Ectopic accumulation of lipid droplets in non-adipose tissues correlates with the degree of insulin resistance in these tissues. Emerging evidence indicates that lipid droplets are specialized organelles that participate in lipid metabolism and intracellular trafficking. These properties are thought to derive from the lipid droplet-associated PAT protein family (perilipin, ADFP, and Tip47). The functions of the ubiquitously distributed adipose differentiation-related protein (ADFP) and Tip47 remain unknown. To evaluate the roles of ADFP and Tip47 in lipid biogenesis and metabolism, ADFP null and wt mice, respectively. In ADFP null cells, Tip47 was identified as the sole lipid droplet-associated protein from the PAT family by mass spectroscopy, which was further confirmed by immunoblotting and immunocytochemistry. Following incubation with oleic acid, ADFP null cells were able to form lipid droplets to the same extent as wt cells. No statistical differences between the two cell types were observed in NEFA uptake or lipolysis. Small interference RNAs (siRNAs) against Tip47 were found to down-regulate protein levels for Tip47 by 85%. ADFP null cells treated with Tip47 siRNA retained the ability to form lipid droplets but to a lesser extent and shunted the utilization of exogenously added NEFA from triglycerides to phospholipids.

Ectopic fat deposition, the accumulation of lipids in lipid droplets in tissues other than adipose, develops in obese patients and is now recognized as a strong prognostic factor for the development of metabolic syndrome (1–3). The molecular mechanisms regulating the formation and metabolism of lipid droplets in non-adipose tissues and their dysfunction in pathophysiological states are not well understood. Recent proteomic studies indicate that lipid droplets are surrounded by a protein coat that provides an interface for lipid metabolic processes, including transport, lipogenesis, and lipolysis (4–8). Even more importantly, these studies identify a proteome “signature” for lipid droplets that consistently includes at least one member of the PAT protein family (originally named for perilipin, ADFP and Tip47). A PAT protein is always present and generally represents the most abundant lipid droplet protein, suggesting at least an important structural role for this class of proteins in lipid droplet machinery.

The mammalian PAT family includes five members: perilipin, ADFP, tail interacting protein of 47 kDa (Tip47), S3-12, and PAT-1 (9). The PAT proteins are defined by primary sequence homology and are well conserved within the family and across species (10). Recent proteomic studies revealed heterogeneity and tissue-specific differences in droplet-associated proteins (4–8). PAT protein distribution is clearly tissue-dependent. Perilipin and S3-12 are confined to adipose and steroidogenic tissues, while ADFP and Tip47 are ubiquitously distributed (11–14). To date, a functional role regulating lipolysis has been shown for perilipin, the principal lipid droplet protein in adipose cells, mainly from studies of the perilipin null mouse that exhibits a lean phenotype (15, 16), but little is known about the function of the other PAT proteins, including the proteins associated with lipid droplets in non-adipogenic tissues, such as ADFP and Tip47.

ADFP has long been identified for association with the lipid droplet surface (13, 17). Discovered by Serrero and co-workers (18) as an early gene of adipocyte differentiation, the ADFP
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protein occurs only transiently in adipocytes. ADFP is present at the surface of nascent lipid droplets in early stages of adipocyte differentiation but reduced dramatically after the onset of perilipin expression, whereas ADFP mRNA remains elevated. In mature adipocytes ADFP is replaced by perilipin as the major lipid droplet protein. Thus it is not surprising that adipocyte lipid droplets in perilipin null mice are coated with ADFP. However, while adipocyte differentiation in perilipin null mice is not compromised (15, 16), the protein kinase A regulation of lipolysis is lost (16). Despite its strong primary sequence homology to perilipin, ADFP is not phosphorylated by protein kinase A and ADFP does not supplant the protein kinase A-regulated function of perilipin (16, 19).

Despite numerous attempts, we have failed to detect any effect of overexpression of ADFP mRNA on lipid metabolism in various types of cells. On the other hand, we have reported that protection of ADFP from proteosomal degradation with the inhibitor, MG-132, is accompanied by a substantial increase (170%) in lipid content compared with control cells untreated with MG-132, suggesting that ADFP, like perilipin, protects neutral lipids from degradation by lipases (20, 21).

A recent addition to the pool of known lipid droplet surface proteins is Tip47. This member of the PAT family was first identified as a possible cargo protein involved in the trafficking of the mannose-6-phosphate receptor and mainly studied as such (22). Tip47 is also known as placental protein pp17b (23) and closely related in primary sequence to ADFP (40%), prompting studies looking for its subcellular localization on lipid droplets (10, 14). Indeed, Tip47 was found to have increased association with the fat cake fraction when HeLa cells were incubated with oleic acid. Subsequently, its localization at the lipid droplet surface was confirmed in various types of cells (23, 24). Tip47, like ADFP, is widely distributed among non-adipogenic tissues but unlike both perilipin and ADFP; it is present in both lipid droplet and cytosolic compartments. This unique property of solubility among the PAT proteins permitted the solution crystal structure of the COOH-terminal portion of Tip47 (25). ADFP null mice exhibit few phenotypic alterations, except for mild resistance to high fat diet-induced fatty liver (26). We report herein that lipid droplets in fibroblastic cells derived from ADFP null mice are coated with Tip47. Using these ADFP null MEF cells, we show that knockdown of Tip47 in these cells with siRNA decreased lipid storage, demonstrating the critical role of PAT proteins in the packaging and storage of neutral lipids in non-adipose cells.

EXPERIMENTAL PROCEDURES

Establishment of Fibroblastic Cell Lines from knock-out and wt Mice—Embryonic cells from ADFP null and wt mice were obtained from 16-day-old embryos, and primary cultures were obtained as described previously (19). To generate immortalized fibroblastic cell lines from these embryos, we followed the method used originally to produce the 3T3-L1 cell line (27). Trypsin digest of seven ADFP null and seven wt embryos were plated in individual T-175-cm² flasks with Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose supplemented with 10% calf serum (Invitrogen), 100 µg/ml streptomycin, and 100 units/ml penicillin (Biofluids, Inc.). Cells were trypsinized every 3rd day for 3 months consecutively for a total of 30 repetitions. Cells divided actively during the first month in culture and then entered a latent phase. Some cells became spontaneously immortalized and began to actively grow again. Several such clonal populations of immortalized cells obtained from ADFP null and wt cultures were frozen in liquid nitrogen and constituted the established ADFP null and wt cell lines used in the present study.

Cell Lines and Cell Culture Conditions—wt and ADFP null fibroblastic cells were studied in parallel. For siRNA experiments, cells were plated in 6- or 24-multiwell dishes (Costar) and later used for metabolic studies, immunoblot and protein assays. For immunocytochemical studies, cells were plated on Petri dishes containing glass bottoms (MatTek Corp.) or 4–8-well chamber slides (Labtech). For isolation of lipid droplets by subcellular fractionation, cells were grown in 100 × 20-mm dishes (Costar). CHO-K1 cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum (HyClone), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Biofluids, Inc.). Clones of CHO-K1 cells overexpressing Tip47-GFP fusion protein or ADFP-GFP fusion protein were selected in the presence of 600 µg/ml G418. For cellular lipidolysis or lipogenesis studies, cells were plated in 24-multiwell dishes (Startest) at a concentration of 1 × 10⁴ cells/well. All cells were cultured in a 5% CO₂ atmosphere at 37 °C.

Oligonucleotides—siRNAs were designed and purchased from Qiagen (HP guaranteed siRNA), and transfection methods for fibroblastic cell lines were used according to the manufacturer’s instructions. The ability to down-regulate Tip47 was tested in both ADFP null and wt cells. Positive siRNAs for mitogen-activated protein kinase-1 and a rhodamine fluorescent oligonucleotide (Qiagen) were used to help establish efficient conditions for transfection.

siRNA Construction and Transfection—The siRNAs directed against mouse Tip47 cargo protein were designed by Qiagen. Cells were plated in antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum at a concentration of 1 × 10⁴ cells per well in a 24-multiwell dish. During the next 2 days, cells were transfected with siRNAs (20 nM) using RNAiFect (Qiagen) according to the manufacturer’s instructions. Among six siRNAs for our target gene, the sequences specific for Tip47 cargo protein sens (5’-AACAGCACA-GAGAAUGAGGAG-3’) and sens (5’GAGAAUGAGGAGUAUUAA-3’) and were selected based upon their potency to inhibit target gene expression. Equal amounts of two positive siRNAs were used. An ineffective siRNA was used as a negative control, sens (5’-GGCGUGUCCAAUUGGUGUUAUAUA-3’). For the lipogenesis experiments, wt and ADFP cells were transfected using Hyperfect (Qiagen) according to the manufacturer’s instructions, cells were plated at a concentration of 1 × 10⁴ cells per well with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a 24-multiwell dish, and transfection with siRNA was performed the next day. In all experiments, cells were used for Western blotting and immunocytochemistry on day 5 after plating unless otherwise stated, since preliminary experiments have shown that day 5 was opti-
mal for Tip47 protein down-regulation (effect of Tip47 siRNA measured by Western blotting was decreased after day 7 post-plating).

Full-length Tip47 and ADFP cDNA constructs were fused in-frame with the 3' end of eGFP in pEGFP-C2 (Clontech) as described previously (10). CHO cells were transfected with Lipofectamine, using either ADFP-GFP or Tip47-GFP fusion protein constructs. Clonal cell lines were established as described previously (19). Briefly, immediately following infection, CHO cells were plated under selection in 600 μg/ml G418, and cells after 8 days in selection medium were re-plated in 96-multiwells at a concentration of 2 cells/ml. Positive “green” clonal cells were identified by microscopy and further expanded.

Fat Cake Preparation—Cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS), scraped into PBS, and pelleted by low speed centrifugation. Lipid droplet isolation was performed as described (14).

Antibodies and Fluorescent Dyes—Antibodies used include the following: anti-Tip47, which was raised in rabbits against the COOH-terminal ~200 amino acids of murine Tip47, and anti-ADFP, which was raised in rabbits against a peptide comprising the 26 amino-terminal amino acids of murine ADFP. Antibody specificity was confirmed with the use of two clonal CHO-K1 cell lines, stably transfected with the pEGFP-C2 vector containing coding murine ADFP and Tip47 sequences. The presence of ADFP and Tip47 in fat cake or total cell extract was analyzed by immunoblotting by loading 20 μg of total protein per lane onto 4–12% BisTris gradient mini gels (NuPAGE, Invitrogen). Immunostaining revealed that the ADFP and Tip47 antibodies do not cross-react (supplemental Fig. 1). Bodipy 558/568 and Bodipy 493/503 (Invitrogen) were used to visualize intracellular neutral lipid droplets. Alexa Fluor 488 and Alexa Fluor 594-conjugated donkey anti-rabbit (1:1000 dilution) and Alexa Fluor 594-conjugated donkey anti-goat (1:500 dilution) were used in different combinations and dilutions as primary antibodies. Spots were scraped and radioactivity determined.

RESULTS

Cellular Lipolysis and Lipogenesis—Lipolysis was performed according to Tansey et al. (16) and Szتلryd et al. (19) where re-esterification of fatty acids in CHO-K1 cells was largely prevented by the inclusion of 5 μM triacsin C (Biomol, Plymouth Meeting, PA), an inhibitor of acyl-coenzyme A synthetase, in the medium. Given our earlier published methods, and published work from another laboratory using this level of triacsin C to block fatty acid esterification into triglycerides (TAGs) (16, 19, 38), 5 μM triacsin C was used without confirmatory measurements that re-esterification and TAG synthesis were suppressed. Triplicate wells were tested for each condition.

Lipid Extraction and Thin Layer Chromatography—The cell monolayer was washed with ice-cold PBS and scraped into 1 ml of PBS. The lipids were extracted by the method of Bligh and Dyer (28) in the presence of an internal neutral lipid standard mix containing 10 μg each of monolein, diolein, triolein, free cholesterol, oleic acid, and cholesteryl oleate (Supelco) for band identification. Thin layer chromatography extractions were performed as described previously (29, 30). In some experiments, the phospholipid spot remaining at the origin in our solvent system was scraped and re-extracted twice with chloroform methanol (1/2) and dried down under nitrogen before being kept at ~80 °C. Polar lipid species were separated on silica gel G (Analtech) plates using a one-dimensional double-development procedure from Igal et al. (38). Briefly, phospholipids were separated in a one-dimensional solvent system. First, the plate was developed with chloroform-methanol-30% ammonium hydroxide-H2O (70:25:3.5:1.5, v/v) to 3 cm from the top. The residual solvents on the plate were evaporated, and the plate was rerun in the same direction in chloroform:methanol:glacial acetic acid:H2O (80:10:2:0.75, v/v). Pure lipid standards: phosphatidylcholine, phophoinositol, and phosphoethanolamine were run in parallel (Avanti lipids) and were added to the samples 10 μg each for identification purpose. Spots were scraped and radioactivity determined.

Statistical Analysis—Statistical significance was tested using a one-way analysis of variance analysis (GraphPad Software Inc.) or a two-tailed Student’s t test.

RESULTS

Tip47 Is the Predominant PAT Protein on Lipid Droplets of Fibroblasts of ADFP Null Cells—The minimal phenotype in ADFP null mice suggested the presence of a compensatory PAT protein at the surface of the lipid droplet. To test for such a possibility, we isolated fat cakes from primary cultures of MEF cells grown in the presence of NEFA and submitted the resulting fat cake to an alkali carbonate wash, pH 10, to eliminate a number of proteins loosely associated with the surface of the lipid droplet (14). After SDS-PAGE, the most abundant band was analyzed by mass spectroscopy, which identified 14 peptides comprising 51% of the 437 amino acids of murine Tip47 (Fig. 1). Mass spectrometry also revealed that the second most abundant band, which migrated near the 45-kDa marker, was vimentin.

Up-regulation of Tip47 in ADFP Null MEFs—To explore the possibility of compensatory mechanisms regulating lipid droplet formation in non-adipogenic cells and their functional consequences, we established MEF cell lines from 16-day embryos...
of ADFP null and wt mice, as described under “Experimental Procedures.” Immunofluorescent staining and immunoblot analysis (Fig. 2) confirmed the absence of ADFP in established ADFP null cell lines. More importantly, Bodipy staining of neutral lipids clearly indicated that ADFP null cells could accumulate lipid droplets in response to fatty acid loading. With the use of our recently developed Tip47 antibody (see “Experimental Procedures” and supplemental Fig. 1), further analyses showed higher expression of Tip47 mRNA and protein in the ADFP null than wt cells (Fig. 3). Furthermore, immunofluorescence staining showed that Tip47 coated the lipid droplets of ADFP null cells (Fig. 4). In addition, we confirmed the higher expression of Tip47 protein in liver fat extracts from ADFP null mice compared with wt mice (Fig. 5).

**Lipid Metabolism in Fibroblastic Cell Lines from wt and ADFP Null Embryos**—To determine whether the replacement of ADFP on the lipid droplets with Tip47 altered lipid metabolism, both lipolysis and NEFA uptake were compared in wt and ADFP null cells (Fig. 6). To measure lipolytic activity, cells were loaded with $[^{3}H]$oleic acid as described previously (29, 30), after which the efflux of $[^{3}H]$oleic acid to the medium was monitored. Triacsin C was included in all incubations during the efflux phase to prevent re-utilization of fatty acids released from hydrolyzed triglycerides (TAG). Replacement of ADFP with Tip47 in the ADFP null cells did not affect their ability to release NEFA (Fig. 6A). Furthermore, there was no significant difference in the amount of oleic acid incorporated into the lipids (Fig. 6B). The absence of ADFP did not compromise the ability of the ADFP null cells to accumulate lipids, even in the presence of exogenous lipids. Thus, no metabolic differences were found between ADFP null and wt cells as measured by lipolysis and NEFA uptake. It is possible that the presence of Tip47 at the lipid droplet surface may be a com-
compensatory mechanism that accounts for the lack of a clear phenotype in the ADFP null mice. These experiments raise important questions: what will be the phenotype of fibroblastic cells in the absence of both PAT proteins, ADFP and Tip47, and will such cells be able to form lipid droplets?

**FIGURE 4.** Tip47 is localized to the lipid droplet surface of ADFP null cells. **Upper panels,** co-immuno-staining with Tip47 and Bodipy 568 of ADFP null cells. Cells were stained with polyclonal anti-Tip47 antibody and Alexa Fluor 488-conjugated secondary antibody. Bodipy preferentially stained neutral lipid. Fluorescent and phase images were generated by LSM510 confocal laser microscopy; bar: 10 μm. **Lower panels,** subcellular lipid clusters; bar: 1 μm.

**FIGURE 5.** Absence of ADFP results in increased Tip47 protein at the lipid droplet in ADFP null mice liver. Immunoblots of fat cakes from liver extracts from four ADFP null and four wt mice fed a high fat diet were performed.

**FIGURE 6.** Lipolysis and total NEFA uptake into lipids are not compromised in ADFP null cells. **A,** lipolysis of wt cells (filled square) and two ADFP null cell lines (empty symbols). Cells were loaded overnight with 400 μM [3H]oleic acid, and the efflux of [3H]oleic acid was tracked over 3 h in the presence of 5 μM triacsin C. Values represent the mean ± S.E. of triplicate determinations of cpm released per μg of protein at times 0, 15, 30, 60, 120, and 180 min. Values are from three separate experiments in which incubations were performed in quadruplicate (n = 12). **B,** NEFA incorporation into total lipids after 1-h incubation with 400 μM [3H]oleic acid. Data are mean ± S.E. from five separate experiments; each experiment was performed in quadruplicate (n = 20).

Down-regulation of Tip47 in ADFP Null Cells: Decreased Lipid Droplet Formation and Altered Lipid Metabolism ～To establish siRNA conditions for modifying PAT protein expression in our MEF cell lines, we first tested a positive control siRNA for mitogen-activated protein kinase-1 in both wt and the ADFP null cell lines (supplemental Fig. 2). With the use of optimized transfection conditions, we tested siRNA for Tip47 obtained from Qiagen’s siRNA design algorithm. We identified three active siRNA sequences (of eight tested, supplemental Fig. 3). For all subsequent experiments we used an equal mix of siRNA 5 and siRNA 6. Fig. 7A shows the down-regulation of Tip47 mRNA in ADFP null cells, and Fig. 7B reveals the loss of Tip47 protein in the same cells. As judged by the unchanged levels of actin, the siRNA transfection protocol imposed minimal cellular toxicity.

Down-regulation of Tip47 by siRNA reduced the number of lipid droplets formed after 12 h of incubation with 400 μM oleic acid (Fig. 7C). Although both ADFP and Tip47 were lacking or reduced, these cells continued to accumulate some lipid droplets as evidenced by Bodipy neutral lipid staining. Lipid droplets in ADFP null cells did not appear to differ in size from those in control cells, but most lipid droplets lacking both Tip47 and ADFP at their lipid droplet surface appear as a single droplet (supplemental Fig. 4). To confirm the observation of reduced lipid droplet accumulation in the absence of both ADFP and Tip47, we determined the metabolic fate of exogenous NEFA. ADFP null cells treated with Tip47 and control siRNA were exposed to 400 μM oleic acid, supplemented with a trace of [3H]-labeled oleic acid. Total lipids were extracted after 1, 6, and 12 h, and TLC was used to separate NEFA, monoacylglycerides, diacylglycerides, triglycerides (TAG), and phospholipids (29, 30). No statistical difference was observed in NEFA uptake into total lipids (Fig. 8), but the distribution of [3H] oleic acid among the phospholipid and TAG fractions was altered by siRNA inhibition of Tip47 (Fig. 8 and supplemental Table 1).

By 12 h, cells lacking both ADFP and Tip47 accumulated nearly 40% less TAG, leading to the conclusion that in the absence of both ADFP and Tip47, metabolism of NEFA storage shifted from the TAG to the phospholipid fraction. This suggests that Tip47 helps direct influx of NEFA to incorporation in TAG. In addition, we examined whether lack of Tip47 could perturb the composition of phospholipids species but found that both ADFP null cells treated with control siRNA or Tip47 siRNA preferentially incorporated oleic acid in the phosphatidylcholine fraction (supplemental Table 2). Tip47 siRNA increased the lipolysis of newly synthesized TAG (determined in the presence of triacsin C) only at early time points, 30 min and 1 h (Fig. 9).

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FIGURE 8. Top panel, NEFA uptake into total cellular lipids is not affected in cells lacking both ADFP and Tip47. wt cells and ADFP null cells were transfected with negative control siRNA (open and hatched squares, respectively) or ADFP null cells were transfected with Tip47 siRNA (solid squares) and 4 days later exposed overnight to 400 μM oleic acid. The following day cells were fixed and co-stained with Bodipy 568, a neutral lipid stain (red), and Tip47 antibody (green). Fluorescent images were generated by LSM510 confocal laser microscopy. Bar: 10 μm. This experiment was performed three times; a representative picture for each experiment is shown.

DISCUSSION

In this paper, we demonstrate that fibroblastic cells lacking ADFP retain the ability to accumulate lipids in intracellular lipid droplets. ADFP null cells do not differ from wt cells in their ability to process lipids via fatty acid uptake, lipogenesis, and lipolysis. The up-regulation of another PAT protein, Tip47, was able to mainly compensate for the loss of ADFP. Down-regulation of Tip47 in ADFP null cell line by siRNA did not affect total lipid synthesis but shifted the utilization of [3H]oleate away from TAG toward phospholipids. The presence of only Tip47 or mostly ADFP at the surface of the lipid droplet did not appear to affect the apparent size or number of lipid droplets in this fibroblastic cell culture system which suggests that ADFP and Tip47 have similar abilities to package lipids.

ADFP is always found at the lipid droplet surface and serves as a convenient marker for non-adipogenic fat depositions (31). However, its role in non-adipogenic tissues has been difficult to characterize.

Numerous hypothetical roles have been proposed for ADFP in non-adipogenic cells. First, it was suggested that ADFP increases cellular NEFA uptake. ADFP overexpression in COS-7 cells leads to increased uptake of NEFA and the atypical presence of ADFP at the plasma membrane (32). By contrast, subsequent experiments in all other types of cells have found ADFP exclusively associated with the lipid droplet (13). Evidence has been reported that ADFP can bind long chain NEFA and it has been postulated that the protein recruits NEFA to the lipid droplets to be rapidly processed into TAG (32). Furthermore, like perilipin, ADFP was suggested to protect against lipolysis but in non-adipogenic tissue (33).

In mammary epithelial cells, it may participate in the formation and secretion of milk lipid droplets (17) or in transporting TAG to supply NEFA to type II epithelial cells for the production of lung surfactant.
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Fragmentary data implicate ADFP with the formation of ectopic fat deposits that occur with insulin resistance in liver and muscle. Indeed, ADFP was found to be up-regulated in steatotic livers from dyslipotrophic AZIP mice (35) and from obese mice (36), and it has been suggested that ADFP plays a role in the pathology of non-alcohol mediated fatty liver disease (36). More recently, in mammalian cells and insect cells it was proposed that ADFP and other PAT proteins may play a role in moving lipid droplets within the cytosolic compartment via binding to motor proteins (37). Unfortunately, in our fibroblastic cellular system, ADFP loss of function did not result in a defined phenotype.

Importantly, the present data seem to indicate that the lack of phenotype in ADFP null cells may be explained by the compensatory overexpression at the surface of the lipid droplet of another PAT protein, Tip47. First, isolated lipid droplets from primary cultured fibroblasts from ADFP null mice revealed that the predominant protein at the surface of the lipid droplet was Tip47, suggesting a functional compensation. ADFP and Tip47 are the two most closely related mammalian PAT proteins (9). In addition, we also observed both increased Tip47 levels for mRNA and protein in cellular fat cake extracts from ADFP null fibroblastic cells, when compared with the wt cells.

It has been previously reported that Tip47 behaves differently than ADFP in that Tip47 is able to exist both in the cytosol as well as at the surface of the lipid droplet (10, 14, 24). ADFP mainly targets the lipid droplet surface, and unbound ADFP protein is rapidly degraded (20). These differences in lipid droplet binding characteristics and/or stability in aqueous environment may be responsible for the lack of Tip47 overexpression observed in the ADFP null mouse tissues studied (26). Indeed, Tip47 Western blots in the earlier study were performed using total cellular homogenate and not protein extracts from isolated fat cake. This interpretation is further supported by the presence of increased amount of Tip47 in ADFP null liver fat cake. Two manuscripts are now reporting a fifth member of the PAT family protein (10, 39), which is specifically expressed in oxidizing tissues. This fifth PAT protein is highly expressed in heart and red muscle and induced in fasting liver. We have checked by Western blotting that this protein was not expressed in the ADFP null fibroblasts (results not shown), but we predict that it will be overexpressed in heart, muscle, and in fasted liver of the ADFP null mice, as a compensatory mechanism. By analyzing what has been so far published in the field of PAT proteins, it is becoming increasingly clear that the PAT protein family function to enhance the lipid droplet formation by protecting it against lipolysis. The variety of existing PAT proteins, five to date, may therefore reflect the lipid metabolism utilization of a particular cell type. Fibroblastic cells are not particularly adapted to oxidize NEFA or to store them, but in cell culture, they use NEFA to build essentially membranes and among the PAT proteins only Tip47 and ADFP are necessary to coat the lipid droplets. Muscle, liver, and heart utilize NEFA for oxidation and may require at various times a different protein coating at the surface of the lipid droplets, and finally adipose tissue, which is the major organ for lipid storage, expresses specifically perilipin, tightly regulating the flux of NEFA. In conclusion, the NEFA utilization and storage requirements in specific cell types may dictate the type of the PAT protein coating on the lipid droplet.

Our data further support the role of Tip47 in lipid droplet metabolism and lead us to hypothesize that in absence of ADFP, Tip47 may compensate functionally for ADFP and that these proteins provide redundancy in metabolic pathways. The presence of Tip47 or ADFP at the surface of the lipid droplet did not appear to affect the apparent size or number of lipid droplets in this fibroblastic cell culture system which suggests that ADFP and Tip47 have similar abilities to package lipids.

It could also be hypothesized that corresponding to what has been described in perilipin null mice adipocytes (i.e. in the absence of perilipin, ADFP re-appears on the droplets) in non-adipogenic cells in the absence of ADFP, Tip47 appears on the droplets. These observations may reflect a hierarchy in the lipid droplet affinities of the various PAT proteins, from perilipin, the most tightly bound to the droplet, to ADFP and finally Tip47.

It was suggested that in adipogenic 3T3-L1 cells, lipid droplet coating will vary as a function of time and subcellular location (24); we demonstrate that in the absence of ADFP, Tip47 will remain at the lipid droplet surface in non adipogenic cells, independently of time and subcellular location.

Our data support strongly the hypothesis that Tip47, in the absence of ADFP, plays an important role in packaging lipids. However, it is important to note that our experimental procedures prevented us from following the kinetics of lipid droplet formation and that our present data might be hampered by the stability of the PAT protein when bound to the surface of the lipid droplet. Indeed to obtain maximum inhibition of Tip47, cells have to be studied after 5 days of plating, and such an experimental procedure prevents us from following the fate of the lipid droplets into a specific cell during that time. Hence, we do not know if the observed lipid droplets observed at day 5 in cells lacking both ADFP and Tip47 are actually droplets that were formed at a time when Tip47 was still binding to the droplet, and then by the day 5 time point, Tip47 disappeared from the lipid droplet, leaving intact the already formed droplet. It is also possible that nascent lipid droplets fused together during these 5 days, and the resultant observed droplets are just trying to offer minimum surface area to maintain themselves in the cytosol.

In conclusion, the data herein reveal that fibroblastic cells derived from ADFP null mice are still able to form lipid droplets and that their ability for FA uptake and lipolysis remains unchanged as Tip47 becomes the most abundant protein at the surface of the lipid droplet. Lack of both ADFP and Tip47, however, resulted in altered lipid metabolism. Cells retained the ability to form lipid droplets but to a much lesser extent and shunted the utilization of exogenously added NEFA from triglycerides to phospholipids, the mechanism for such a switch remaining unknown. These data support the hypothesis that Tip47 plays an important role in lipid metabolism.

Our results validate the use of siRNA to inhibit expression of PAT family proteins and induce a loss of function phenotype in

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3 ADFP null adipocytes (~2K Dalen, T. Dahl, E. Holter, B. Arntsen, C. Londos, C. Sztalryd and H. I. Nebb, submitted for publication.)
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a non-adipogenic cell type. Moreover, they have begun to give information about the role of Tip47 in lipid droplet formation in a fibroblastic cell. Our data suggest that in pathophysiological states such as obesity and diabetes when the supply of fatty acids is high, the relative abundance of Tip47, ADFP, and now Gavrilova, O., Reitman, M. L., Deng, C. X., Li, C., Kimmel, A. R., and Londos, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6494–6499

17. Heid, H. W., Schoenholzer, M., and Keenan, T. W. (1996) Biochem. J. 320, 1025–1030

18. Jiang, H. P., and Serrero, G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7856–7860

19. Sztalryd, C., Xu, G., Dorward, H., Tansey, J. T., Conterras, J. A., Kimmel, A. R., and Londos, C. (2003) J. Cell Biol. 161, 1093–1103

20. Xu, G., Sztalryd, C., Lu, X., Tansey, J., Gan, J., Dorward, H., Kimmel, A. R., and Londos, C. (2005) J. Biol. Chem. 280, 42841–42847

21. Xu, G., Sztalryd, C., and Londos, C. (2006) Biochim. Biophys. Acta 1761, 83–90

22. Diaz, E., and Pfeffer, S. R. (1998) Cell 93, 433–443

23. Than, N. G., Sumegi, B., Kelly, S., Berk, S., Szekeres, G., Janaky, T., Szigeti, A., Bohn, H., and Than, G. N. (2003) Eur. J. Biochem. 270, 1176–1188

24. Wolinski, N. E., Quanier, B. K., Skinner, J. R., Schoenfish, M. J., Tzekov, A., and Bickel, P. E. (2005) J. Biol. Chem. 280, 19146–19155

25. Hickenbottom, S. J., Kimmel, A. R., Londos, C., and Hurley, J. H. (2004) Structure (Camb.) 12, 1199–1207

26. Chang, B. H., Li, L., Paul, A., Taniguchi, S., Nannegari, V., Heid, W. C., and Chan, L. (2006) Mol. Cell. Biol. 26, 1063–1076

27. Green, H., and Kehinde, O. (1975) J. Cell Sci. 11, 2601–2611

28. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917

29. Sztalryd, C., Levacher, C., and Picon, L. (1989) Cell. Mol. Biol. 35, 81–88

30. Tansey, J. T., Huml, A. M., Vogt, R., Davis, K. E., Jones, J. M., Fraser, K. A., Brasaemle, D. L., Kimmel, A. R., and Londos, C. (2003) J. Biol. Chem. 278, 8401–8406

31. Mishra, M., Emanipator, S. N., Miller, C., Kern, T., and Simonson, M. S. (2004) Am. J. Physiol. 286, F913–F921

32. Gao, J., and Serrero, G. (1999) J. Biol. Chem. 274, 16825–16830

33. Phillips, S. A., Choe, C. C., Ciarelli, T. P., Greenberg, A. S., Kono, A. P., Baxi, S. C., Christiansen, L., Mudalia, S. R., and Henry, R. R. (2005) Obes. Res. 13, 1321–1329

34. Schultz, C. J., Torres, E., Londos, C., and Torday, J. S. (2002) Am. J. Physiol. 283, 1288–1296

35. Gavrilova, O., Haluzik, M., Matsusue, K., Cutsum, J. J., Johnson, L., Dietz, K. R., Nicol, C. J., Vinson, C., Gonzalez, F., and Reitman, M. L. (2003) J. Biol. Chem. 278, 34268–34276

36. Schadinger, S. E., Bucher, N. L., Schreiber, B. M., and Farmer, S. R. (2005) Am. J. Physiol. 288, E1195–E1205

37. Martin, S., Driessen, K., Nixon, S. J., Zerial, M., and Parton, R. G. (2005) J. Biol. Chem. 280, 42325–42335

38. Igal, R. A., and Coleman, R. A. (1996) J. Biol. Chem. 271, 16644–16651

39. Yamagushi, T., Matsushita, S., Motojima, K., Hirose, F., and Osumi, T. (2006) J. Biol. Chem. 281, 14232–14240

REFERENCES

1. Meigs, J. B. (2002) Am. J. Manag. Care 8, S283–SS292

2. Eckel, R. H., Grundy, S. M., and Zimmet, P. Z. (2005) Lancet 365, 1415–1428

3. Shulman, G. I. (2000) J. Clin. Invest. 106, 171–176

4. Brasaemle, D. L., Dolios, G., Shapiro, L., and Wang, R. (2004) J. Biol. Chem. 279, 46835–46842

5. Liu, P., Ying, Y., Zhao, Y., Munday, D. I., Zhu, M., and Anderson, R. G. W. (2004) J. Biol. Chem. 279, 3767–3792

6. Wu, C. C., Howell, K. E., Neville, M. C., Yates, J. R., III, and McManaman, J. L. (2000) Electrophoresis 16, 3470–3482

7. Fujimoto, Y., Itabe, H., Sakai, J., Makita, M., Noda, J., Mori, M., Higashi, Y., Kojima, S., and Takano, T. (2004) Biochim. Biophys. Acta 1644, 47–59

8. Ozeki, S., Cheng, J., Tauchi-Sato, K., Hatano, N., Taniguchi, H., and Fujimoto, T. (2005) J. Cell Sci. 118, 2601–2611

9. Lu, X., Gruia-Gray, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., London, C., and Kimmel, A. R. (2001) Mamm. Genome 9, 741–749

10. Miura, S., Gao, J. W., Bzdostowski, J., Parisi, M. J., Schultz, C. J., London, C., Oliver, B., and Kimmel, A. R. (2002) J. Biol. Chem. 277, 32253–32257

11. Blanchette-Mackie, E. J., Dwyer, N. K., Barber, T., Coxey, R. A., Takeda, T., Rondinone, C. M., Theodorakis, J. L., Greenberg, A. S., and London, C. (1995) J. Lipid Res. 6, 1211–1226

12. Servetnick, D. A., Brasaemle, D. L., Gruia-Gray, J., Kimmel, A. R., Wolf, J., and London, C. (1995) J. Biol. Chem. 270, 16965–16973

13. Brasaemle, D. L., Barber, T., Wolins, N. E., Serrero, G., Blanchette-Mackie, E. J., and London, C. (1997) J. Lipid Res. 38, 2249–2263

14. Wolins, N. E., Rubin, B. O., and Brasaemle, D. L. (2001) J. Biol. Chem. 276, 5101–5108

15. Martinez-Botas, J., Anderson, J. B., Tessier, D., Lapillonne, A., Chang, B. H., Quast, M. J., Gorenstein, D., Chen, K. H., and Chan, L. (2000) Nat. Genet. 4, 474–479

16. Tansey, J. T., Sztalryd, C., Gruia-Gray, J., Roush, D. L., Zee, J. V., Gavrilova, O., Reitman, M. L., Deng, C. X., Li, C., Kimmel, A. R., and Londos, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6494–6499

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