Posttranscriptional control of the expression and function of diacylglycerol acyltransferase-1 in mouse adipocytes

(Running title: Overexpression of DGAT1 in 3T3-L1 adipocytes)

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Abbreviations:

DGAT, acyl-CoA:diacylglycerol acyltransferase; TG, triglyceride; DAG, diacylglycerol; 
GFP, green fluorescent protein; ALLN, N-acetyl-leu-leulnorleucinal; TCID50, 50% 
tissue culture infectious dose; RT-PCR, reverse transcription-polymerase chain reaction; 
TLC, thin-layer chromatography; PID, post-induction day.
Summary

Acyl-CoA:diacylglycerol acyltransferase-1 (DGAT1) catalyzes the final step of triglyceride synthesis in mammalian cells. Data obtained from DGAT1-knockout mice have indicated that this enzyme plays an important role in energy homeostasis. We investigated the regulation of the expression and function of DGAT1 in mouse 3T3-L1 cell as a model for mammalian adipocytes. We demonstrated that the DGAT1 protein level increased by ~90-fold following differentiation of 3T3-L1 into mature adipocytes, a change which was accompanied by ~7-fold increase in DGAT1 mRNA. On the other hand, forced overexpression of DGAT1 mRNA by >20-fold via a recombinant-adenovirus only resulted in ~2-fold increase in DGAT1 protein in mature adipocytes, and little increase in preadipocytes. These results indicated that gene expression of DGAT1 in adipocytes is subjected to rigorous posttranscriptional regulation, which is modulated significantly by the differentiation status of 3T3-L1 cells. Protein stability is not a significant factor in the control of DGAT1 expression. The steady-state levels of DGAT1 were unaffected by blockage of proteolytic pathways by ALLN. However, translational control was suggested by sequence analysis of the 5'-UTR of hDGAT1 mRNA. We found that the level of DGAT1 activity was predominantly a function of the steady-state level of DGAT1 protein. No significant functional changes were observed when the conserved tyrosine-phosphorylation site in hDGAT1 was mutated by a single base-pair substitution. Despite only a ~2-fold increase in DGAT1 protein caused by recombinant-viral transduction, a proportionate increase in cellular triglyceride synthesis resulted
without affecting triglyceride lipolysis rate, leading to >2-fold increase in intracellular triglyceride accumulation. No change in adipocyte morphology, or in the expression levels of LPL, PPAR-\(\gamma\), and aP2 was evident secondary to DGAT1-overexpression at different stages in 3T3-L1 differentiation. These data suggest that dysregulation of DGAT1 may play a role in the development of obesity, and manipulation of the steady-state level of DGAT1 protein may offer a potential means to treat or prevent obesity.

**Supplementary key words:** DGAT, posttranscriptional control, tyrosine-phosphorylation, triglyceride biosynthesis, 3T3-L1 adipocytes, recombinant adenovirus.
Adiposity, or the amount of triglyceride (TG) stored in adipocytes, is fundamentally a net result of two opposing metabolic processes, i.e., lipogenesis and lipolysis. These are highly regulated processes influenced by various hormones, cytokines, nutrients and other factors (c.f. (1)) that may either serve as substrates or function as signaling molecules for one or more specific metabolic pathways. Acyl-CoA:diacylglycerol acyltransferase (DGAT; EC2.3.1.20) is thought to be a key enzyme in controlling the synthetic rate of TG in adipocytes (2). It catalyzes the last step in the de novo TG synthetic pathway employed by various types of cells, producing TG from its two substrates, diacylglycerol (DAG) and fatty acyl-CoA (for review c.f. (3,4)). Two DGAT genes have been identified. DGAT1 is a member of the ACAT (acyl-CoA cholesterol acyltransferase) gene family, and was cloned from human and mouse tissues through a homology search for ACAT-like sequences (5,6). More recently, DGAT2 was cloned from animals and fungi (7,8); DGAT2 bears no sequence homology with DGAT1.

The function of DGAT1 gene in TG metabolism has been assessed in knockout mice (9,10). Mice deficient in DGAT1 are viable and appear to have histologically normal adipose tissue (9). However, these mice have decreased adiposity, and less TG content in muscle and the liver (10). They also display a significant defect in TG synthesis in the mammary and sebaceous glands (9,11). Reexamination of DGAT1 deficient mice revealed that as much as half normal levels of DGAT activity were present in adipose tissue. This remaining DGAT activity was more readily displayed in an in vitro assay at
lower magnesium concentrations, and was thought to be mediated by DGAT2 (7). Interestingly, DGAT1 deficient mice showed increased insulin and leptin sensitivity (10), and they had significant resistance to diet-induced obesity due to increased energy expenditure (9). These experiments point to a significant role of DGAT1 in systemic energy homeostasis.

Despite much existing data concerning the biology of DGAT, how DGAT’s expression and function are regulated in mammalian adipocytes remains poorly understood. Furthermore, although insect cells have been used for exogenous expression of the mammalian DGAT1 gene, with resultant increases in intracellular TG synthesis (6,12), studies of the effects of overexpressing DGAT1 gene in mammalian adipocytes are lacking. In the present paper, we report our studies of the regulation of DGAT1 expression and function in mouse 3T3-L1 cells in relation to their differentiation status. We demonstrated that DGAT1 expression is not only regulated transcriptionally as previously shown, but also strictly controlled posttranscriptionally. This posttranscriptional mechanism is heavily modulated by the differentiation status of 3T3-L1 cells. We further showed that DGAT1 activity and its effect on the rate of intracellular TG accumulation depend primarily on the steady-state levels of DGAT1 protein. Mutation at the putative tyrosine-phosphorylation site in DGAT1 causes no significant changes in its activity or overall rates of TG accumulation. Finally, we showed that overexpression of DGAT1 in 3T3-L1 cells does not alter the course of adipocyte differentiation.
Experimental Procedures

Materials: [2-3H]glycerol (1.0 mCi/mmol), [1-14C]palmitoyl-coenzyme A (55.0 mCi/mmol) and ECL Western blotting detection reagents were purchased from Amersham Life Science. [32P]dCTP was from NEN Life Science Products. Thin-layer-chromatography (TLC) plates were obtained from Merck. Culture media, supplements and antibiotics were all purchased from Life Technologies. Triglyceride assay kits (Trig/GB) were from Roche Diagnostics. Other chemicals and reagents were mostly purchased from Sigma with the highest purity available.

Recombinant adenovirus: To make the recombinant adenovirus, a full-length hDGAT1 (ARGP-1) cDNA sequence, which includes a 244 bp 5′-untranslated region (5′-UTR) and a 273 bp 3′-UTR containing a poly A signal AATAAA, (5) was first subcloned into a shuttle plasmid, pAd.CMV-link.1, at the HindIII and KpnI (repaired) sites. This plasmid contains a stretch of 5′-end adenoviral DNA sequence flanking the insert DNA, hDGAT1, that enables subsequent homologous viral recombination needed to make the recombinant adenovirus. pAd.CMV-link.1-hDGAT was linearized with NheI, and cotransfected with ClaI-digested defective adenoviral DNA (lacking the 5′ end ITR sequence) into 293 cells. 293 cells support replication and packaging of the defective adenovirus by providing trans-expression of an early gene product (E1) of the adenovirus, which is lacking in the defective adenovirus. As a result of homologous recombination between pAd.CMV-link.1-hDGAT1 and the ClaI-digested adenoviral
DNA, recombinant adenoviruses containing hDGAT1 in place of E1 gene were selected as viral plaques on 293 monolayers. We confirmed the presence of the insert DNA of hDGAT1 in the recombinant virus by PCR. After two rounds of plaque purification, the recombinant hDGAT1-adenovirus (Ad-hDGAT1) was further amplified by repeated infections in 293 cells. A control recombinant virus containing the cDNA for green florescence protein (GFP) was constructed similarly. Lysates were prepared from the infected monolayer 293 cells by freeze-and-thaw methods, and purification of viral particles was achieved using CsCl gradient centrifugation as previously described (13,14). Viral titers were determined by TCID50 (15,16).

**Mutation of hDGAT1 at the conserved tyrosine phosphorylation site.** The point mutation converting Tyr^{316} to Phe, a structurally related amino acid residue, was made using a PCR-based strategy. A pair of PCR primers was made commercially: the forward 5’ primer corresponding to nucleotide sequence 978-997 in hDGAT1 and the reverse 3’ primer corresponding to nucleotide sequence 1179-1221 with a base substitution from A to T at position 1190. This substitution converts the codon TAC for Tyr to the codon TTC for Phe. The resultant PCR fragments was digested, in preparation for subsequent ligation, with Eco47III and HaeII, which are located near the 5’ and 3’ ends of the fragment, respectively. The full-length hDGAT1 in pcDNA3.1 was excised with Eco47III (which cuts hDGAT1 sequence at position 1069) and XbaI (which cuts at the polylinker region of the vector, 3’ to the insert sequence). While the larger Eco47III/XbaI fragment (fragment A) from this digestion containing the vector sequence and part of the
DGAT sequence was saved for re-ligation, the smaller Eco47III/XbaI fragment was further digested with HaeII (position 1209) to produce a 3' HaeII/XbaI DGAT fragment (fragment B). Finally, a 3-piece re-ligation was carried out between fragments A and B and the Eco47/HaeII PCR fragment to generate pc(T316F). This plasmid, which contains a full-length hDGAT1 with phenylalanine substitution for tyrosine, was used to generate the recombinant adenovirus, Ad-T316F, similarly as described (above).

Cell culture, differentiation and adenoviral infection: 3T3-L1 preadipocytes were maintained in high glucose (25mM) DMEM with 10% bovine calf serum. Medium was changed every other day, and cells were always split and passed before they reached full confluence. To induce differentiation, confluent cells were cultured in maintenance medium for 48 hours before changing to an induction medium of high glucose DMEM containing 10% bovine fetal serum, 1 µM dexamethasone and 5 µg/ml insulin. Cells were routinely cultured in induction medium for 10 days, unless otherwise indicated, to obtain full differentiation. Again, medium was changed every other day. To infect cells with recombinant adenovirus, cells were first incubated with viruses (typically 30 pfu/cell) in serum-free DMEM for 2 hours. In most experiments, polylysine at concentration of 0.75 ug/ml was added during the incubation to facilitate viral transduction (17). An equal volume of DMEM medium containing 4% bovine calf serum (final concentration 2%) was then added, and the cells were incubated for another 12 hours before the medium was changed. Assays were carried out at least 48 hours after viral infection.

Oil-red-O staining for intracellular TG: Oil-red-O (0.4%) in isopropanol solution was
freshly made. Fine particles were allowed to precipitate out after standing at 23°C for
10 min. Following a brief spin, clear supernatant was transferred to a new tube and mixed
with one half volume of H2O. After 10 min at 23°C, the dye was spun again, and the
clear supernatant was used for staining immediately. To stain for TG, cells in the
monolayer were first washed with PBS buffer three times, then fixed in 37% formaldehyde solution for 30-60 min without shaking. Next, formaldehyde was washed
off with 6 washes in PBS buffer. Fixed cells devoid of formaldehyde were stained with
the freshly prepared oil-red-O solution for 10 min at 23°C, followed by extensive
washes with H2O (6 times).

**RT-PCR:** RNA was prepared from cultured cells with TriZol reagent using the protocol
provided by the manufacturer (Life Technologies). Reverse transcription was carried out
with a reverse transcriptase and random hexamer primers. One to two ul of the reaction
mixture containing reverse transcripts was used for subsequent PCR reaction using Taq
polymerase and sequence-specific oligonuclear primers. To amplify a DNA fragment of
less than 500-bp, thirty-five cycles were typically used as follows: denaturing 94°C/15
sec; annealing 55°C/15 sec; polymerization 72°C/30 sec. The relative RNA levels were
determined by “quantitative RT-PCR”. For this assay, primers for small PCR fragments
of about 150 bp were designed. Amplification was pre-titrated within a linear range,
using a limited number of cycles and 32P-dCTP labeling for quantification (18,19).

**Northern blot:** Fifteen µg RNA was applied to each lane in a 1% agarose gel with
formaldehyde, and Northern analysis was performed based on standard methods (20).

The probes used for hybridization were generated by RT-PCR labeling with $^{32}$P-dCTP using RNAs isolated from mouse adipose tissue as initial templates. Specific oligonucleotide primers used in the PCR labeling were made of about 20 base-pairs based on gene bank database sequences for specific adipocyte differentiation marker proteins described in the text.

**Western blot:** This analysis was performed according to standard methods (21). The primary polyclonal antibodies against DGAT were generated from rabbits using MAP-conjugated synthetic peptides corresponding to the first 20 amino acid residues of mouse DGAT1. Specific protein bands were visualized using ECL immunodetection system (Amersham Life Science) after blotting with horse radish peroxidase-conjugated second antibodies against rabbit IgG. In some experiments, $^{131}$I-labeled mouse anti-rabbit antibodies were used and specific protein bands were visualized after autoradiographic exposure.

**In vitro DGAT assay:** Membrane fractions containing DGAT1 were prepared from the post-mitochondrial fraction of 3T3-L1 adipocytes essentially according to published protocols with minor modifications. Briefly, cells in monolayer were washed twice with cold PBS, then scraped into ice-cold homogenization buffer (20 mM HEPES, pH 7.4, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM DTT and a cocktail of protease inhibitors (PIs) containing Trasylol, PMSF, leupeptin and pepstatin). Cells were allowed to swell on ice for 10 min before homogenization (ball-bearing homogenizer, 10 strokes). One-fourth
volume of 30% sucrose was added to the sample immediately following homogenization. The homogenization mixture was then centrifuged at 1,500 x g for 10 min at 4°C. The supernatant was then spun at 150,000 x g for one hour at 4°C. The membrane pellet was homogenized and resuspended in a buffer containing 20 mM HEPES, pH 7.4, 0.25 M sucrose and PIs. Protein concentration was determined using BCA Protein Assay Reagent Kit (Pierce).

To measure DGAT1 activity, typically 10 µg membrane protein was used in a 200 ul reaction mixture containing 100 mM Tris, pH 7.5, 250 mM sucrose, 1 mg/ml bovine serum albumin, 150 mM MgCl₂, 0.8 mM EDTA, 0.25 mM 1,2-Dioleoyl-sn-Glycerol and 25 µM Palmitoyl-CoA containing 0.3 µCi [¹⁴C] radioactivity. In some experiments, 10 mM MgCl₂ was used to assess a wider range of DGAT activity. The reaction was carried out at 37°C for 5 min, and stopped by adding 0.75 ml lipid extraction solvents (chloroform/ methanol in a ratio of 2:1). After adding 0.375 ml acidic solution (1 mM H₂SO₄/ 17 mM NaCl), the organic phase was separated out and dried under a stream of N₂. Lipids were re-dissolved in 20 µl hexane, and then analyzed by thin-layer-chromatography (TLC) as described (22) except that plates were developed in hexane-diethyl ether-glacial acetic acid 70:30:1 (v/v/v). Chromatographic bands containing TG were cut out after iodine staining, and quantitated by scintillation counting.

[^H]glycerollabeling: To measure TG synthetic rates in 3T3-L1 adipocytes, cells were labeled in high glucose DMEM with[^H]glycerol (10 µCi in 2 ml medium) for one hour.
Cells were washed 3 times with PBS buffer. Lipids were then extracted from the monolayer cells with organic solvents made of hexane and isopropanol in 3:2 ratio. The extraction was carried out either at 23°C for 2 hours or at 4°C overnight. The extraction mixture was dried under a liquid nitrogen stream, re-dissolved in 20 µl hexane, and then analyzed by TLC. TG was quantitated by scintillation counting. To study TG turnover, cells were labeled with a larger amount of [3H]glycerol (30 µCi in 2 ml medium) for 2 hours. At the end of labeling, cells were washed 3 times with PBS before changing to chase medium (high glucose DMEM without radioactive tracers). Lipids were extracted by hexane/isopropanol) at each time point, and [3H]TG was quantitated by scintillation counting after TLC separation.

**Total TG mass**: Cellular lipids were extracted using hexane/isopropanol (3:2) as described above. TG mass was determined enzymatically using colorimetric kits (Trig/GB from Roche Diagnostics). TG standards were used for quantification.

**Statistical analysis**: Statistica V6.0 was used for all analyses. The significance of differences was determined using student’s t test. A two-tailed p value <0.05 was considered to indicate statistical significance.
Results

DGAT1 expression is regulated at the posttranscriptional level, and the protein level of DGAT1 is determined predominantly by the differentiation status of 3T3-L1 cells. To investigate how the expression and function of DGAT1 are regulated in mammalian adipocytes, we used a well-characterized mouse cell line, 3T3-L1. 3T3-L1 cells are preadipocytes, which undergo adipocyte-differentiation when induced by insulin/dexamethasone (I/D). Compared to undifferentiated 3T3-L-1 cells, there was a 7-fold increase in DGAT1 mRNA levels in full differentiated 3T3-L1 adipocytes (post-induction day 10, PID 10) (Figure 1A, lanes a and a’), consistent with the transcriptional regulation of this gene. To determine the protein levels of DGAT1, we raised rabbit polyclonal antibodies against synthetic peptides corresponding to the first 20 amino acid residues of mouse DGAT1 (mDN20). Since mouse and human DGAT1 share a high degree homology in amino acid sequence within the first 20 amino acid residues, the antibodies (anti-mDN20) recognize both human and mouse DGAT1 as a protein band of ~58 kD in 3T3-L1 adipocytes. While the level of DGAT1 protein increased concomitantly with DGAT1 mRNA during adipocyte- differentiation in 3T3-L1 cells, the increase in DGAT1 protein far exceeded the increase in DGAT1 mRNA, (Figure 1B, lane d and d’; note that the amount of proteins loaded in lane d’ was 1/10 that in lane d). An estimated 90-fold increase in DGAT1 protein level was observed (9-fold densitometric difference between lanes d and d’ x 10-fold difference in protein loading). This marked disparity between the increases in DGAT1 protein and mRNA indicates that
the expression of DGAT1 gene was regulated by an additional posttranscriptional mechanism during normal differentiation of 3T3-L1 cells.

To further characterize this posttranscriptional regulation, 3T3-L1 cells were directed to produce high levels of full-length DGAT1 mRNA from an exogenous human DGAT1 (hDGAT1) gene containing the entire 5' and 3' untranslated regions, and the resultant DGAT1 protein levels were followed. Forced high-level expression of hDGAT1 mRNA was achieved by using a recombinant adenovirus carrying a hDGAT1 expression cassette (Ad-hDGAT1) under the control of the cytomegalovirus promoter/enhancer (pCMV).

We first transduced fully-differentiated 3T3-L1 adipocytes (PID 10) with Ad-hDGAT1. As shown in Figure 1A, Ad-hDGAT1 transduction gave rise to a 20-fold increase in the steady-state level of DGAT1 mRNA in mature 3T3-L1 adipocytes (hDGAT1), relative to the control cells either transduced with recombinant adenovirus carrying pCMV-GFP (GFP), or mock-transduced (-) (compare lanes c' with b' or a'). In contrast, only a 2.6-fold increase in DGAT1 protein was observed in the same set of cells (Figure 1B, compare lanes f' with e' or d'). As expected, the overexpressed DGAT1 was concentrated in the membrane (Mb) fraction. Less than 2% of the protein was detected in the cytosol (Cyt) fraction, which most likely resulted from membrane contamination during cell fractionation. We next examined the effect of Ad-hDGAT1 transduction on the mRNA and protein levels of DGAT1 in undifferentiated 3T3-L1 cells. If a strict posttranscriptional mechanism exists in these preadipocytes that do not store TG, little increase in DGAT1 protein would be predicted even when the cells produce a high level of DGAT1 mRNA from the exogenous gene. Indeed, we found only a 2.4-fold relative
increase in DGAT1 protein above the basal levels in the membrane fraction (Mb) of the hDGAT1-transduced preadipocytes (Figure 1B, compare lanes f with e or d); no DGAT1 protein was detected in the cytosol faction (Cyt) of the cells. Note that the 2.4-fold relative increase in DGAT1 protein represents only an insignificant absolute-increase over a very low basal level, which is in sharp contrast to a ~ 40-fold increase in DGAT1 mRNA level in the same cells (Figure 1A, lanes c vs b or a). Taken together, the above experiments indicate that a high steady-state level of DGAT1 mRNA may be necessary (transcriptional regulation), and certainly is permissive, for a much higher level of expression of DGAT1 protein during adipocyte differentiation. However, a similar high level of DGAT1 mRNA is insufficient for the expression of DGAT1 protein in undifferentiated 3T3-L1 cells. In both states, posttranscriptional regulation constitutes an important mechanism in controlling DGAT1 protein expression, and is predominantly differentiation status-dependent.

The steady-state levels of DGAT1 are unaffected by blockage of proteolytic pathways by N-acetyl-leu-leulnorleucinal. In order to further delineate between translational control and posttranslational regulation of protein stability as a potential mechanism for the observed posttranscriptional control of DGAT1 protein expression, we measured steady state levels of DGAT1 protein in the presence and absence of N-acetyl-leu-leu-norleucinal (ALLN). ALLN is an inhibitor that blocks proteolytic activity of both type I calpain and the 26S proteosome. Mature 3T3-L1 adipocytes incubated for 6 hours in the presence of as much as 100 µg/ml ALLN, a concentration at or below which effective inhibition of protein degradation in adipocytes has been demonstrated (23), failed to show
any increase in the steady-state levels of DGAT1 protein (Figure 2A). This was true in both Ad-hDGAT1 transduced 3T3-L1 adipocytes (Figure 2A, compare lane b with d) and the control cells (Figure 2A, lane a vs. c). No more than 5% of total DGAT1 protein was detected in the cytosol fraction under these conditions (data not shown). We next measured the half-life of DGAT1. We treated mature Ad-hDGAT1- and Ad-GFP-adipocytes with cycloheximide (CHX) at the final concentration of 250 µM to block protein synthesis (24). Cells were analyzed for the steady state levels of DGAT1 protein by Western blot immediately before and at various time intervals after CHX treatment (Figure 2B). Relative DGAT1 levels were quantified by densitometric measurement of DGAT1 protein bands, and values plotted against and extrapolated over the time intervals post CHX treatment. Using these methods, DGAT1 half-life was determined to be 18 hours in both Ad-hDGAT1 transduced or control 3T3-L1 adipocytes. No additional DGAT1 pool of rapid-turnover was detected in Ad-hDGAT1 transduced cells. Under the same conditions, the turnover of GFP in Ad-GFP-adipocytes was also investigated (Figure 2B, bottom panel), and its half-life was determined to be 8 hours. The inability of ALLN to affect the steady-state levels of DGAT1 protein, together with the fact that this protein has a relatively long half-life which was not changed in Ad-hDGAT1-adipocytes, strongly argue against a rapid protein turnover as an explanation for the limited increase in the steady-state DGAT1 protein level in these cells when DGAT1 mRNA was increased >20 fold.
steady-state DGAT1 protein level. Despite strong posttranscriptional regulation, we observed 2- to 3-fold increases in steady-state levels of DGAT1 protein in Ad-hDGAT1-transduced 3T3-L1 adipocytes. Therefore, we examined the functional and biological effects of overexpressing DGAT1. DGAT activity was measured in an in vitro assay with microsomal membranes. Compared with undifferentiated 3T3-L1, mature adipocytes exhibited a 100-fold increase in DGAT activity (see Figure 3A, “(-)” in a and b), a level comparable with the increase in protein level (90-fold). Transduction of preadipocytes and mature adipocytes with Ad-hDGAT1 resulted in a 2.7 and 2.9-fold increase in microsomal DGAT1 activity, respectively, as compared with their own controls (Figure 3A, left and right panels, respectively). These increases in DGAT activity were comparable with the 2.4 and 2.6-fold increases in DGAT1 protein levels in respective cells (Figure 1B). Thus, the increases in the enzymatic activity were accounted for, almost fully, by the increases in DGAT1 protein in these cells. To determine the functionality of these changes in DGAT1 activity, the cellular incorporation rate of [3H]Glycerol into TG, and the total intracellular TG accumulation, were determined in cultured cells. Compared with the mock-transduced cells (-) or cells transduced with Ad-GFP (GFP), a 2.2-fold increase in cellular TG synthesis rate (Figure 3B) was observed. The increase in the rate of cellular TG synthesis is consistent with the 2.6-fold increase in DGAT1 protein and the 2.9-fold increase in microsomal DGAT1 capacity measured in vitro. Finally, there was a 2.3-fold increase in total intracellular TG mass in cells transduced with Ad-hDGAT1 (Figure 4C, compare “hDGAT1” with “(-)” and “GFP”).
We next examined TG-turnover rates in hDGAT-1 transduced 3T3-L1 adipocytes. In these experiments, [3H]-labeled TG was chased for up to 72 in both Ad-GFP and Ad-hDGAT1 transduced mature adipocytes. As shown in Figure 4, a slight, statistically insignificant decrease in the fractional turnover rate was observed in Ad-hDGAT1 transduced cells, as compared with that in Ad-GFP transduced cells. These results further substantiated our conclusion that the >2-fold increase in TG accumulation in hDGAT1-transduced adipocytes (Figure 3C) was due directly to an elevated TG synthesis rate; there was no significant change in TG lipolysis rate in these cells.

**Mutation at the putative tyrosine-phosphorylation site in DGAT1 has no significant effect on DGAT1 activity and overall cellular TG accumulation.** Previous biochemical studies suggested that DGAT activity is regulated by phosphorylation of the enzyme (25-28). Since these data were all obtained before the cloning of the DGAT genes, it is unclear which DGAT was particularly modified in particular experiments. Lau and Rodriguez reported that DGAT isolated from rat adipocytes was bound and inactivated by an ATP-dependent tyrosine kinase; this process could be reversed by incubating the sample with a crude preparation containing protein phosphatase(s) (28). Both human and mouse DGAT1 contain a single conserved sequence for tyrosine-phosphorylation (5,6). By contrast, the cloned DGAT2 contains several putative protein kinase C phosphorylation sites (7). To verify potential tyrosine-phosphorylation regulation of DGAT1, we made a single point mutation at the conserved tyrosine-phosphorylation site, converting tyrosine-316 to phenylalanine (Figure 5A). Clearly, this mutant hDGAT1
would be unable to serve as a substrate for a tyrosine kinase.

The mutant hDGAT1 (T316F) was introduced into 3T3-L1 adipocytes using the same technique employing recombinant adenovirus. Viral transduction gave rise to a comparable high-degree expression of the mutant mRNA, T316F, compared with the wild type mRNA (Figure 5B, compare lanes b with c). Similarly, the protein levels of T316F and the wild-type hDGAT1 were increased by 2- and 2.5-fold, respectively, over the control (Figure 5C, Mb), suggesting that the T316F mutant was subjected to the same posttranscriptional control as the wild-type. Little DGAT1 protein was found in the cytosol in either instance (Figure 5C, Cyt), suggesting that the mutation did not alter subcellular distribution of hDGAT1 protein either. Furthermore, microsomal membranes isolated from the T316F-transduced cells (T316F) exhibited a 1.7-fold increase in DGAT activity over the control (GFP); in the same set of experiments, the wild-type hDGAT1 caused a 2.1-fold increase in DGAT activity (Figure 5D, left panel). These results suggest that the mutant hDGAT1 is functionally active. However the “nonphosphorylated” enzyme did not result in higher enzymatic activity more than what might be accounted for by the increased protein expression of the mutant hDATG1. This was in contrast to what might be expected if the “nonphosphorylated” form of this enzyme represents a more active DGAT1, a scenario suggested previously for “overall” DGAT activity being subjected to tyrosine-phosphorylation modification (28). Under the above assay condition (with 150 mM MgCl2), DGAT2 activity is largely suppressed (7). In order to assess if any compensatory changes in DGAT2 activity occur in DGAT1-
overexpressing cells, experiments were repeated at 10 mM MgCl2 to optimally display DGAT2 activity (7). Under such a condition, a fuller range of DGAT activity (both DGAT1 and DGAT2) was measured. As shown in Figure 5D, right panel, higher DGAT activities (a difference by the factor of 2-2.5 between the two assay conditions) were observed. However, there was no significant difference in the relative increase among the three specimens. These results suggested that DGAT2 levels were preserved in all these cells, and there was no down-regulation of DGAT2 in cells transduced with Ad-hDGAT1 or Ad-T316F in response to the increased DGAT1 expression and function. Finally, consistent with the increase in total DGAT activity in wild-type hDGAT1- and mutant T316F-expressing cells adipocytes, a 2.6- and 2.3-fold increase, respectively, in total intracellular TG content were obtained 5 days after viral transduction (Figure 5E).

DGAT1 overexpression has no significant effect on adipocyte differentiation. Since the expression of endogenous DGAT1 increases during 3T3-L1 differentiation, we also examined if overexpression of the exogenous hDGAT1 would in turn affect the course of differentiation. Potential effects of overexpression of DGAT activity on adipocyte-differentiation were assessed first by examining the gross morphology of the adipocytes. Oil-red-O was used to stain neutral lipids in these cells. The expression levels of differentiation marker genes were also determined to assess the differentiation status at the molecular level. Potential effects on differentiation was assessed by transducing 3T3-L1 cells with Ad-hDGAT1 at two morphologically distinct stages during their differentiation, post-induction day 4 (PID 4), at which time no significant TG droplets had yet formed, and post-induction day 10 (PID 10), at which time a full lipid-laden
morphology had been achieved. Figure 6A shows representative examples of oil-red-O staining of preadipocytes (a), mature adipocytes (PID day 12) without viral transduction (b), mature adipocytes (PID 12) transduced with Ad-hDGAT1 on PID 4 (c), mature adipocytes (PID 12) transduced with Ad-hDGAT1 on PID 10 (d). All the cells on PID 12 (b-d) reached a similar degree of maturity as judged by their gross morphology and oil-red-O staining. They all showed a distinctly round, adipocyte-cell type, as opposed to the typical fibroblast-cell type of the preadipocytes, which also did not stain with oil-red-O (a). There were no gross differences between Ad-hDGAT1-transduced cells and the control cells. Neither were there differences between cells transduced at an early and a late stage in the differentiation process (compare b, c and d).

The mRNA levels of three differentiation marker genes, lipoprotein lipase (LPL), proxisomal proliferation activating receptor-gamma (PPAR-γ), and a fatty acid binding protein, aP2, were assessed by Northern blotting in the above adipocytes to further evaluate their differentiation status. As shown Figure 6B, vial transduction at both stages (PID 4 and PID 10) resulted in marked increases in DGAT mRNA levels, although the increase of DGAT mRNA level was greater in cells transduced on PID10 (20-fold, compare lanes a’ with b’ and c’) relative to those transduced on PID 4 (5-fold, compare lanes a with b and c). This difference in relative increase was likely a result of a greater decay of adenoviral expression in cells transduced earlier, as cells transduced on PID 4 were assayed 8 days after viral infection, as opposed to 2 days after infection in cells transduced on PID 10. In either case, there were no significant differences in the mRNA levels of LPL, PPAR-γ, or aP2 between Ad-hDGAT1 transduced adipocytes and their
two controls, Ad-GFP cells and mock-transduced cells (-) (compare lanes a with b and c; lanes a’ with b’ and c’). In fact, 3T3-L1 cells were able to differentiate normally even when they were transduced with Ad-hDGAT1 before induction; no significant acceleration or deceleration was observed during the subsequent differentiation process (data not shown). As expected, mRNA levels for LPL, PPAR-γ, or aP2 were not detectable in undifferentiated 3T3-L1 cells (Figure 6B, lane d).
Discussion

Mammalian TG biosynthesis is thought to be accomplished mainly through the glycerol-phosphate pathway present in virtually all cell types and the monoacylglycerol pathway functioning primarily in intestinal enterocytes. Both pathways utilize DGAT for the final common step converting DAG to TG. A diacylglycerol transacylase pathway that bypasses DGAT and appears to operate in oilseed maturation may play a limited role in mammalian gastrointestinal system (4,29). Recently, another major TG biosynthetic pathway has been identified in yeast mediated by Lro1, a homolog of human lecithin cholesterol acyltransferase, which esterifies DAG using phosphatidylcholine as the acyl donor, bypassing DGAT (30). It is unclear if this type of TG biosynthesis plays any significant role in mammalian cells. With regard to the glycerol-phosphate pathway, two DGAT genes have been identified, and there may be several members in the DGAT2 family (7,8). DGAT2 activity does not appear to be up-regulated in DGAT1 deficient mice (7). We have no evidence that it is down-regulated in DGAT1 overexpressing adipocytes, since the difference in total DGAT activity resulting from overexpressing DGAT1 is preserved under the assay conditions optimal for DGAT2 (see text). An important issue relates to the regulation of DGAT expression and function. Clearly, a better understanding of these mechanisms would help in subsequent investigations, in which DGAT activity may be manipulated in a controlled manner. Equally important is to determine if alterations in DGAT activity in adipocytes will bring about anticipated changes in TG homeostasis.
The present studies show clearly that the expression of DGAT1 is tightly regulated at both the transcriptional and post-transcriptional levels. While the transcriptional regulation of this gene is closely related to the differentiation status of adipocytes (31), our studies demonstrated that post-transcriptional control is the principal mechanism for the regulation of the expression of this gene. Post-transcriptional control of DGAT gene expression has been suggested (32) based on the disparity between the degrees of increase in DGAT activity and mRNA level; these data were obtained separately from two different laboratories (6,31). Our studies confirmed this observation using a set of cells under the same experimental conditions, and further demonstrated that the disparity lies in DGAT1 mRNA and protein levels; there is no significant difference between the steady-state levels of DGAT1 protein and the enzymatic activity. In fact, in our experiments, while DGAT1 mRNA increased by just more than 7-fold in mature adipocytes compared with preadipocytes, DGAT1 protein increased by 90-fold. A similar increase (~100-fold) in DGAT activity correlated well with the increase in protein. These data suggest that in addition to transcriptional regulation of DGAT1 during adipocyte differentiation, the expression of DGAT1 protein is further enhanced at the posttranscriptional level. On the other hand, the posttranscriptional regulation of DGAT1 protein expression is not unidirectional. Thus, when an inappropriately higher level of mRNA (relative to cell function or differentiation status of the adipocytes) is produced (as occurs in Ad-hDGAT1 transduced cells), the increase in protein expression is curtailed at the posttranscriptional level. This mode of control was demonstrated in both mature and pre-adipocytes, but more profoundly in the latter, undifferentiated cells that
do not store TG. As would be predicted, a >40-fold increase in DGAT1 mRNA in Ad-
hDGAT1 transduced preadipocytes caused only a negligibly small absolute-increase in
gTAG1 protein over a very low basal level in these cells. To extrapolate this finding
further, such posttranscriptional control may be necessary in order to maintain
intracellular TG homeostasis in situations when, speculatively, transcriptional regulation
of this gene fails in any pathological conditions. This posttranscriptional control of
gTAG1 expression does not seem to be caused by a possible species-incompatibility of
hDGAT1 with mouse adipocytes. In fact, no difference was observed when Ad-hDGAT1
was introduced into a human liver cell line (HepG2) or a rat liver cell line (McRH7777).
Both cell lines displayed similar, relatively low levels of increase in DGAT1 protein and
activity as opposed to much higher levels of DGAT1 mRNA in both cells (data not
shown). Of note, posttranscriptional regulation of DGAT1 has also been suggested
recently in the plant, *Brassica napus* (33).

Unlike a previous report (28) suggesting that DGAT activity is significantly regulated by
tyrosine-phosphorylation, our results showed that a site-specific mutation in the
conserved tyrosine-phosphorylation site in hDGAT1 did not result in any enhanced
gTAG activity, as compared with the wild-type hDGAT1. The “nonphosphorylated
gTAG1” is an active form of the enzyme, however, since the mutant hDGAT1, T316F,
displayed an appropriate increase in DGAT activity and intracellular TG accumulation in
Ad-T316F transduced adipocytes, compatible with an increase in its protein level. Our
results further showed that at the whole cell level, TG accumulation was not affected
disproportionately by the presence of the overexpressed “constitutively nonphosphorylated” form of DGAT1. Thus, our data suggest strongly that tyrosine-phosphorylation does not play a significant role in regulating DGAT1 activity. We cannot rule out the possibility, although unlikely, that the conversion of the tyrosine to the phenylalanine residue in our T361F construct caused a significant alteration in DGAT1 conformation. Such a conformational change may not abolish its enzymatic function, but cause a decrease in activity compared with the native “nonphosphorylated” molecules. Another possibility is that a functional tyrosine-phosphorylation site is present in a yet-unidentified DGAT gene. Phosphorylation modification of DGAT activity by cAMP-dependent or Ca2+/calmodulin-dependent protein kinases was also reported (26,27). But these phosphorylations resulted in enhanced, rather than decreased, DGAT activity. Such modification is likely present in DGAT2 since the cloned DGAT2 gene contains several putative protein kinase C phosphorylation sites (7). Nevertheless, the phosphorylation of the serine/threonine residues needs to be verified at specific sites in DGAT2 sequence using site-specific mutation, an approach we have taken to determine the functionality of the tyrosine-phosphorylation site of DGAT1. Consistent with the lack of the tyrosine-phosphorylation regulation in DGAT1 activity is the fact that the DGAT activity and the cellular TG incorporation rate correlate closely only with the steady-state protein level of DGAT1. This is true in both Ad-hDGAT1 and Ad-T316F transduced adipocytes. It is clear that the DGAT1 activity is determined primarily by the steady-state level of DGAT1 protein. It is this protein level that is subjected to the rigorously regulation through a posttranscriptional mechanism, which is modulated by the differentiation status
Posttranscriptional control of gene expression appears to be an important mechanism involved in many aspects of lipid metabolism in response to various physiological stimuli. Gene expression of several major apolipoproteins is controlled at the posttranscriptional level (34-38). The secretion rates of apolipoprotein B (apoB), for example, depend largely on the lipid availability in the ER, which permits continuous synthesis of this protein to be coupled with regulated lipoprotein secretion. In the absence of lipid ligands, either translation of apoB is retarded, or the newly synthesized polypeptides are quickly targeted for proteosomal degradation (c.f. (39,40)). HMG-CoA reductase, the rate limiting enzyme for cholesterol synthesis, is another example of posttranscriptional regulation (41-46). In addition to transcriptional suppression by sterols, this enzyme is further regulated by mevalonate through a posttranscriptional mechanism, primarily by accelerating the degradation of this protein (41,44,45). We do not know at this time how posttranscriptional regulation of the expression of DGAT1 protein is achieved. But a need for a more rapid regulation (as opposed to transcriptional regulation) in response to metabolic stimuli (hormones or lipid intermediates) is conceivable. As demonstrated in enterocytes, DGAT is a component of the “TG synthetase” complex that also contains acyl CoA ligase, acyl CoA acyltransferase and monoacylglycerol acyltransferase (47). If DGAT1 is also part of a protein complex in adipocytes, it is possible that the other components of the complex serve to stabilize DGAT1 protein when it is associated with the complex. Therefore, one could speculate
that when DGAT1 protein is produced in large quantities, it would be in free form and, therefore, targeted for rapid protein degradation. This hypothesis could be further tested by examining and comparing DGAT1 protein levels in the presence and absence of specific protease inhibitors that may block the proteolytic pathway responsible for the targeted DGAT1 degradation. We have assessed DGAT1 protein levels in 3T3-L1 adipocytes in the presence and absence of ALLN, a nonspecific proteosome inhibitor. Our results did not shown any increase in DGAT1 protein in the presence of this protease inhibitor, and do not, therefore, support the notion that posttranslational protein degradation, at least via ALLN-sensitive pathways, plays a significant role in controlling steady-state levels of DGAT1 protein. Consistent with this conclusion was the fact that DGAT1 has a relatively long protein half-life of 18 hours, and this half-life was not affected by overexpression of DGAT1. If rapid protein degradation of an excess of newly synthesized DGAT1 (which could be in a different subcellular pool other than the membrane fraction) constitutes a significant regulatory pathway, then a much shorter protein half-life would be expected. In addition, we did not detect any significant amount of DGAT1 in subcellular fractions other than the membrane fraction either in the presence or absence of ALLN (data not shown), indicating that a distinct subcellular pool of rapidly degrading DGAT1 does not exist. Our approach to determine protein half-life may not be precise, since in the presence of CHX, mild cytotoxicity was observed after 10 hours of treatment. Therefore, the decrease in DGAT1 at later times post CHX treatment may, in part, be attributed to some degree of cell toxicity. However, this effect of CHX should have been similar in both DGAT1 and GFP induced cells. A better
approach awaits the availability of suitable antibodies capable of immunoprecipitating DGAT1, so that a pulse-chase experiment can be performed, and the fate of newly synthesized DGAT1 polypeptides can be followed by radio-labeling.

In the absence of evidence for protein stability as an explanation for the posttranscriptional control of DGAT1 protein, we speculate that DGAT1 expression is controlled at the translational level. Translational regulation of protein expression has been described for various types of mammalian proteins (48-51) including ApoB (52,53). Although the efficiency of protein translation may be controlled both at the initiation and elongation, translation initiation has been more extensively studied. In this regard, the primary and secondary structures of the 5′-untranslated region (5′-UTR) of the mRNA may constitute important cis-acting regulatory elements. The cap structure at the 5′ terminus (54) and the internal ribosome-entry sites (IRESes) (55), the oligopyrimidine tracts at the extreme 5′ terminus (56), the Kozak sequence about the initiation codon (57), the presence or absence of secondary structures (58) or upstream AUGs (uAUGs) (51,59) have all been shown to influence the efficiency of translation initiation. hDGAT1 has a 244 bp 5′-UTR with a potential to form extensive secondary structures because of its high GC content (74% GC overall, and above 80% in several stretches of sequences; c.f. (5)); it also contain an uAUG. These features make it possible that the initiation of hDGAT1 translation is inefficient at baseline, and may, thus, be subjected to significant translational regulation. Of note, a recent study of hDGAT1 gene in Turkish women revealed sites of polymorphism in the 5′-UTR that appear to link to
the efficiencies of protein expression (60). These data are supportive of the importance of the 5′-UTR of the hDGAT1 gene in regulating hDGAT1 protein expression. Although the 3′-UTR of an mRNA may be important for RNA stability in general, it may also significantly modulate translation efficiency, as has been demonstrated by the presence of an AU-rich sequence in the 3′-UTRs of many cytokines and proto-oncogenes, capable of repressing the translation of mRNA molecules in which they are represented (61,62). We do not know if there are any elements in the 3′-UTR of hDGAT1 that may function to influence its translation efficiency, albeit no similar AU-rich sequence was found. The hypothesis that translational initiation control of hDGAT1 plays a significant role in protein expression will have to be formally tested in future experiments, in which both the 5′-UTR and the 3′-UTR may be systematically investigated under controlled experimental conditions; specific cis-acting elements may be identified and mapped with the use of a report gene (e.g., the CAT or luciferase gene) linked immediately downstream or upstream of the hDGAT1 5′-UTR or 3′-UTR, respectively.

Although DGAT1-deficient mice have less fat mass, they are not lipoatrophic (9) because of the redundancy in TG biosynthesis. Thus, even when one enzyme is permanently absent, adequate TG biosynthesis can still be sustained by another enzyme(s). TG homeostasis may, therefore, be regulated by controlling the expression and functions of the involved enzyme(s). In addition to transcriptional regulation, we have demonstrated a rigorous posttranscriptional mechanism that appears to control the protein level of DGAT1. This control is not absolute, and a significant amount of
fluctuations in protein expression can occur. Importantly, any changes in the level of DGAT1 protein appear to be effectively linked to metabolic changes. This has been demonstrated here with a >2-fold increase in TG accumulation secondary to ~2-fold increase in DGAT1 protein in 3T3-L1 adipocytes. This degree of increase in TG stores is certainly significant with regard to energy homeostasis. If such an increase were to occur in transgenic mice, the weight gain due to excessive fat accumulation would be substantial. Compared with the downward deviation in TG homeostasis, in which only about one third of normal TG stores is affected due to DGAT1 deficiency in the knockout mice (10), it appears that the upward deviation in adipocyte TG mass is relatively easier when DGAT1 protein level is increased. These phenomena may be explained by the evolutionary pressure on selection for mechanisms that ensure adequate TG store for survival and successful breeding, but less pressure on selection against over-storage of fat. Similar ideas have been suggested when the role of leptin in energy homeostasis is examined (cf. (63)).

The fact that increased protein levels of DGAT1 can result in substantial increases in TG mass in adipocytes may point to a potential role of this enzyme in the development of obesity. Further identification of mechanism(s) or potential molecular mediator(s) for posttranscriptional control of DGAT1 expression may allow us to develop therapeutic interventions to correct defects related to the activity of this enzyme. A transgenic mouse with adipose tissue-specific DGAT1 overexpression, which is being created in our
laboratory, would be a suitable animal model for such undertakings.
Acknowledgments

This work was supported by the Lucille P. Markey Research Fellowship from Columbia University, an Endocrine Fellow Foundation Grant, National Institutes of Health Grant K08 DK60530-01 (Y-H. Y), and National Institutes of Health Grants HL55638 and T32 HL07343 (H. N. G).
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Figure legends

Figure 1. Disparity of hDGAT1 expression in mRNA and in protein. 3T3-L1 cells were grown to confluence. Cells were either fully differentiated in an induction medium, or proceeded for viral transduction as preadipocytes. Mock treatment [(-)], or viral transduction with Ad-GFP (GFP) or with Ad-hDGAT1 (hDGAT1), was conducted in mature adipocyte (PID 10) or preadipocytes. On post-transduction day 3, RNA was extracted from one set of the cells for Northern analysis. Another set of cells was subjected to cell fractionation, and the membrane (Mb) and cytosol (Cyt) fractions were analyzed in Western blots. A. Northern blot: 15 µg RNA in each lane. B. Western blot. Cyt: 40 µg protein in each lane; Mb: 2 µg protein for mature adipocytes, and 20 µg protein for preadipocytes.

Figure 2. DGAT1 protein stability and its half-life. Fully differentiated 3T3-L1 adipocytes were transduced with either Ad-hDGAT1 or Ad-GFP (as indicated) on PID 10. Forty-eight hours after viral infection, cells were either subjected to ALLN treatment (100 ug/ml x 6 hours) followed by assessment of changes in steady-state levels of DGAT1 protein, or cycloheximide (CHX) treatment (250 µM) followed by assessment of protein half-lives of DGAT1 and GFP. A. Western Blot after ALLN treatment. B. Western Blot after CHX treatment.

Figure 3. Effects of DGAT1-recombinant adenoviruses on TG metabolism in 3T3-L1
cells. 3T3-L1 preadipocytes or fully differentiated cells were treated with recombinant adenoviral infection (or mock-infection) as described in Fig 1. Analyses were carried out 48 hours after viral transduction for DGAT activity (A) and TG biosynthesis (B), but 5 days after transduction for total TG mass determination (C). The results shown in this figure represent 3 experiments for each specimen. The differences between the DGAT1-overexpressing cells and the mock-infected, and Ad-GFP transduced cells, are statistically significant in each analysis. Asterisk, p<0.01.

**Figure 4. Intracellular TG turnover in differentiated 3T3-L1 cells infected with recombinant adenoviruses.** Mature 3T3-L1 adipocyte (PID 10) were infected with either Ad-GFP (GFP) or Ad-hDGAT1 (hDGAT1). Forty-eight hours after infection, cells were labeled with [3H]glycerol for 2 hours, washed and cultured in a chase medium. Lipids were extracted from separate sets of cells (3 for each specimen) immediately after labeling (0 hr), or 24 hr and 72 hr after chase. After TLC separation, the level of [3H]TG was quantified by scintillation counting. The difference between hDGAT1 and GFP was not significant statistically at each point (p>0.05).

**Figure 5. The effects of mutation at the conserved tyrosine-phosphorylation site in hDGAT1 on gene expression, enzymatic activity and cellular TG accumulation.** A. DNA sequence of the wild-type hDGAT1 and T316F, showing the point mutation converting Tyr316 to Phe. B. Northern blot: 15 µg RNA each. Cells were viral transduced on PID
10, and assays were carried out 2 days after infection. C. Western blot: 2 µg protein each from the cytosol (Cyt) or membrane (Mb) fraction. Cells as in B. D. Microsomal DGAT activity assays at either 150 mM MgCl₂ or 10 mM MgCl₂ as indicated. Cells as in B. E. Total cellular TG mass. Cells as in B, except that assays were done 5 days after viral transduction. Asterisk, p<0.01; double asterisks, p<0.05.

**Figure 6. The cell morphology and the differentiation status of 3T3-L1 adipocytes with or without Ad-hDGAT1 transduction.** Viral transduction with Ad-hDGAT1 (hDGAT1) or Ad-GFP (GFP), or mock-treatment was carried out in 3T3-L1 cells either on post-induction day 4 (PID 4) or on post-induction day 10 (PID 10). In each case, analyses were performed on post-induction day 12. Preadipocytes were used as controls. A. Oil-red-O staining: a, preadipocytes; b-c, adipocytes with mock-treatment, Ad-hDGAT1 transduction on PID 4, and Ad-hDGAT1 transduction on PID 10, respectively. B. Northern blot for DGAT1 (probes with sequence homology for both hDGAT1 and mDGAT1), LPL, PPAR-γ and aP2. The 28S and 18S rRNA are shown in the bottom panels for loading controls.
Figure A demonstrates the expression of GFP and hDGAT1 under two conditions: - ALLN and + ALLN. The lanes labeled 'a' and 'b' show the expression in the absence of ALLN, while 'c' and 'd' show the expression in the presence of ALLN. The arrow indicates the location of DGAT1.

Figure B illustrates the expression of Ad-hDGAT1 and Ad-GFP over time post CHX treatment. The lanes are labeled '0', '0.5', '4', '10', and '36' corresponding to different time points. The arrow indicates the location of DGAT1 and GFP.
A. Microsomal DGAT1 activity:

- **a** Preadipocytes
  - TG formed (Pmol/min/mg protein)
  - ( ) GFP hDGAT1

- **b** Mature adipocytes
  - ( ) GFP hDGAT1

B. Cellular TG biosynthetic rate:

- ( ) GFP hDGAT1

C. Cellular TG accumulation:

- ( ) GFP hDGAT1
**A**

Wild-type

| Tyr 316 | hDGAT1 | T316F |
|---------|--------|-------|
| GGACATGGACTTACTCAGCATCATCGAGCGCCTCCTGAAGC | GGACATGGACTTTCTCAGCATCATCGAGCGCCTCCTGAAGC |
| Phe | T316F |

**B Northern blot**

- **DGAT1**
  - a, b, c

**C Western blot**

- DGAT1
  - a, b, c
- Cyt
- Mb

**D Microsomal DGAT activity**

- **150 mM MgCl₂**
  - GFP
  - hDGAT1
  - T316F

- **10 mM MgCl₂**
  - GFP
  - hDGAT1
  - T316F

**E Cellular TG accumulation**

- Intracellular TG mass
  - GFP
  - hDGAT1
  - T316F

*Significant differences*
Posttranscriptional control of the expression and function of acyl-CoA:diacylglycerol acyltransferase-1 in mouse adipocytes
Yi-Hao Yu, Yiying Zhang, Peter Oelkers, Stephen L. Sturley, Daniel J. Rader and Henry N. Ginsberg

J. Biol. Chem. published online October 28, 2002

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