Perilipin A Increases Triacylglycerol Storage by Decreasing the Rate of Triacylglycerol Hydrolysis*

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The perilipins are the most abundant proteins at the surfaces of lipid droplets in adipocytes and are also found in steroidogenic cells. To investigate perilipin function, perilipin A, the predominant isoform, was ectopically expressed in fibroblastic 3T3-L1 pre-adipocytes that normally lack the perilipins. In control cells, fluorescent staining of neutral lipids with Bodipy 493/503 showed a few minute and widely dispersed lipid droplets, while in cells stably expressing perilipin A, the lipid droplets were more numerous and tightly clustered in one or two regions of the cytoplasm. Immunofluorescence microscopy revealed that the ectopic perilipin A localized to the surfaces of the tiny clustered lipid droplets; subcellular fractionation of the cells using sucrose gradients confirmed that the perilipin A localized exclusively to lipid droplets. Cells expressing perilipin A stored 6–30-fold more triacylglycerol than control cells due to reduced lipolysis of triacylglycerol stores. The lipolysis of stored triacylglycerol was 5 times slower in lipid-loaded cells expressing perilipin A than in lipid-loaded control cells, when triacylglycerol synthesis was blocked with 6 μM triacsin C. This stabilization of triacylglycerol lipase was not due to the suppression of triacylglycerol lipase activity by the expression of perilipin A. We conclude that perilipin A increases the triacylglycerol content of cells by forming a barrier that reduces the access of soluble lipases to stored lipids, thus inhibiting triacylglycerol hydrolysis. These studies suggest that perilipin A plays a major role in the regulation of triacylglycerol storage and lipolysis in adipocytes.

Lipid droplets in adipocytes store the body’s major energy reserves as triacylglycerols. These structures contain a large core of neutral lipid, primarily triacylglycerol, covered by a phospholipid monolayer. The intracellular mechanisms that control the storage and release of triacylglycerols are largely uncharacterized, yet are likely to be fundamental to understanding the regulation of energy metabolism in the body. Recent studies have shown that lipid droplets are covered with a proteinaceous coat; the functions and identities of the component proteins have not been fully elucidated. The first identified lipid droplet-specific proteins are the perilipins (1–7), a family of proteins coating the surfaces of lipid droplets of adipocytes and steroidogenic cells of adrenal cortex, testes, and ovaries, but lacking in other types of cells and in other cellular compartments. The perilipins are encoded by a single copy gene that gives rise to multiple mRNAs by alternative splicing mechanisms; these mRNAs are translated to yield the three described protein isoforms (2, 4). Perilipin A is the predominant isoform in both adipocytes and steroidogenic cells, perilipin B is found primarily in adipocytes, and perilipin C is unique to steroidogenic cells. Perilipin A is the most abundant protein on highly purified lipid droplets isolated from fully differentiated cultured 3T3-L1 adipocytes and from murine primary adipocytes, as assessed by Coomassie staining and silver staining of lipid-droplet proteins separated on denaturing gels. Based on the localization of perilipins at the surfaces of lipid droplets, we hypothesized that the perilipins may function in regulating the packaging and storage of neutral lipids.

The mechanisms of lipid droplet formation are poorly understood. Early studies showed that all cells in culture take up free fatty acids and lipoproteins provided by serum in the culture medium, and use the lipids as a source of energy and building components for membrane synthesis (8). Many cells have been observed to accumulate tiny cytoplasmic inclusions of triacylglycerols or cholesterol esters during exposure to lipid-rich medium (8, 9), while cultured adipocytes form relatively huge droplets (3) that occupy the majority of the cell volume. Additionally, analysis of histological sections reveal enormous lipid droplets in adipocytes and smaller lipid droplets in various non-adipose tissues including liver, heart, adrenal gland, testes, ovary, muscle, intestine, kidney, and mammary gland (10–12). Nonetheless, few details of how or where lipid droplets are formed are known. Enzyme activities for neutral lipid synthesis have been isolated in microsomes from fractionated cells, thus implying localization to the endoplasmic reticulum (ER). Both diacylglycerol acyltransferase, which catalyzes the final step in triacylglycerol synthesis, and acyl coenzyme A-cholesterol acyltransferase, which adds fatty acids to cholesterol,

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2 N. E. Wolins and C. Londos, unpublished data.

3 The abbreviations used are: ER, endoplasmic reticulum; CHO, Chinese hamster ovary; DEUP, diethylumbelliferyl phosphate; E600, diethyl-p-nitrophenyl phosphate; PCMB, p-chloromercuribenzoate; TNF-α, tumor necrosis factor-α.
have been localized to ER by subcellular fractionation (13, 14) and immunofluorescence microscopy (15, 16). The mechanisms leading to the nucleation of lipid droplet formation are currently uncharacterized; a hypothetical model includes the formation of a lens of neutral lipids between the luminal and cytoplasmic leaflets of the membrane bilayer of the ER that either is retained as a bleb associated with the ER (17), or pinches off and takes a cytoplasmic location.

Assuming that lipid droplets are formed within the ER membrane, unique to lipid droplets may originate as integral ER proteins that selectively target to the patches of the ER that contain the growing accumulation of neutral lipids, or alternatively, may be translated on free ribosomes and then inserted into the lipid droplet following its dissociation from the ER. Several sterol and phospholipid biosynthetic enzymes and fatty acyl-coenzyme A ligases in yeast are found on both the ER and isolated lipid droplets (18–21), and hence may be examples of lipid droplet-associated proteins that are synthesized on the ER; similarly, phosphatidylethanolamine N-methyltransferase has been localized to lipid droplets isolated from rat hepatocytes (22), as well as to an ER-like compartment termed the mitochondria-associated membrane fraction (23), that is enriched in numerous lipid biosynthetic enzymes (24). By contrast, the perilipins are translated on free, and not ER-bound, ribosomes, and thus, traffic to and associate with lipid droplets post-translationally. Although the perilipins appear to be associated only with lipid droplets, their functions are uncharacterized.

To study the function of perilipins in lipid metabolism, perilipin A was ectopically and stably expressed in fibroblastic 3T3-L1 pre-adipocytes that lack endogenous perilipins prior to differentiation. The ectopic perilipin A was found exclusively on tiny lipid droplets in these cells, which stored significantly more triacylglycerol than control cells lacking perilipins. The metabolic basis for the increased storage of triacylglycerol was investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fetal bovine serum, fatty acid-free bovine serum albumin, triolein, oleic acid, diethyl-p-nitrophenyl phosphate, and p-chloromercuribenzoate were purchased from Sigma. Geneticin was purchased from Life Technologies, Inc. or Mediatech, Inc. (Herndon, VA). [9,10-3H] oleic acid and [9,10-3H]triolein were purchased from PerkinElmer Life Sciences. Trias cin C (Biomol, Plymouth Meeting, PA) was generously donated by Dr. Peter Gillies and Dr. Sandie Germain at DuPont (Wilmington, DE). Diethylumbelliferyl phosphate was synthesized by Chem-Master International, Inc. (East Setauket, NY). Ammonium sulfate-impregnated silica gel H thin layer chromatography plates were purchased from Analtech (Newark, DE). An anti-calnexin polyclonal antibody was purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Bodipy 493/503 and Alexa Fluor 546 goat anti-rabbit tagged amino-terminal peptide of perilipin A (29), and an Alexa Fluor secondary antibody. Neutral lipids were stained with Bodipy 493/503 (30). Cells were viewed with a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu Orca digital camera interfaced with a Power Macintosh G4 computer; images were processed using Improvision Openlab software.

**Subcellular Fractionation of Cells and Characterization of Fractions**—Confluent monolayers of 3T3-L1 pre-adipocytes stably expressing perilipin A were incubated with 400 μM oleic acid complexed to fatty acid-free bovine serum albumin (6.1, moles of oleate:moles of albumin) in culture medium for 16 h to increase the storage of triacylglycerols. Cells were harvested and lysed in a hypotonic medium containing 10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 0.1 μg/ml leupeptin, 1 mM benzamidine, and 100 mM [4-(2-aminomethyl)-benzyl]-imidazolyl] hydrochloride for 10 min at 4 °C, followed by 10 strokes in a Teflon/glass homogenizer. The homogenate was centrifuged for 10 min at 1000 × g at 4 °C, and the supernatant was adjusted to 35% sucrose, and layered over a 0.5-mL cushion of 50% sucrose. A linear sucrose gradient was then layered over the density-adjusted supernatant, and the tubes were centrifuged for 4 h at 154,000 × g at 4 °C. The floating lipid droplet layer was harvested in approximately 1 mL by slicing off the top of the tube using a Beckman tube slicer; 11 additional fractions were collected. Equal portions of each fraction were solvent-extracted for lipid analysis, as described previously (7); the proteins in an additional portion of each fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose membranes and immunoblotted with antibodies against perilipin A and calnexin, an ER integral membrane protein; enhanced chemiluminescence was used to detect bound antibodies. Two separate fractionations of cells were compared and found to be nearly identical.

**Lipid Analysis**—Cellular lipid content was determined following solvent extraction of cells, as described previously (7). Lipid content was expressed relative to total cellular protein content measured by the bicinchoninic acid method (Pierce; Ref. 31).

**Measurement of the Rate of Triacylglycerol Hydrolysis in Intact Cells**—Confluent monolayers of 3T3-L1 pre-adipocytes stably expressing perilipin A and control cells were incubated with 400 μM oleic acid complexed to bovine serum albumin for 16 h to increase the storage of triacylglycerols. The medium was then removed and 6 μM triacsin C (from a 1 mg/ml stock in Me₂SO) was added in fresh culture medium without supplemental fatty acids. Cells were rinsed with phosphate-buffered saline and harvested by scraping at various times; lipids were extracted and quantified (7), and the mass of triacylglycerol was expressed relative to cell protein (31). Confluent monolayers of 3T3-L1 pre-adipocytes were used for these experiments since cell division is inhibited by contact inhibition in these cells, and the cell number remained constant throughout the chase incubation. Triacsin C, an acyl-coenzyme A synthetase inhibitor, was added to the chase medium to inhibit the re-utilization of fatty acids released from hydrolyzed triacylglycerols (32, 33). To test for the effectiveness of triacsin C in the inhibition of triacylglycerol synthesis, 3T3-L1 cells stably expressing perilipin A and control cells were incubated with 400 μM oleate complexed to albumin for 16 h in the presence or absence of 6 μM triacsin C; lipids were solvent-extracted and quantified (7).

**Measurement of Cellular Lipolytic Activity against Triacylglycerols in Vitro**—Twenty 100-mm dishes of confluent 3T3-L1 pre-adipocytes stably expressing perilipin A or control cells were rinsed with phosphate-buffered saline, harvested by scraping, and pooled. Cells were centrifuged at 500 × g for 10 min, followed by homogenization in a cold Teflon/glass homogenizer. Homogenates were centrifuged at 15,000 × g for 15 min at 4 °C. Tripletic supernatant fractions of 200 μL, each corresponding to approximately 2.8 mg of total cellular protein, were assayed for the ability to hydrolyze emulsified [1H]triolein by the methods of Khoo and Steinberg.
panels B and C were cultured in standard low lipid-mM DEUP to inhibit triacylglycerol hydrolysis. The cells were incubated with perilipin A and control cells; both types of cells were incubated with 0.6 Ci/60-mm culture dish) complexed to fatty acid-free bovine serum albumin, harvested at various times, and the cellular lipids extracted and analyzed by thin-layer chromatography (31). A paired Student's t test was used to assess differences in triacylglycerol synthesis between samples from cells expressing perilipin A relative to samples from control cells.

RESULTS

The Ectopic Expression of Perilipin A Causes Lipid Droplets to Cluster in One or Two Regions of the Cytoplasm in Fibroblastic Cells—To facilitate study of the functions of perilipins in lipid metabolism, perilipin A was ectopically and stably expressed in fibroblastic 3T3-L1 pre-adipocytes using an efficient retroviral expression system (25). Untransfected and transfected control cells normally lack perilipins, but store small quantities of cholesterol esters and triacylglycerols; the staining of neutral lipids with Bodipy 493/503 (30) revealed that control cells have a few minute lipid droplets dispersed throughout the cytoplasm (Fig. 1A). When oleic acid was added to the culture medium to provide substrate for triacylglycerol synthesis, more numerous lipid droplets were observed dispersed throughout the cytoplasm, but excluded from a perinuclear region of the control cells (Fig. 1B). Expressed perilipin A targets to and associates with lipid droplets; immunostaining for perilipins revealed tightly aggregated clusters of tiny spherical structures in one or two areas of the cytoplasm (Fig. 1C); the cores of these spherical structures were stained with Bodipy 493/503, thus identifying the structures as lipid droplets. Perilipin staining was either coincident with the neutral lipid staining or appeared as distinct rings around the perimeters of minute lipid droplets (Fig. 1C), due, at least in part, to the imaging of the clustered lipid droplets at various focal planes. When oleic acid was added to the culture medium, the number and sizes of lipid droplets in the clusters increased, and a distinct staining pattern of rings of perilipin surrounding cores of neutral lipid became apparent (Fig. 1D). No staining for perilipins was observed in control 3T3-L1 pre-adipocytes when the cells lacked an expression construct (see Fig. 8I in Ref. 29), or were infected with a retroviral vector lacking the cDNA for the coding sequence of perilipin A (Fig. 1, A and B). Comparable results were obtained in Chinese hamster ovary (CHO) fibroblasts stably expressing perilipin A and control cells (data not shown); the expression of perilipin A altered the distribution of lipid droplets to one or two tight clusters of numerous lipid droplets covered with perilipin A, compared...
lipid storage droplets containing the majority of cellular triacylglycerol on sucrose gradients of cell homogenates. 3T3-L1 pre-adipocytes stably expressing ectopic perilipin A were incubated for 16 h with 400 μM oleic acid complexed to albumin before being harvested, homogenized, and fractionated by the centrifugation of 0–30% sucrose gradients; 12 equal fractions were collected following centrifugation. The top panel shows a thin layer chromatography plate of lipid extracts from each of the 12 fractions developed to separate phospholipids, cholesterol, triacylglycerols, and cholesterol esters, as indicated. The lower panel shows a single immunoblot of proteins from each of the 12 fractions resolved by SDS-polyacrylamide gel electrophoresis, and probed with polyclonal antibodies raised against perilipin A and calnexin, a marker for endoplasmic reticulum.

**Figure 2.** Ectopic perilipin A localizes exclusively to buoyant lipid storage droplets containing the majority of cellular triacylglycerol on sucrose gradients of cell homogenates. 3T3-L1 pre-adipocytes stably expressing ectopic perilipin A were incubated for 16 h with 400 μM oleic acid complexed to albumin before being harvested, homogenized, and fractionated by the centrifugation of 0–30% sucrose gradients; 12 equal fractions were collected following centrifugation. The top panel shows a thin layer chromatography plate of lipid extracts from each of the 12 fractions developed to separate phospholipids, cholesterol, triacylglycerols, and cholesterol esters, as indicated. The lower panel shows a single immunoblot of proteins from each of the 12 fractions resolved by SDS-polyacrylamide gel electrophoresis, and probed with polyclonal antibodies raised against perilipin A and calnexin, a marker for endoplasmic reticulum.

**Table 1**
The expression of perilipin A in 3T3-L1 pre-adipocytes selectively increases the cellular storage of triacylglycerols, and not cholesterol esters

| Sample          | Lipid content (μg/mg cellular protein) |
|-----------------|----------------------------------------|
|                 | Triacylglycerol | Cholesterol Ester |
| Experiment 1: control | 5.1 ± 0.9      | ≈0.5               |
| Experiment 1: + perilipin A | 32.0 ± 6.3    | ≈0.5               |
| Experiment 2: control | ≤0.5          | ≤0.5               |
| Experiment 2: + perilipin A | 16.5 ± 5.9    | ≤0.5               |

with a dispersed arrangement of a few minute lipid droplets in control CHO cells lacking perilipin A.

**Ectopic Perilipin A Localizes Exclusively to Lipid Droplets**—To confirm the localization of ectopic perilipin A on lipid droplets, homogenates of 3T3-L1 pre-adipocytes stably expressing perilipin A were fractionated on sucrose gradients. The isolation of lipid droplets in the most buoyant fraction was confirmed by the detection of greater than 99% of the total cellular triacylglycerol in the uppermost fraction of the sucrose gradients (Fig. 2). Immunoblotting of the proteins in the gradient fractions revealed that perilipin A was quantitatively collected in the buoyant lipid droplet fraction (Fig. 2). Massive overexposure of the immunoblots failed to reveal perilipin A in any other subcellular fractions (data not shown). The nitrocellulose membranes were also probed for calnexin, an integral ER protein. Most of the calnexin was found in fractions of intermediate density that also contained the highest levels of cholesterol, another marker for cellular membranes (Fig. 2); however, approximately 3% of the total calnexin was found in the floating lipid droplet fraction, thus indicating potential contamination of this fraction with a small amount of ER.

**Ectopic Perilipin A Drives Triacylglycerol Storage in 3T3-L1 Pre-adipocytes**—Since cells expressing perilipin A showed more staining with Bodipy 493/503 than control cells, the neutral lipid content was quantified in control and perilipin A-expressing 3T3-L1 pre-adipocytes. Cells expressing perilipin A showed a higher triacylglycerol content than control cells, while showing no detectable differences in the extremely low levels of stored cholesterol esters (Table I). The triacylglycerol content of both control and perilipin A-expressing cells varied depending upon when the samples were harvested for lipid analysis after the addition of fresh culture medium containing serum lipids; when the lipid content was measured 24 h after feeding the cells, 3T3-L1 pre-adipocytes expressing perilipin A stored 6.4-fold, or more than 30-fold, more triacylglycerol than control cells in two separate transfection experiments. Similar results were obtained when comparing CHO fibroblasts stably expressing perilipin A and control cells (data not shown); cells expressing perilipin A stored 6–7-fold more triacylglycerol than control cells, while cholesterol ester levels were significantly higher than the levels found in 3T3-L1 pre-adipocytes and were unaltered by perilipin expression.

We reasoned that the increased storage of triacylglycerol in cells expressing perilipin A was due to either increased synthesis of triacylglycerols, or decreased hydrolysis of stored triacylglycerols by cytosolic lipases. Given the observations that triacylglycerols are synthesized on the ER (13) and that perilipins coat the surfaces of lipid droplets, and have not been found on the ER, we hypothesized that the most likely mechanism for the increased storage of triacylglycerols in cells expressing perilipin A was via the decreased turnover of stored triacylglycerols, since the hydrolysis of triacylglycerol most likely occurs at the surfaces of the lipid droplets.

**The Expression of Perilipin A Inhibits the Hydrolysis of Triacylglycerols**—To assess the consequences of the expression of perilipin A on the hydrolysis of triacylglycerols without complications due to the recycling of newly released fatty acids back into triacylglycerols, we used triacsin C, an inhibitor of acyl-coenzyme A synthetase (32, 33). To test the efficacy of triacsin C, 3T3-L1 pre-adipocytes expressing perilipin A and control cells were incubated with 400 μM oleate complexed to albumin in the presence or absence of 6 μM triacsin C for 16 h,
The expression of perilipin A does not alter the activity of soluble lipases in cells expressing perilipin A and control cells. Post-mitochondrial supernatants from both cell types were prepared from 3T3-L1 pre-adipocytes expressing perilipin A and control cells. The cell extracts were incubated with $[3\text{H}]$triolein emulsified with gum arabic, and the released fatty acids were quantified. In two experiments, extracts from both cell types were demonstrated to hydrolyze exogenous triacylglycerol equally (Table II); thus, the expression of perilipin A has no effect on the total activity of lipases in the cells.

**Triacylglycerol Hydrolysis in 3T3-L1 Pre-adipocytes Is Inhibited by DEUP and E600, but Not by PCMB**—Cells stably expressing ectopic perilipin A expressed significantly more triacylglycerol than control cells when incubated with 400 μM oleic acid for 16 h (Fig. 3). We investigated whether this increased storage of triacylglycerol was due solely to the increased rate of turnover of triacylglycerols in the control cells, or could be due, in part, to an increased rate of triacylglycerol synthesis in the cells expressing perilipin A. To measure triacylglycerol synthesis without the complications of simultaneous turnover, we sought conditions to inhibit triacylglycerol hydrolysis.

Since the soluble triacylglycerol hydrodases of 3T3-L1 pre-adipocytes have not yet been identified or characterized, we tested several known inhibitors of triacylglycerol or cholesterol ester hydrolysis (36–39) for the ability to inhibit the hydrolysis of triacylglycerols in 3T3-L1 pre-adipocytes. To increase stored triacylglycerols, the cells were incubated for 16 h with 400 μM oleate complexed to albumin (Fig. 5A, “lipid-loaded” conditions). Supplemental fatty acids were withdrawn and chase medium containing 6 μM triacsin C and PCMB, E600, DEUP, or medium without hydrolysis inhibitors was added to the cells for an additional 15 h. The cells were harvested, lipids extracted, and the remaining triacylglycerol quantified. Over 15 h, the triacylglycerol content of cells incubated with chase medium lacking hydrolysis inhibitors was reduced by 75% (Fig. 5A), consistent with previous observations (Fig. 4). PCMB failed to effectively inhibit the hydrolysis of triacylglycerols when tested at concentrations up to 50 μM (Fig. 5A); higher concentrations of PCMB led to cell death. Both E600 and DEUP inhibited triacylglycerol hydrolysis; almost complete inhibition of cellular lipases was observed with 0.6 mM E600 or DEUP in the chase media. DEUP (0.6 mM) inhibited greater than 90% of triacylglycerol hydrolysis for up to 18 h (Fig. 5B). No obvious signs of toxicity were observed when cells were incubated with E600 or DEUP up to 0.8 mM for 24 h (data not shown).

**Cells Expressing Perilipin A Do Not Synthesize Significantly More Triacylglycerol than Control Cells**—The incorporation of...
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**Figure 5.** Triacylglycerol hydrolysis is inhibited by E600 and DEUP, but not PCMB, in 3T3-L1 pre-adipocytes. Confluent monolayers of 3T3-L1 pre-adipocytes were incubated with 400 μM oleic acid complexed to albumin for 16 h to increase stored triacylglycerols. A, supplemental fatty acids were removed and chase medium containing 6 μM triacsin C and PCMB, E600, or DEUP at the indicated concentrations was added for an additional 15 h before cells were harvested and the triacylglycerol quantified. Data are the means and standard deviations of triplicate determinations; where error bars are not visible, they are contained within the symbol.

**Figure 6.** 3T3-L1 pre-adipocytes stably expressing perilipin A (○, □) do not synthesize significantly more triacylglycerol than control cells lacking perilipins (●, △). Confluent monolayers of 3T3-L1 pre-adipocytes stably expressing perilipin A and control cells were incubated with [3H]oleic acid complexed to albumin in the presence (○, □) or absence (●, △) of 0.6 mM DEUP to inhibit triacylglycerol hydrolysis. Cells were harvested at various times, the lipids extracted, and the incorporation of [3H]oleate into triacylglycerols determined, as a measure of triacylglycerol synthesis. Data represent the means and standard deviations of the average values from quadruplicate samples from three separate experiments; where error bars are not visible, they are contained within the symbol.

DISCUSSION

Ectopically expressed perilipin A associates exclusively with lipid droplets in cultured 3T3-L1 pre-adipocytes. Although most cultured cells store a small mass of neutral lipids in a few minute lipid droplets when provided with serum containing fatty acids and cholesterol, few cells express the perilipins. To date, the perilipins have been found only in differentiated, but not undifferentiated, adipocyte cell lines, such as 3T3-L1 adipocytes (1, 3, 4), and steroidogenic Y-1 adrenal cortical cells (4, 7) and MA-10 Leydig cells (4, 29); additionally, perilipins coat the lipid droplets of adipocytes in white and brown adipose tissue, and in mammary tissue (3). Previous studies have demonstrated that epitope-tagged perilipin A targets to lipid droplets when expressed in Y-1 adrenal cortical cells (7) that have a background of perilipins A and C (4, 7). Furthermore, it is probable that exogenous perilipins A and B expressed via an adenoviral expression system targeted to lipid droplets in fully differentiated 3T3-L1 adipocytes (40), although these constructs lacked an epitope tag to distinguish the expressed perilipins from endogenous perilipins in immunoblotting and microscopy experiments. The present study is the first to demonstrate the expression of perilipin A in cells that lack all perilipins. Clearly, these cells contain all of the cellular machinery necessary for newly synthesized perilipin A to target to and assemble onto lipid droplets. Additionally, ectopic perilipin A targets to and assembles onto lipid droplets in CHO fibroblasts, human Hep G2 hepatoma cells and rat McArH7777 hepatoma cells (data not shown). Thus, many cultured cells are able to synthesize lipid droplets containing perilipins when provided with the appropriate expression constructs; hence, the assembly of perilipins onto lipid droplets appears to require no cell-specific machinery. Furthermore, we have found no evidence of perilipins associated with subcellular fractions con-
Activity has been shown to be 19-fold higher than that of differentiated 3T3-L1 adipocytes where triacylglycerol lipase ectopic perilipin A, the perilipins are naturally abundant in pre-adipocytes is inhibited by E600 and DEUP, but not by now report that the hydrolysis of triacylglycerols in 3T3-L1 cells (48). The sulfhydral reagent PCMB inhibits the long chain skin fibroblasts that cleaves triacylglycerols with short and organophosphorous reagents such as E600 and DEUP. The lipid droplets and in microsomal subcellular fractions from and the cultured CHO cell line (45); and 2) an unrelated triacylglycerol lipase, which hydrolyzes stored triacylglycerols, with no detectable change in the mass of adipocytes, and steroidogenic cells. Perilipin A has been shown to regulating lipolysis and the storage of triacylglycerols in adipocytes. We hypothesize that perilipin A shields stored triacylglycerol from soluble, cytosolic lipases, yet the cytosolic lipases of 3T3-L1 pre-adipocytes and most other cells are uncharacterized. The existence of cytosolic neutral lipid hydrolases in cells such as fibroblasts has long been postulated as an essential element in the control of cholesterol homeostasis to regulate the flux of cholesterol between an esterified storage pool in lipid droplets and free cholesterol in membranes. Likewise, fatty acids released from triacylglycerol-rich lipid droplets, such as those of skeletal muscle, provide a source of energy, yet the mechanisms controlling this lipolysis have not been elucidated. To date, only two candidate lipases have been identified: 1) hormone-sensitive lipase, which hydrolyzes stored triacylglycerols in response to the stimulation of cell surface β-adrenergic receptors in adipocytes, and has also been found at low abundance in cells of the adrenal cortex, testes, and ovaries, as well as in heart, skeletal muscle, peritoneal macrophages (41–44), and the cultured CHO cell line (45); and 2) an unrelated triacylglycerol hydrolase that has recently been found on isolated lipid droplets and in microsomal subcellular fractions from liver, and in homogenates of kidney and intestine (22, 46, 47). Many unidentified lipases appear to be members of the serine esterase class of enzymes and are irreversibly inhibited by organophosphorous reagents such as E600 and DEUP. The activity of the newly identified liver triacylglycerol hydrolase (22, 46, 47) is inhibited by E600, as is a hydrolase of human skin fibroblasts that cleaves triacylglycerols with short and medium chain fatty acids (37). DEUP has been reported to irreversibly inhibit the cholesterol esterase of homogenates of rat Fuf5Ah hepatoma cells (38, 39) and murine MA10 Leydig cells (48). The sulhydral reagent PCMB inhibits the long chain triacylglycerol hydrolase of human skin fibroblasts (37). We now report that the hydrolysis of triacylglycerols in 3T3-L1 pre-adipocytes is inhibited by E600 and DEUP, but not by PCMB.

Although the present study demonstrates reduced hydrolysis of triacylglycerols in 3T3-L1 pre-adipocytes stably expressing ectopic perilipin A, the perilipins are naturally abundant in differentiated 3T3-L1 adipocytes where triacylglycerol lipase activity has been shown to be 19-fold higher than that of undifferentiated cells (49). Support for a role for the perilipins in the protection of adipose triacylglycerol stores from lipolysis derives from a recent study investigating the lipolysis induced by the chronic treatment of cultured 3T3-L1 adipocytes with tumor necrosis factor-α (TNF-α) (40). In this study, TNF-α increased glycerol release, and hence lipolysis, during 24-h treatments concomitant with a reduction in the expression of perilipins A and B. The expression of perilipins A or B in the cultured 3T3-L1 adipocytes using an adenoviral expression vector prevented the TNF-α-induced increase in glycerol release while maintaining perilipin levels on the lipid droplets. The authors propose that the overexpression of perilipins limited TNF-α-mediated lipolysis (40). Here, we have used a completely different approach of determining the rates of triacylglycerol hydrolysis and synthesis in the presence and absence of perilipin A, yet we have also concluded that perilipin A protects triacylglycerol from lipolysis. The high levels of soluble lipase activity in adipocytes may necessitate the presence of the perilipins at the surfaces of lipid droplets to protect the vast stores of triacylglycerol from hydrolysis.

The aggregation of lipid droplets into tight clusters in cells expressing perilipin A, but not in control cells, implies that perilipins may play a role in bringing small lipid droplets together, potentially through protein-protein interactions between perilipins, or between perilipins and other proteins, on adjacent lipid droplets. This aggregation of lipid droplets may serve an important function in differentiating adipocytes where many small lipid droplets appear to fuse into a few larger droplets that eventually coalesce into a single droplet with reduced surface area relative to volume. Although perilipins may play a role in droplet fusion, perilipins alone appear to be insufficient to mediate fusion, since in all cells that we have investigated to date, the addition of fatty acids to cells ectopically expressing perilipin A results in the formation of larger clusters of relatively small lipid droplets, rather than the formation of very large droplets such as those found in adipocytes.

We do not yet know the significance of the selective increase in triacylglycerol content in cells ectopically expressing perilipin A. Although the lipid droplets of control 3T3-L1 pre-adipocytes contain primarily triacylglycerol, the droplets of control CHO fibroblasts are relatively enriched in cholesterol esters. The serum-containing culture medium provides both fatty acids and cholesterol for the synthesis of neutral lipids; however, the droplets of both 3T3-L1 pre-adipocytes and CHO fibroblasts expressing ectopic perilipin A become selectively enriched in triacylglycerols, with no detectable change in the mass of stored cholesterol esters. Future studies will determine whether the perilipin A binds preferentially to, or selectively protects, droplets containing a triacylglycerol core.

These studies suggest that the perilipins play a major role in regulating lipolysis and the storage of triacylglycerols in adipocytes and steroidogenic cells. Perilipin A has been shown to be a major substrate for cAMP-dependent protein kinase in lipolytically stimulated adipocytes (1, 50). Further study is required to determine whether or not the phosphorylation of the six consensus sites for cAMP-dependent protein kinase of perilipin A (2) following the lipolytic stimulation of adipocytes plays a role in attenuating the barrier to lipolysis to permit hormone-sensitive lipase access to the lipid droplet.

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