The Phosphorylation of Serine 492 of Perilipin A Directs Lipid Droplet Fragmentation and Dispersion*

Received for publication, January 6, 2006, and in revised form, February 17, 2006. Published, JBC Papers in Press, February 17, 2006, DOI 10.1074/jbc.M600171200

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Perilipin A is a key regulator of triacylglycerol storage and hydrolysis in adipocytes; phosphorylation of perilipin A by protein kinase A facilitates maximal lipolysis. Chronic stimulation of lipolysis in 3T3-L1 adipocytes causes large perinuclear lipid droplets to fragment into myriad dispersed perilipin A-covered microlipid droplets. In cultured fibroblasts stably expressing ectopic perilipin A, clustered lipid droplets disperse throughout the cytoplasm upon incubation of the cells with forskolin and isobutylmethylxanthine (IBMX) to elevate levels of cAMP and activate protein kinase A, mirroring events observed in adipocytes. Furthermore, diethylumbelliferyl phosphate inhibits stimulated lipolysis but not the dispersion of lipid droplets, suggesting that products of lipolysis are not required for this remodeling process. We hypothesized that protein kinase A-mediated phosphorylation of perilipin A triggers the remodeling of lipid droplets. The mutation of serine 492 of perilipin A to alanine prevented the dispersion of clustered lipid droplets in fibroblasts stably expressing the mutated perilipin upon incubation with forskolin and IBMX. In contrast, the substitution of serines 81, 222, 276, or 433 with alanine, either singly or in combinations, did not affect the protein kinase A-mediated remodeling of lipid droplets. Interestingly, substitution of serines 433, 492, and 517 of perilipin A with glutamic acid residues blocked the dispersion of clustered lipid droplets in cells incubated with forskolin and IBMX, indicating that the addition of a negative charge does not mimic a phosphate group. We conclude that protein kinase A-mediated phosphorylation of serine 492 of perilipin A drives the fragmentation and dispersion of lipid droplets.

Adipose tissue contains the largest energy reserve in the body, stored as triacylglycerol in the intracellular lipid droplets of adipocytes. Triacylglycerol stores are hydrolyzed to mobilize energy during fasting and extended exercise. Catecholamines bind to β-adrenergic receptors on the plasma membranes of adipocytes to initiate a G-protein-mediated signaling cascade that activates adenyl cyclase, thus increasing intracellular cAMP levels. Protein kinase A (or cAMP-dependent protein kinase) is then activated and phosphorylates several proteins required for the hydrolysis of triacylglycerol and consequent mobilization of fatty acids. Following phosphorylation by protein kinase A, hormone-sensitive lipase translocates from the cytosol onto lipid droplets (1–5), where it hydrolyzes triacylglycerol and diacylglycerol (6–8). Perilipin A is another major substrate for protein kinase A (9, 10).

Perilipin A localizes to lipid droplets in adipocytes and plays roles in facilitating both the storage and hydrolysis of triacylglycerol. In adipocytes, two isoforms of perilipin are derived from the alternate splicing of mRNA transcribed from a single gene (10, 11); perilipin A is the predominant protein isoform, whereas perilipin B is a minor isoform. Perilipins A and B share a common sequence through the first 405 amino acids of the amino terminus that contains three consensus sequences for protein kinase A; perilipin A has a unique carboxyl terminus with three additional consensus sequences for protein kinase A (10, 11). In adipocytes, perilipin A forms a barrier at the surfaces of lipid droplets that restricts the access of cytosolic lipases (12–17), including hormone-sensitive lipase (18, 19), to the lipid droplet, thus promoting triacylglycerol storage under basal conditions. Consequently, perilipin null mice have a dramatically reduced mass of triacylglycerol in adipose tissue when compared with wild-type mice, and adipocytes isolated from perilipin null mice have a significantly elevated rate of basal lipolysis when compared with that of adipocytes isolated from wild-type mice (13, 14).

Perilipin B does not protect stored triacylglycerol from hydrolysis by cytosolic lipases (16, 19), suggesting that the unique 112-amino-acid carboxyl-terminal sequence of perilipin A is critical for this barrier function. Furthermore, studies with truncated forms of perilipin A have shown that sequences in both the amino and carboxyl termini are required for the protection of triacylglycerol stores (17).

When cAMP levels rise in adipocytes, perilipin A is phosphorylated by protein kinase A (9) on as many as six serines (10, 11); the barrier function of perilipin A is attenuated, and lipolysis increases. Perilipin null mice show a blunted lipolytic response to β-adrenergic agonists (13, 14), indicating that phosphorylated perilipin plays a critical role in facilitating maximal lipolysis. Phosphorylation of one or more of three amino-terminal protein kinase A sites of perilipin A is required to assist docking of hormone-sensitive lipase onto lipid droplets (4) and to obtain maximal lipolysis (18, 19). Additionally, the protein kinase A-mediated phosphorylation of ectopic perilipin A on both amino and carboxyl-terminal consensus sites facilitates lipolysis in fibroblasts that do not express hormone-sensitive lipase (16, 18, 19), suggesting that phosphorylated perilipin A promotes lipolysis by other cytosolic lipases that may include the recently identified adipose triglyceride lipase, also called desnutrin (20–22). Clearly, there are uncharacterized mechanisms by which phosphorylated perilipin A facilitates lipolysis.

Continuous stimulation of β-adrenergic receptors over several hours induces the large perinuclear lipid droplets of 3T3-L1 adipocytes to fragment into microlipid droplets and disperse throughout the cytoplasm (23–25). A similar remodeling of lipid droplets has been observed in rat adipocytes following infusion of an agonist for β-3 adrenergic receptors into a subcutaneous fat pad or electroporation of cDNA encoding a constitutively active β-1 adrenergic receptor into the fat pads of live rats (26). The mechanisms that promote the remodeling of lipid droplets in adipocytes are uncharacterized. In this study, we test the hypothesis that the fragmentation and dispersion of lipid droplets in lipolytically stimulated adipocytes is triggered by the phosphorylation of perilipin A by protein kinase A.*

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*This work was supported by National Institutes of Health Grant DK54797 and a Research Award from the American Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and geneticin were obtained from Mediatech, Inc. (Herndon, VA). Forskolin, 3-isobutyl-1-methylxanthine (IBMX),2 fetal bovine serum, and goat anti-rabbit immunoglobulin G peroxidase conjugate were purchased from Sigma. Fatty acid-free bovine serum albumin (BSA) was purchased from either Sigma or Biocell Laboratories, Inc. (Rancho Dominguez, CA). Diethylumbelliferyl phosphate (DEUP) was synthesized by Chem-Master International, Inc. (Stony Brook, NY). Triacsin C was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503 were obtained from Molecular Probes, Inc. (Eugene, OR; catalog number D-3922). Rabbit anti-phosphoserine IgG was purchased from Research Diagnostics, Inc. (Flanders, NJ; catalog designation RDI-PHOSSEArbr), and rabbit anti-phospho-(Ser/Thr) protein kinase A substrate IgG was purchased from Cell Signaling Technology, Inc. (Beverly, MA; catalog number 9621). A polyclonal antibody raised against calnexin was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Polyclonal antibodies raised against adipose fatty acid-binding protein were kindly donated by Dr. Judith Storch (Rutgers University, New Brunswick, NJ). Anti-serum raised against an amino-terminal peptide of mouse adipophilin (27) was kindly donated by Dr. Constantine Londos (National Institutes of Health, Bethesda, MD). Pf remarkably DNA polymerase was purchased from Stratagene, Inc. (La Jolla, CA).

Cell Culture—For most experiments, 3T3-L1 preadipocytes were used as a model of fibroblasts and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, as described previously (12). 3T3-L1 fibroblasts stably expressing intact perilipin A delivered by a retroviral expression system, or the retroviral expression vector without a cDNA insert (control cells), were maintained in 0.6 mg/ml geneticin (12).

Confocal Microscopy of Adipocytes—Monolayers of 3T3-L1 preadipocytes were grown in culture medium with 8 μg/ml bovine insulin, and upon reaching confluence, were induced to differentiate into adipocytes by the daily addition of 10 μg/ml insulin, 10 μM dexamethasone, and 0.5 mM IBMX in fresh culture medium for 3 days, followed by the removal of these agents and the daily addition of fresh culture medium without additives (28). Six days following the initiation of differentiation, the cells were rinsed with phosphate-buffered saline, released with 0.25% trypsin and 1 mM EDTA, and seeded into culture dishes containing glass coverslips; cells from one 100-mm culture dish were seeded into three or four new 100-mm dishes. The cells were cultured for 24 h and then incubated in culture medium, either without additives or with 10 μM isoproterenol and 0.5 mM IBMX for 2, 4, or 8 h before fixation with 3% paraformaldehyde in phosphate-buffered saline, and prepared for microscopy (29). Fixed cells were probed with polyclonal antibodies raised against a recombinant amino-terminal peptide of perilipin A (28) followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503 to detect neutral lipids (30). Images of cells were captured in monochrome using a Zeiss LSM510 Meta confocal laser scanning microscope and processed using a Zeiss LSM image browser.

Subcellular Fractionation of Adipocytes on Sucrose Gradients—Confluent 3T3-L1 preadipocytes in 100-mm culture dishes were induced to differentiate for 6 days, and adipocytes were then incubated in control medium or medium containing 10 μM isoproterenol and 0.5 mM IBMX for 6 h. Cells were harvested, lysed, and fractionated, as described previously (31). Briefly, post-mitochondrial supernatants were adjusted to 35% sucrose using a solution of 70% sucrose and then layered over 1-ml 50% sucrose cushions. Thereafter, 8 ml of a 0–30% linear sucrose gradient were layered over each sample, and gradients were centrifuged at 154,000 x g in a Beckman SW41Ti rotor for 4 h at 4 °C. The floating lipid droplet fractions were collected by slicing off the top portions of the tubes with a Beckman tube slicer. The remainder of each gradient was collected in 1-ml fractions. Proteins from equivalent volumes of each fraction were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with polyclonal antibodies raised against perilipin (28), calnexin, and adipose fatty acid-binding protein, followed by horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were detected using enhanced chemiluminescence reagents (Amersham Biosciences).

Expression of Perilipin A cDNA Containing Alanine or Glutamic Acid Substitutions for Serines within Protein Kinase A Consensus Sequences—Perilipin A cDNA was amplified using polymerase chain reaction with Pfica polymerase and oligonucleotide primers complementary to the 5’ and 3’ ends of the coding sequence of perilipin A. Point mutations were introduced into the six protein kinase A consensus sequences using internal primers complementary to 20–36 nucleotides flanking and including the sequence encoding each serine; the codon for serine was altered to encode either alanine or glutamic acid. The mutated forms of the perilipin A cDNA were ligated into the unique HindIII site of the pSRaMSVtkneo retroviral expression vector (32). All mutations were confirmed by sequencing the inserted cDNA. The retrovirus for each mutated form of perilipin A was packaged in 293T cells and used to transduce 3T3-L1 fibroblasts (12). 3T3-L1 fibroblasts stably expressing the mutated forms of perilipin A were selected by growth in 0.6 mg/ml geneticin (12, 33).

Incubation of 3T3-L1 Fibroblasts to Study Lipid Droplet Morphology and Lipolysis—Prior to experiments, 3T3-L1 fibroblasts stably expressing either perilipin A, a mutated form of perilipin A, or the retroviral expression vector without an added cDNA sequence (control cells) were incubated with culture medium containing oleic acid complexed to fatty acid-free BSA at a 6:1 molar ratio (12) for 16–18 h (lipid-loading). The cells were incubated with 200 μM oleate for fluorescence microscopy experiments; for all other experiments, the cells were incubated with 400 – 600 μM oleate. The latter lipid-loading conditions were selected to fully stabilize ectopic perilipin A (34) but also increased protein levels of endogenous adipophilin on lipid droplets. To increase intracellular levels of cAMP, 10 μM forskolin in 0.1% dimethyl sulfoxide (Me2SO) and 0.5 mM IBMX were added to the cells in fresh culture medium. In some experiments, the cells were incubated with 800 μM DEUP added in 0.5% Me2SO without further additions or for 1 h prior to and during incubation with 10 μM forskolin and 0.5 mM IBMX. For each experiment, all cells were incubated with the same concentration of Me2SO.

Fluorescence Microscopy of 3T3-L1 Fibroblasts—Control 3T3-L1 fibroblasts and cells expressing perilipin A or a mutated form of perilipin A were cultured on glass coverslips, lipid-loaded overnight, and then incubated in the experimental medium described above prior to fixation with 3% paraformaldehyde in phosphate-buffered saline and preparation for microscopy (29). Fixed cells were probed with antibodies raised against perilipin, followed by Alexa Fluor 546-conjugated goat anti-rabbit IgG and Bodipy 493/503 to detect neutral lipids (30). Cells were viewed with a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu Orca digital camera interfaced with a Power Macintosh G4. Images were captured in monochrome and processed using Improvision Openlab software. Cells co-stained with Alexa Fluor 546

2 The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; DEUP, diethylumbelliferyl phosphate; BSA, bovine serum albumin.
secondary antibodies and Bodipy 493/503 are depicted in colors opposite to those observed for esthetic reasons.

Measurement of the Rate of Triacylglycerol Hydrolysis in 3T3-L1 Fibroblasts—Confluent monolayers of cells expressing perilipin A and control cells were lipid-loaded for 16–18 h. Exogenous fatty acids were removed, and the cells were incubated in medium containing 6 μM triacsin C (12, 35), 1% fatty acid-free BSA to bind released fatty acids, and either forskolin and IBMX, DEUP, or forskolin and IBMX with DEUP. Cells were harvested, total lipids were extracted (36), and triacylglycerol was quantified by thin layer chromatography, as described previously (34).

Detection of Phosphorylation of Unmodified and Mutated Forms of Perilipin A—Confluent monolayers of control 3T3-L1 fibroblasts and cells stably expressing perilipin A or mutated forms of perilipin A were lipid-loaded and then incubated in control medium or medium containing forskolin and IBMX in the presence or absence of DEUP. Cells from six 100-mm culture dishes were collected and pooled for each condition and disrupted by homogenization in a hypotonic solution containing 10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 10 μg/ml leupeptin, 1 mM benzamidine, and 100 μM 4(2-aminoethyl)benzenesulfonfylfluoride) hydrochloride (lysis solution), as described previously (33). The lysate was adjusted to 20% sucrose by dilution with 60% sucrose in lysis solution, transferred to an ultracentrifuge tube, overlaid with 9 ml of 50% sucrose in lysis solution, and then centrifuged for 30 min at 26,000 × g at 4°C in a Sorvall TH-641 rotor. The floating lipid droplet layer was collected after slicing off the top portions of the tubes with a Beckman tube slicer. Lipid droplet fractions were delipidated (33) and the proteins solubilized in 2-fold-concentrated Laemmli’s sample buffer (37). Proteins were separated by SDS-PAGE and transferred electro- phoretically to nitrocellulose membranes. Immunoblots were probed with antibodies raised against phosphorylated serine and horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by enhanced chemiluminescence detection. Blots were stripped and reprobed with polyclonal antibodies raised against phosphoserine within a protein kinase A consensus sequence (RRXS), perilipin (28), and adipophilin (27).

Northern Blot Analysis—Total RNA was extracted from 3T3-L1 fibroblasts using RNeasy minicolumns (Qiagen) following the manufacturer’s protocol, separated on 1% agarose gels using NorthernMax-Gly reagents (Ambion, Inc.), and then transferred electrophoretically to MagnaCharge nylon membranes (Osmonics). Membranes were hybridized with 32P-labeled cDNA probes for perilipin A and β-actin.

RESULTS

Lipolytic Stimulation of 3T3-L1 Adipocytes Promotes the Dispersion of Microlipid Droplets Coated with Perilipin—Under basal conditions, cultured 3T3-L1 adipocytes have primarily large, centrally located lipid droplets that are covered with perilipin (Fig. 1, A, D, and G). When the cells are incubated with isoproterenol (a β-adrenergic agonist) and IBMX (a phosphodiesterase inhibitor) to activate sustained lipolysis, the lipid droplets fragment and disperse. Significant fragmentation of the large lipid droplets is detectable by 2 h (not shown) and is more pronounced by 4 h (Fig. 1, B, E, and H). By 8 h, the fragmentation is essentially complete; large lipid droplets are absent, and the cytoplasm of each cell is filled with microlipid droplets (Fig. 1, C, F, and I). Co-staining for perilipin and neutral lipid shows a coincident punctate staining pattern, indicating that perilipin remains associated with microlipid droplets containing neutral lipid.

Subcellular Fractionation of Lipolytically Stimulated 3T3-L1 Adipocytes Confirms That Perilipin A Remains Buoyant on Microlipid Droplets—The observation that perilipins remain associated with microlipid droplets in lipolytically stimulated adipocytes conflicts with previous reports of a diffuse staining pattern for perilipin following the stimulation of lipolysis that was interpreted to indicate that perilipin exits lipid droplets and becomes soluble in the cytoplasm (3, 8, 15, 38, 39). To further investigate the subcellular localization of perilipin, we used sucrose gradients to fractionate lysates from untreated 3T3-L1 adipocytes and cells treated with isoproterenol and IBMX for 6 h. Most importantly, the cell lysates were adjusted to 35% sucrose and layered beneath 0–30% sucrose gradients so that soluble cytosolic proteins remained in the lower fractions of the gradients during centrifugation, whereas more buoyant membrane fractions floated to the appropriate densities. Immunoblotting of proteins in fractions collected from the gradients revealed that the cytosolic adipose fatty acid-binding protein remained in the dense fractions for both basal and lipolytically stimulated adipocytes (Fig. 2), whereas calnexin (a marker for endoplasmic reticulum) floated to intermediate density fractions (Fig. 2). Significantly, perilipin A was recovered only in the most buoyant fractions of gradients containing lysates from both basal (Fig. 2A) and lipolytically stimulated (Fig. 2B) adipocytes, indicating that perilipin A remains associated with lipid droplets and is not released into the cytoplasm.

Incubation of Fibroblasts Expressing Ectopic Perilipin A with Forskolin and IBMX Disperses Clustered Lipid Droplets—We hypothesized that perilipin A (but not the subcellular environment of the adipocyte) is required for remodeling of lipid droplets. To test this hypothesis, we stably expressed perilipin A in 3T3-L1 fibroblasts that lack perilipins. Under basal conditions, the stable expression of ectopic perilipin A promoted the clustering of numerous small lipid droplets into one or two areas of the cytoplasm (Fig. 3A, micrograph B), as shown previously (12, 16, 18, 33). When forskolin and IBMX were added to the cells to activate adenyl cyclase and sustain intracellular levels of cAMP, perilipin A-coated lipid droplets dispersed throughout the cytoplasm (Fig. 3A, micrograph D). Following the addition of forskolin and IBMX, we observed three patterns of distribution of lipid droplets in cells expressing perilipin A, including 1) densely clustered (Fig. 4A), 2) partially or
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loosely clustered with some dispersed droplets (Fig. 4B), and 3) fully dispersed (Fig. 4C); cells expressing perilipin A were scored according to these patterns (Fig. 3B). In contrast, the few small lipid droplets of control fibroblasts lacking perilipins were scattered throughout the cytoplasm under basal conditions and following the addition of forskolin and IBMX (Fig. 3A, micrographs A and C, respectively). Thus, ectopic perilipin A, in the absence of other adipocyte-specific proteins, directed the dispersion of clustered lipid droplets in response to the activation of adenylyl cyclase.

Incubation of Fibroblasts Expressing Ectopic Perilipin A with Forskolin and IBMX Promotes Phosphorylation of Perilipin A and Increases Lipolysis—Previous studies have shown that the activation of protein kinase A promotes the phosphorylation of serine residues of perilipin A and control cells lacking perilipins but treated with retrovirus were lipid-loaded for 16–18 h and then incubated for 4 h in medium containing 10 μM forskolin and 0.5 mM IBMX (C and D) or no additions (A and B). Additionally, lipid-loaded cells were incubated with 800 μM DEUP for 1 h prior to and during 4 h of incubation with forskolin and IBMX (E and F). Cells were fixed with paraformaldehyde and probed with polyclonal antibodies against perilipin (green) and Bodipy 493/503 for neutral lipid staining (red). Images were captured in monochrome and then colorized. The individual cells depicted are representative of hundreds of cells observed in three or more experiments. Scale bar = 10 microns. B, cells expressing perilipin A were incubated under normal culture conditions (Basal), with forskolin and IBMX (F+I), DEUP (DEUP), and forskolin, IBMX, and DEUP (F+I+DEUP) and then scored for the distribution of lipid droplets according to the three patterns depicted in Fig. 4: densely clustered (Clustered), partially or loosely clustered (Intermediate), and fully dispersed (Dispersed). Data are the average of 100-cell counts for each of two independent experiments.

FIGURE 2. Perilipin associates with a buoyant lipid droplet fraction in lysates of basal and lipolytically stimulated 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated in control medium (A) or medium containing 10 μM isoproterenol and 0.5 mM IBMX (B) for 6 h followed by lysis and homogenization of the cells. Post-mitochondrial supernatants were adjusted to 35% sucrose and layered over a 1-ml 50% sucrose cushion; 9-ml 0–30% linear sucrose gradients were then layered over the samples. Gradients were centrifuged, and 1-ml fractions were collected. Proteins from equal portions of each fraction were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies raised against perilipin A, calnexin, and adipose fatty acid-binding protein (AFABP). Fraction 1 was collected from the top of the gradient and contains all detectable perilipin. Calnexin, a protein integral to membranes of the endoplasmic reticulum, was detected primarily in fractions 6–10. The cytosolic protein adipose fatty acid-binding protein was detected in fractions 9–12. The blots depicted are from one experiment of two comparable experiments.

FIGURE 3. Clustered perilipin A-coated lipid droplets disperse throughout the cytoplasm following incubation of 3T3-L1 fibroblasts with forskolin and IBMX. 3T3-L1 fibroblasts stably expressing perilipin A and control cells lacking perilipins but transfected with retrovirus were lipid-loaded for 16–18 h and then incubated for 4 h in medium containing 10 μM forskolin and 0.5 mM IBMX (C and D) or no additions (A and B). Additionally, lipid-loaded cells were incubated with 800 μM DEUP for 1 h prior to and during 4 h of incubation with forskolin and IBMX (E and F). Cells were fixed with paraformaldehyde and probed with polyclonal antibodies against perilipin (green) and Bodipy 493/503 for neutral lipid staining (red). Images were captured in monochrome and then colorized. The individual cells depicted are representative of hundreds of cells observed in three or more experiments. Scale bar = 10 microns. B, cells expressing perilipin A were incubated under normal culture conditions (Basal), with forskolin and IBMX (F+I), DEUP (DEUP), or forskolin, IBMX, and DEUP (F+I+DEUP) and then scored for the distribution of lipid droplets according to the three patterns depicted in Fig. 4: densely clustered (Clustered), partially or loosely clustered (Intermediate), and fully dispersed (Dispersed). Data are the average of 100-cell counts for each of two independent experiments.

FIGURE 4. Patterns of lipid droplet distribution in cells. Three general patterns of lipid droplet distribution were observed in 3T3-L1 fibroblasts stably expressing perilipin A. Lipid droplets were densely clustered (A), lipid droplets were partially or loosely clustered with some individually dispersed droplets (B), or lipid droplets were fully dispersed with no observable cluster of lipid droplets (C). These three patterns of lipid droplet distribution were used for cell counts summarized in Figs. 38 and 78. Fixed cells were probed with antibodies raised against perilipin.

protein kinase A. Lipid-loading conditions were selected to optimize stabilization of ectopic perilipin A (34) but also promoted the stabilization of endogenous adipophilin on lipid droplets. Thus, adipophilin lev-
els were used as a loading control for lipid droplet proteins on immunoblots; adipophilin levels were similar across all samples (Fig. 5C).

The turnover of triacylglycerol was measured in 3T3-L1 fibroblasts stably expressing perilipin A and control cells stably expressing the retroviral vector without a cDNA insert. The cells were lipid-loaded for 16–18 h to increase the synthesis and storage of triacylglycerol. Exogenous fatty acids were removed, and the cells were incubated with fresh culture medium containing triacsin C to inhibit acyl-CoA synthetase (35) and with or without forskolin and IBMX. In the absence of forskolin and IBMX, the mass of triacylglycerol decreased at a slower rate in cells stably expressing perilipin A ($t_{1/2} = 16.7$ h) than in control cells lacking perilipins ($t_{1/2} = 5.1$ h) (Fig. 6A). Thus, under basal conditions, ectopic perilipin A protects triacylglycerol from hydrolysis, as shown previously (12–14). Following the addition of forskolin and IBMX, the mass of triacylglycerol decreased with $t_{1/2} = 9.7$ h in cells expressing perilipin A (Fig. 6A), providing confirmation that the barrier against lipolysis was attenuated. In contrast, the rate of lipolysis did not increase in control cells (triacylglycerol $t_{1/2} = 7.3$ h) (Fig. 6A).

**Phosphorylation of Perilipin A Disperses Lipid Droplets**—We next asked whether an increase in lipolysis is required for the dispersion of perilipin A-coated lipid droplets. DEUP is an inhibitor of serine esterase activity that reduces lipolysis under basal conditions (12, 40, 41). We tested whether DEUP prevents the increase in lipolysis that accompanies the activation of protein kinase A in cells expressing ectopic perilipin A. All cells incubated with 800 μM DEUP had significantly reduced rates of triacylglycerol hydrolysis (Fig. 6, B and C). In cells stably expressing perilipin A, triacylglycerol content decreased with $t_{1/2} = 54.6$ h when the cells were incubated with DEUP compared with $t_{1/2} = 16.6$ h in the absence of DEUP (Fig. 6B), indicating that DEUP reduced the low level of basal lipolysis. Importantly, triacylglycerol content was preserved ($t_{1/2} = 70.9$ h) when DEUP, forskolin, and IBMX were added to the cells expressing perilipin A (Fig. 6B), suggesting that lipolysis did not increase in response to the elevation of cAMP. DEUP also inhibited lipolysis in control cells; triacylglycerol decreased with $t_{1/2} = 36.0$ h when DEUP was added, and $t_{1/2} = 42.7$ h when DEUP, forskolin, and IBMX were added compared with $t_{1/2} = 6.0$ h for untreated
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control cells (Fig. 6C). Thus, DEUP inhibits lipolysis in both the presence and absence of forskolin and IBMX. Interestingly, upon incubation of perilipin A-expressing cells with forskolin, IBMX, and DEUP, the clustered lipid droplets dispersed (Fig. 3A, micrograph F), indicating that significant lipolysis is not required for the remodeling of lipid droplets. DEUP had no major effect on the clustered arrangement of perilipin A-coated lipid droplets when added in the absence of forskolin and IBMX (Fig. 3B). Furthermore, DEUP had no effect on the scattered arrangement of lipid droplets in control cells under basal conditions (data not shown) or following the addition of forskolin and IBMX (Fig. 3A, micrograph E). Finally, DEUP did not affect the protein kinase A-mediated phosphorylation of perilipin A or the levels of perilipin A associated with lipid droplets (Fig. 5).

Mutation of Serine 492 to Alanine Prevents the Dispersion of Lipid Droplets—We hypothesized that the phosphorylation of perilipin A by protein kinase A drives the dispersion of lipid droplets. Perilipin A has six serines within consensus sequences for protein kinase A at positions 81, 222, 276, 433, 492, and 517, which are referred to as protein kinase A sites 1–6 in this study. To test the hypothesis, these serines were mutated to alanines individually and in combinations; the mutated forms of perilipin A were stably expressed in 3T3-L1 fibroblasts, and the arrangement of lipid droplets was examined under basal conditions and following the addition of forskolin and IBMX.

In basal conditions, the majority of cells expressing either ectopic perilipin A or a mutated form of perilipin A displayed clustered lipid droplets (Fig. 7). When forskolin and IBMX were added to cells expressing unmodified perilipin A or mutated forms of perilipin A with alanine substitutions for serines 81, 222, and 276 (S1,2,3A) or serine 433 (S4A), the lipid droplets dispersed throughout the cytoplasm (Fig. 7). In contrast, cells expressing perilipin A with alanine substitutions for serine 492 (S5A) or serines 433 and 492 (S4,5A) displayed clustered lipid droplets under basal conditions that failed to disperse following the addition of forskolin and IBMX. Thus, the phosphorylation of serine 492 of perilipin A triggers the dispersion of lipid droplets, whereas phosphorylation of protein kinase A sites 1, 2, 3, or 4 is not required. The effect of an alanine substitution for serine 517 (protein kinase A site 6) was not examined due to difficulty in expressing this form of mutated perilipin A in 3T3-L1 fibroblasts.

Glutamic Acid Cannot Substitute for Phosphorylated Serine 492 of Perilipin A in Driving the Dispersion of Lipid Droplets—The phosphorylation of serines adds negative charges to the protein. We hypothesized that the substitution of a glutamic acid residue for serine 492 may mimic phosphorylation and promote the dispersion of lipid droplets in cells in basal conditions. We tested the hypothesis by mutating serines within all six of the protein kinase A consensus sites in combinations. Surprisingly, cells expressing mutated forms of perilipin A with glutamic acid substitutions for serines 81, 222, and 276 (S1,2,3E) or serines 433, 492, and 517 (S4,5,6E) displayed clustered lipid droplets in basal conditions (Fig. 7). Similarly, lipid droplets of cells expressing a single glutamic acid substitution for serine 492 (S5E) were clustered (data not shown). Thus, the addition of a negative charge to perilipin A is insufficient to disperse clustered lipid droplets without the activation of protein kinase A. Furthermore, following incubation with forskolin and IBMX, lipid droplets of cells expressing the S1,2,3E mutated form of perilipin A dispersed, whereas lipid droplets containing the S4,5,6E mutated form of perilipin A did not (Fig. 7). These findings confirm that phosphorylation of serines 81, 222, and 276 is not required for lipid droplet remodeling and also suggest that the addition of a negative charge to amino acid 492 cannot mimic phosphorylation. Additionally, perilipin A containing a single glutamic acid substitution for serine 517 was stably expressed in 3T3-L1 fibroblasts (data not shown); the lipid droplets of these cells were clustered in the basal state and dispersed following incubation of the cells with forskolin and IBMX, suggesting that phosphorylation of serine 517 is not required for remodeling of lipid droplets.

Confirmation of the Expression and Phosphorylation of Mutated Forms of Ectopic Perilipin A—Northern blot analysis showed that mRNA levels of unmodified perilipin A and mutated forms of perilipin A were similar in cells selected to stably express these constructs (Fig.
8A). Immunoblotting of proteins from isolated lipid droplets revealed similar levels of mutated forms of perilipin A relative to unmodified ectopic perilipin A (Fig. 8B). Phosphorylation of these forms of perilipin A was assessed by probing the immunoblots with antibodies specific for phosphorylated serine and for phosphorylated serine within the context of at least two experiments. B, immunoblots of lipid droplet proteins from control cells lacking perilipin (control) and cells expressing ectopic perilipin A (Peri A) or a mutated form of perilipin A (S123A, S4A, S5A, S45A, S123E, S456E) that were incubated in control medium or medium containing 10 μM forskolin and 0.5 mM IBMX for 1 h, as indicated. Membranes were probed with anti-phospho-(Ser/Thr) protein kinase A substrate antibodies (P04-RRXS) and then stripped and reprobed with anti-phosphoserine antibodies (P04-Ser), antibodies raised against adipophilin (Adipophilin). Data shown are representative of at least two experiments.

**DISCUSSION**

Following activation of the β-adrenergic signaling pathway of adipocytes, the phosphorylation of perilipin A by protein kinase A facilitates lipolysis through multiple mechanisms. Stimulation of lipolysis leads to the fragmentation of large central lipid droplets into myriad tiny dispersed microlipid droplets over a period of several hours (23–26). The major finding of this study is that the protein kinase A-mediated phosphorylation of serine 492 of perilipin A triggers the fragmentation and dispersion of lipid droplets. By contrast, protein kinase A-mediated phosphorylation of serines 81, 222, 276, 433, or 517 is not required for this remodeling of lipid droplets. Importantly, perilipin A remains associated with fragmented microlipid droplets and does not disperse into the cytoplasm or to another subcellular compartment when lipolysis is stimulated. The mass of perilipin A does not increase commensurate with the vastly increased surface area of the microlipid droplets, indicating that the relative surface coverage of perilipin thins during lipid droplet remodeling. Consequently, this remodeling process may promote increased access of cytosolic lipases to stored triacylglycerol while reducing the barrier function of perilipin A at the surfaces of lipid droplets. In support of this idea, fibroblasts expressing a mutated form of perilipin A lacking serines in all three carboxyl-terminal protein kinase A consensus sites (S4,5,6A) showed reduced lipolysis following the addition of forskolin when compared with cells expressing unmodified perilipin A (19). Therefore, we suggest that lipid droplet remodeling following the phosphorylation of perilipin A on serine 492 facilitates lipolysis, although the phosphorylation of serines 433 and 517 may also promote lipolysis through additional mechanisms. Interestingly, the substitution of serine 492 with glutamic acid to insert a negatively charged residue into this site failed to trigger constitutive dispersion of lipid droplets and prevented dispersion in response to the activation of protein kinase A, suggesting that unique properties of the phosphate group are required for lipid droplet remodeling.

The phosphorylation of perilipin A facilitates lipolysis by at least one additional mechanism. Protein kinase A-mediated phosphorylation of one or more of serines 81, 222, and 276 promotes the docking of hormone-sensitive lipase on perilipin A-coated lipid droplets (4) and maximal lipolysis (19). Phosphorylation of one or more of these sites also facilitates lipolysis in the absence of hormone-sensitive lipase (16, 19), suggesting that other lipases, which may include adipose triglyceride lipase (20–22), gain increased access to perilipin A-coated lipid droplets. Furthermore, the phosphorylation of perilipin A drives both rapid and delayed (but sustained) events. In adipocytes, the translocation of hormone-sensitive lipase from the cytoplasm to the surfaces of perilipin A-coated lipid droplets is rapid and essentially complete within 5 min (1). The secretion of glycerol and free fatty acids into the culture medium initiates after a brief delay and then increases steadily over hours. The fragmentation and dispersion of lipid droplets becomes evident much later. In this study, significant fragmentation of adipocyte lipid droplets was observed by 2 h after the addition of β-adrenergic agonists, and the conversion of large lipid droplets into microlipid droplets was complete by 8 h. Mechanisms that contribute to these changes in lipid droplet structure are not yet understood. Interestingly, the inhibi-
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Phosphorylation of perilipin on serine 492 of perilipin A may promote the recruitment of motor proteins or other factors required for this motility to the surfaces of lipid droplets. Previous studies have shown that lipid droplets move along microtubule tracks (42–44) using dynein motors (45, 46). Additionally, compounds that either elevate cellular levels of cAMP, thus activating protein kinase A, or inhibit serine/threonine phosphatases 1 and 2A increase the movement of cargo vesicles along microtubule tracks (47); these observations suggest that phosphorylation events may regulate the assembly of motor proteins with cargo or increase the efficiency of the movement of cargo along microtubules. Recent studies have shown that caveolin-1 is required for the protein kinase A-mediated phosphorylation of perilipin A and for full stimulation of lipolysis in adipocytes (48) and that caveolin-1 localizes to lipid droplets following β-adrenergic stimulation of 3T3-L1 adipocytes (24). Furthermore, a dominant negative form of caveolin prevents the movement of lipid droplets along microtubules in transfected Vero cells (49). Thus, caveolins likely play a role in lipid droplet remodeling and motility. Additionally, recent proteomics initiatives have identified various Rab GTPases in preparations of isolated lipid droplets (24, 42, 50, 51), suggesting a role for GTP hydrolysis in the trafficking of molecules through the lipid droplet compartment. Confirmation of the association of Rab18 with lipid droplets (52, 53) and particularly the increased association of Rab18 with lipid droplets following β-adrenergic stimulation of 3T3-L1 adipocytes (52) implies a role for Rab18 in lipid droplet remodeling and motility. Finally, in exciting recent findings, the protein kinase A-mediated phosphorylation of perilipin homologs LSD1 and LSD2 of insects has been shown to increase lipolysis (55) and the motility of insect lipid droplets (54), suggesting that these mechanisms contributing to the control of lipolysis are highly conserved throughout evolution.

Acknowledgments—We thank Dr. Norika Kane-Goldsmith for assistance with confocal microscopy of adipocytes. We thank Dr. Judith Storch for donating antibodies raised against adipose fatty acid-binding protein and Dr. Constantine Kontos for donating antibodies raised against adipophilins. We thank Dr. Michael Wolfe for useful discussions and Drs. Nathan Wold and Vidya Subramanian for useful discussions and critical review of the manuscript. We thank Boris Rubin, Sucharita Bhattacharyya, Dr. J. Matias Cavigilia, Anna Sekowski, Dr. Jose Pérez-Jiménez, Alexis Rothenberg, Terry Yin, Maryellen Sepelya, Bill DeMartini, Ingrid Harten, Paul Jansen, and Florence Fudalan for technical support. Finally, we thank two anonymous reviewers for their suggestions to improve the manuscript.

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