [^{3}H]glycerol into total lipids.** Preconfluent DGAT-A and control SV40 cells grown in 10% FBS, MEM were labeled with 5 μCi of [^{3}H]-glycerol in the presence of 100 μM oleate for 24 h. Total cell lipids were isolated and their radioactivity levels were determined by scintillation counting. Triacylglycerol (*TAG*), PC, and PE (*inset*) were separated by TLC. Each *bar* represents the mean ± S.D. for three 60-mm dishes.

acids, for the synthesis of new membrane phospholipids (2). In Chinese hamster ovary cells, the accumulation of triacylglycerol contributes to protect the cells against saturated fatty acid-induced apoptosis (33). On the other hand, the accumulation of triacylglycerols in pancreatic islet cells has been linked to a dysregulation of insulin secretion (26) and programmed cell death (34, 35). Nevertheless, the mechanisms by which triacylglycerols modulate these functions are poorly understood.

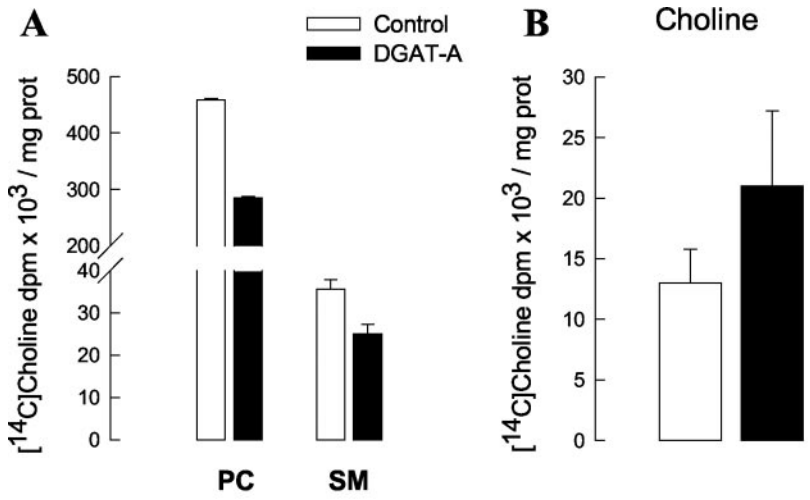
Another aspect of the non-energetic functions of triacylglycerols, mainly related to cellular signaling and membrane formation, is the potential role that DGAT might play in controlling DAG levels within the cell. We have shown that DAG released from plasma membrane of Chinese hamster ovary cells is mainly used as a substrate for DGAT in triacylglycerol synthesis (22). However, this new regulatory role for DGAT has not been directly tested. The isolation of cDNAs encoding two DGAT isoforms (23, 24) provides molecular tools to evaluate the role of triacylglycerol synthesis as a modulator of DAG levels and as a possible mechanism to sequester excess DAG signal into triacylglycerol stores. To study these potential roles of DGAT, we developed a stable DGAT1-overexpressing mammalian cell model based on SV40-transformed human lung fibroblasts, which contain high levels of intracellular DAG (12) and enhanced lipid synthesis for building new membranes.[2] The stable transfection of SV40 cells with mouse DGAT cDNA resulted in a severalfold increase in both expression and activity of DGAT, and a 3-fold increase in triacylglycerol content. Interestingly, in addition to this small accumulation of triacylglycerol, DGAT overexpression depressed the synthesis of membrane phospholipids, presumably by preferentially channeling lipid substrates into triacylglycerol instead of phospholipid. Our data indicate that DGAT, indeed, can control the flow of lipid substrates used for the synthesis of both triacylglycerol and phospholipids, thereby regulating the balance between the formation of neutral and polar lipids for energetic, structural and signaling purposes. This conclusion is based on the fact that the great accumulation of triacylglycerol promoted by DGAT overexpression concurred with decreased levels of 1,2-DAG and PC, demonstrating that DGAT was segregating DAG destined to phospholipid synthesis. Furthermore, the massive synthesis of triacylglycerol that took place in DGAT cells (2.3- and 5.5-fold more [^{14}C]oleic acid incorporated into triacylglycerols compared with mock-transfected SV40 cells) occurred in parallel to a deficient production of phospholipids. Thus, while phospholipid synthesis from [^{14}C]oleate was significantly decreased in DGAT cells, the incorporation of [^{14}C]oleic acid into total lipids remained essentially similar between the two cell groups, indicating that a 3-fold increase in DGAT activity could regulate the synthesis and remodeling of phospholipid without altering the rate of uptake of exogenous fatty acids. Additionally, [^{3}H]glycerol-labeled lipid substrates were preferentially incorporated into triacylglycerol in DGAT cells, indicating that DGAT was also capable of regulating the partitioning of endogenously synthesized lipids. This observation also confirms the existence of pools of lipid substrates, originated from either endogenously or exogenously sources, that are equally available for both polar and neutral lipid synthesis. In our DGAT-overexpressing SV40 cells, the redirection of lipid intermediates toward triacylglycerols appears to lead to substrate deprivation for phospholipid synthesis. Thus, DGAT cells formed less PC from [^{14}C]choline than controls and accumulated CDP-choline, suggesting that the amount of DAG available was unable to sustain the terminal reaction for PC synthesis. The significant decrease in 1,2-DAG content together with the lower levels of [^{14}C]oleate-labeled 1,2-DAG found in DGAT-overexpressing cells with respect to mock transfected controls confirms this hypothesis. A similar regulatory effect of DAG levels on the rate of PC synthesis was observed when choline-phosphate cytidylyltransferase was overexpressed in COS cells (36). In the choline-phosphate cytidylyltransferase-overexpressing cells, a 100-fold higher choline-phosphate cytidylyltransferase activity led to only a modest increase in PC synthesis, despite a 12-fold elevation in CDP-choline content; this was attributed to a limiting supply of DAG. Together, these findings corroborate that DGAT, by preferentially using lipid substrates for glycerolipid synthesis, exhausts the source of substrates, mainly DAG, for phospholipid formation.

In association with the significant changes promoted in lipid metabolism, the overexpression of DGAT in SV40 neoplastic cells had profound effects on cell growth. The growth rate of DGAT cells was significantly decreased and was even slower than the normal parental fibroblasts. More strikingly, DGAT overexpression also eliminated anchorage-independent growth, another feature of neoplastic transformation. The altered growth pattern of DGAT-overexpressing SV40 cells (cell division and anchorage-independent growth) are unlikely to be caused solely by accumulation of triacylglycerol. For example, neutral lipid storage disease fibroblasts contain ~20 times more triacylglycerol than normal cells (2) but have similar growth rate.[3] In addition, stable overexpression of mitochondrial GPAT in Chinese hamster ovary cells led to a 4-fold accumulation of triacylglycerol and a reduced formation of phospholipids with no change in the rate of cell proliferation (37).

Both the slow cellular proliferation and loss of the DGAT-overexpressing SV40 cells capacity to grow in soft agar might be caused by either the deprivation of essential DAG substrate

[2] J. M. Caviglia and R. A. Igal, unpublished observations.

[3] R. A. Igal and R. A. Coleman, unpublished observations.



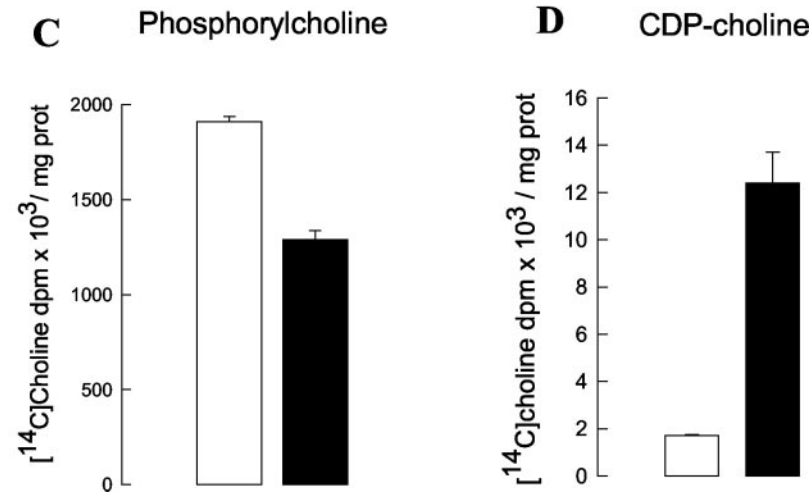


FIG. 4. **DGAT overexpression decreases the synthesis of [^{14}C]choline-labeled PC and SM and promotes the accumulation of [^{14}C]CDP-choline.** Control and DGAT SV40-transformed cells (DGAT-A clone) were grown in 10% FBS, MEM until near confluency and were labeled with 2 μCi/dish of [^{14}C]choline for 6 h in growing media. Aqueous and chloroform phases were extracted and analyzed as stated under "Experimental Procedures." Each *bar* expresses the mean ± S.D. of one experiment performed in triplicate. *Panels A*, [^{14}C]PC and [^{14}C]SM; *B*, [^{14}C]choline; *C*, [^{14}C]-phosphorylcholine; *D*, [^{14}C]CDP-choline.

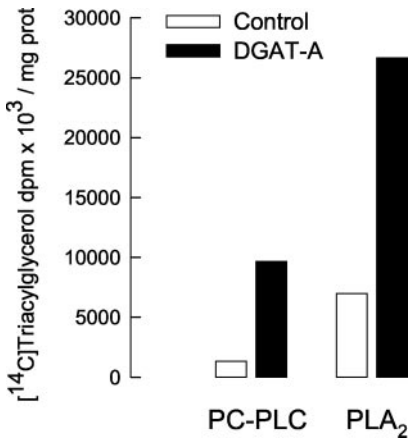


FIG. 5. **DGAT overexpression stimulates the formation of triacylglycerols from DAG and fatty acids released from plasma membrane.** Near confluent control and DGAT-A cells were labeled to steady-state (48 h) with 0.25 μCi/dish of [^{14}C]oleic acid. Next, cells monolayers were treated with 10 units/dish PC-PLC or PLA_2 for up to 6 h. Data are expressed as disintegrations/min of [^{14}C]triacylglycerol formed during the period of phospholipase treatment. Values represent two or three independent determinations, each performed in duplicate. Variability between duplicates was 5–7%.

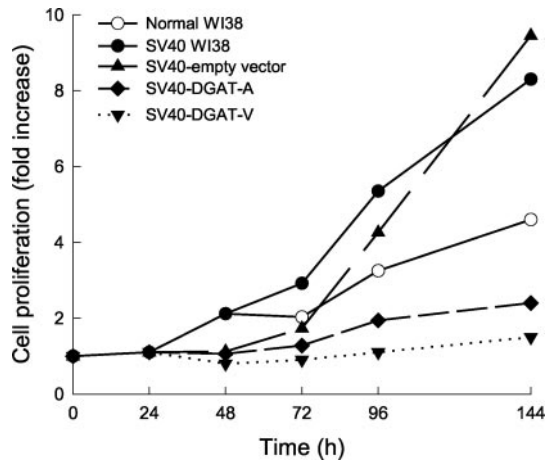


FIG. 6. **DGAT overexpression slows the proliferation rate of SV40-transformed cells.** Normal human lung fibroblasts (WI38) and SV40-transformed WI38 fibroblasts (wild type, stably transfected with either empty vector (Control) or with DGAT cDNA (DGAT-A and DGAT-V)), grown in 10% FBS, MEM, were seeded in 60-mm plates. Cells were then trypsinized and counted at time points up to 144 h. Data represents the average of duplicate determinations and variability between duplicates was less than 10%. Results reported are representative of three independent experiments.

for phospholipid synthesis required to sustain rapid membrane biogenesis and/or by an alteration in the DAG-activated signaling network related to cell proliferation. The synthesis of membrane structural lipids is active in the SV40-transformed human lung fibroblasts; we have observed a 2-fold higher *de novo* formation of phospholipid and cholesterol in the neoplastic cells compared with their precursor WI38 human fibroblasts.[2]

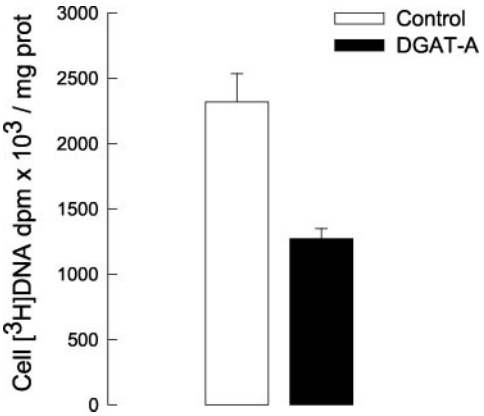


FIG. 7. **[^{3}H]Thymidine incorporation into SV40 cells is inhibited by DGAT overexpression.** Control and DGAT-A cells, grown in 60-mm dishes to 50% confluence, were pulsed with [^{3}H]thymidine (1 μCi/dish) for 3 h at 37 °C. Cell monolayers were then precipitated with 5% trichloroacetic acid and the acid-insoluble material was counted. *Bars* represent the mean ± S.D. of three determinations.

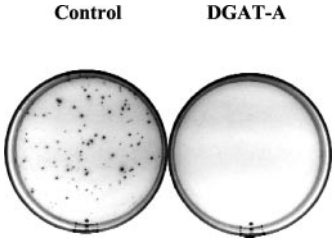


FIG. 8. **Anchorage-independent growth of SV40-transformed human fibroblasts is suppressed by DGAT overexpression.** 1×10^5 control and DGAT-A cells were plated in MEM containing 10% FBS and 0.3% agar over a previously poured layer of 0.6% agar. After 21 days, cell colonies were stained with ethidium bromide and photographed under UV light. A single clone, representative of three independent experiments, is shown for each cell line.

On the other hand, the finding that DGAT-overexpressing cells had a reduced synthesis of the major phospholipids because of a limited supply of DAG is surprising, because the cell media contained a sufficient concentration of the different substrates needed for phospholipid synthesis. Moreover, the inclusion of 100 μM oleate in the incubation media to enhance the formation of DAG substrates for lipid synthesis failed to normalize phospholipid synthesis, thereby indicating that decreased phospholipid formation is not exclusively produced by the lack of DAG and/or fatty acid substrates. In addition, the lower synthesis of [^{3}H]glycerol-labeled lipids in the DGAT cells indicates that these cells were not able to increase the *de novo* formation of DAG to compensate phospholipid synthesis. This observation is intriguing because the high *de novo* synthesis of lipids exhibited by the SV40-transformed cells[3] and other neoplastic cell strains (38) compared with normal cell strains, could provide sufficient substrates for phospholipid synthesis. However, a deficiency of PC formation caused by DGAT overexpression could be responsible for normalizing the transformation phenotype of SV40 cells. In this regard, it was reported that the pharmacological inhibition of PC synthesis promotes a slower cell proliferation rate and loss of actin fiber organization in A549 adenocarcinoma cells (39), as well as inhibition of invasiveness in mouse lymphoma cells (40).

The lack of compensatory up-regulation of *de novo* DAG synthesis for phospholipid formation raises the question of whether DGAT overexpression is altering the cell growth properties by merely redirecting DAG molecules destined to phospholipid synthesis toward triacylglycerol synthesis. Results from our experiments support a role for DGAT in cell regulation by its ability to modulate the levels of intracellular signaling DAG and fatty acids. Neoplastic transformation in SV40 cells is associated with increased DAG levels and low content of ceramide (12). The constitutive elevation in intracellular DAG content during SV40 transformation led to NF-κB activation, probably mediated by DAG-stimulated PKC activity (41). Thus, the PKC system must be dysregulated in DGAT-overexpressing SV40 cells, which exhibit DAG levels lower than mock-transfected SV40 cells. One possible major candidate for down-regulation is PKC-α, a ubiquitous DAG-dependent isoform implicated in several biological functions including cell growth, control of cell cycle and cell shape, and motility (42). In addition, PKC-α activity seems essential to sustain the tumorigenicity of neoplastic cells. In this regard, human lung carcinoma cells that have a high activity of PKC-α have an increased capacity for invasion and antisense PKC-α expression reverses the transformed phenotype of these cells (43). However, other signaling proteins activated by DAG could also be affected by DGAT overexpression. In this regard, it has been reported that DAG kinase ζ phosphorylates specifically localized DAG molecules, thereby controlling the activation of the DAG-activated signaling protein RasGRP and H-Ras (20). DAG kinase ζ isozymes can regulate local DAG signals; whether other enzymes like DGAT or DAG lipases that process DAG molecules participate in regulating DAG-activated signals is unknown.

In summary, we found out that DGAT-overexpressing neoplastic cells show: 1) oversynthesis of triacylglycerol, a decrease in the intracellular levels of DAG, and diminished acylation and *de novo* synthesis of the main phospholipids, PC and PE, and SM; 2) a high rate of incorporation of DAG and fatty acids released from plasma membrane phospholipids by phospholipase treatment into triacylglycerol; 3) decreased DNA synthesis and cell proliferation rate; and 4) total inhibition of anchorage-independent cell growth. Taken as a whole, this study provides strong evidence on a novel role of DGAT in the regulation of cellular growth by modulating membrane biogenesis and terminating intracellular lipid signals.

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Overexpression of Diacylglycerol Acyltransferase-1 Reduces Phospholipid Synthesis, Proliferation, and Invasiveness in Simian Virus 40-transformed Human Lung Fibroblasts

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