Adipocytes serve as the principal energy reservoir of the body; however, the subcellular organization of the machinery regulating lipid trafficking and metabolism is poorly understood. Mobilization of stored triglyceride is thought to be controlled by interactions among intracellular lipases and proteins that coat lipid storage droplets. A major limitation of previous studies of hormone-mediated lipolysis, however, is the use of cultured model adipocytes whose three-dimensional architectures do not resemble those in real adipose tissue. To address this limitation, we investigated the intracellular targeting of perilipin, a major lipid coat protein, and hormone-sensitive lipase in three preparations that exhibit more appropriate morphologies: 3T3-L1 adipocytes grown in three-dimensional matrix, dissociated mature adipocytes from mouse adipose tissue, and adipocytes within intact fat pads. High resolution imaging of native and fluorescently tagged proteins indicate that: 1) perilipin preferentially targets a special class of peripheral lipid storage droplets, but not the major or central lipid storage droplets, 2) the peripheral droplets are the sites of attack by hormone-sensitive lipase, and 3) perilipin and hormone-sensitive lipase are continuously colocalized following lipolytic activation. These results indicate that in white adipose tissue, lipolysis takes place in a specialized subcellular domain that is distinct from the major lipid storage site and is defined by perilipin.

The white adipose tissue plays a central role in energy storage and utilization. Work in the last decade has established perilipin (Plin) as a critical regulator of lipid stores in adipocytes (reviewed in Refs. 1–3). It has been proposed that Plin covers lipid droplet surfaces and serves as a physical barrier that protects stored triglyceride from hydrolysis by cellular lipases (4, 5). Upon lipolytic stimulation, Plin is phosphorylated at multiple sites by PKA, thereby altering its association with the droplet surface and allowing hormone-sensitive lipase (HSL) access to lipid droplet surface to initiate lipolysis.

Although there is strong evidence that Plin is critically involved in regulating access of HSL to stored triglyceride, several observations indicate that a simple barrier/translocation model is insufficient to explain lipolysis in vivo. For example, bulk translocation of HSL and Plin dissociation are not consistent features of lipolytic activation in primary adipocytes (6, 7). Furthermore, lipolysis can be observed in permeabilized fat cells, strongly indicating that the lipolytic machinery is preassembled to such a degree that the soluble pool of HSL may be irrelevant (8–10). Finally, the barrier/translocation model does not explicitly address the issue of whether all lipid storage droplet (LSD) surfaces are coated with Plin, although the model implies that surfaces not coated by Plin should be subject to attack by HSL.

One important difference between the model cells used to develop the barrier/translocation hypothesis and fat cells in their native environment is that normal fat cells contain a single major LSD whereas cultured cells (e.g. 3T3-L1 adipocytes) contain multiple LSDs that rarely exceed 5 microns in diameter. Furthermore, cell shape is known to have important effects on phenotypic gene expression and the organization of signaling pathways in adipocytes (11). In this study, we have therefore investigated the organization of lipolytic signaling in unilocular mature adipocytes using a novel, in vivo "adiporation" and imaging techniques (12). In addition, we employed 3T3-L1 adipocytes that were supported in a three-dimensional matrix. Unlike model adipocytes grown on flat surfaces, cells in these three-dimensional cultures develop 1–3 major lipid droplets (>20 μm in diameter) that more closely resemble true unilocular adipocytes in vivo. Our results indicate that mature adipocytes contain at least two types of LSDs: a major or central LSD that is typically associated with unilocular fat cells, and novel peripheral LSDs that are interposed between the plasma membrane and the central LSD. Analysis of the distribution of Plin and HSL indicates that these proteins are largely colocalized to peripheral LSDs and not the core LSD during lipolytic stimulation. These data indicate that Plin defines the sites of lipase action and is unlikely to act as a requisite barrier of all LSD surfaces, particularly the core LSD. These observations and recent proteomic studies (13, 14) suggest that adipocytes contain a specialized domain devoted to regulated lipolysis and that Plin may play a key role in organizing the structure and function of that domain.

**EXPERIMENTAL PROCEDURES**

*Materials—*Alexa-488-Bodipy was purchased from Molecular Probes (Eugene, OR). Rabbit anti-Plin and rabbit anti-HSL antibodies were kindly provided by Dr. A. Chaudhry (Pfizer, Ann Arbor, MI). Plin antibody was developed against the N-terminal 15 amino acids of rat Plin (12) and the HSL antibody was that described by Syu and Saltiel (13).

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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(15). The specificities of these antibodies were confirmed using 293T cells transfected with plasmids encoding Plin or HSL; in each case, the antibodies produced a positive immunofluorescence signal only from cells that had been transfected with the appropriate construct (data not shown). Rabbit anti-phospho-(Ser-Thr)-PKA substrate antibodies were obtained from Cell Signaling Technology (Beverly, MA), and guinea pig anti-ADRP antibodies were obtained from Research Diagnostics (Concord, MA). Full-length mouse perilipin A was cloned in-frame into the KpnI site of EYFP-N1 (Plin-A-EYFP) and was obtained from Dr. Sucharit Bhakdi (Institute for Medical Microbiology and Hygiene, Mainz, Germany).

Streptolysin-O (SLO) was purchased from Dr. Sucharit Bhakdi (Institute for Medical Microbiology and Hygiene, Mainz, Germany). Fluorescence histochemistry we employed the procedure of Negoescu et al. (16). The slides were washed four times with PBS over a 40-min period, and then incubated for 1 h with 3 µg/ml Cy3-conjugated goat anti-rabbit Fab fragment (Jackson Immunoresearch, West Grove, PA). The slides were washed as before, and then incubated for 45 min with primary antibodies against the second antigen (rabbit anti-Plin at 1:1000 dilution or rabbit anti-phospho-(Ser-Thr) PKA substrate antibody at 1:150 dilution). The slides were again washed and incubated for 45 min with 2 µg/ml Alexa-488-conjugated goat anti-rabbit (Fab) antibodies (Molecular Probes). Washed slides were post-fixed with 1% paraformaldehyde for 15 min. All antibodies were diluted in permeabilization buffer, and incubations were carried out at room temperature. For each pair of antibodies used, control experiments were performed in which one of the primary antibodies was omitted. In all cases, omission of the primary antibody resulted in total elimination of fluorescent signals in the corresponding channel (supplementary Fig. S1, and data not shown), validating this double staining approach.

In experiments where immunofluorescence staining patterns were compared with those of central lipid storage droplets (Figs. 1 and 3), the cells were fixed, permeabilized, and stained with primary antibodies as described above. The coverslips were then incubated for 45 min with 4 µg/ml Alexa-555-conjugated goat anti-rabbit and 0.25 µg/ml Bodipy-493/503 (both from Molecular Probes).

Reconstitution of Lipolysis in Transfected 293T Cells—293T cells plated in 24 wells were transfected with either ECFP-HSL (0.7 µg per well) alone, or a mixture of ECFP-HSL and Plin-A-EYFP (0.7 µg each) using ExGen 500 according to the manufacturer’s instruction (Fermen
tas). A control plasmid (DsRed) was added to yield 2.1 µg of total plasmid per well. Four hours later, the transfection mixture was removed, and the cells were fed with growth medium containing 300 µM oleic acid, 1% BSA, 0.5 µg/ml insulin for 16 h. The cells were rinsed once with Krebs Ringers supplemented with 4% fatty acid-free BSA, and then incubated in this medium (200 µl per well) containing either 1 µM Plin (control) or 10 µM forskolin and 1 mM IBMX (stimulated) for 2 h at 37 °C. Glycerol content of 100 µl of the medium was determined using the glycerol-3-phosphate oxidase technique (Sigma F6428). In parallel experiments, cells cotransfected with ECFP-HSL and Plin-A-EYFP were treated under control and stimulated conditions for 30 min, then fixed with 1% paraformaldehyde and imaged by spinning disc confocal microscopy, as detailed below.

In Vivo Adi
oporation—Adi
oporation was performed according to our published procedure (12). Briefly, 3-month-old male 129/S1VimJ mice were anesthetized with Avertin, and skin in the interscapular region was removed of fur and disinfected with alcohol. A 15-mm incision was made along the midline between and the skin overlying supracapular white adipose tissue (SSWAT) was gently dissected so that an approach to this subcutaneous pad could be easily made on either side. Plasmid DNA was reconstituted in sterile water at a total concentration of 1 µg/µl and 4 injections (7 µl per injection) were made into the SSWAT pad with 30-gauge needle and a Hamilton microlitre syringe. For adi
oporation with both Plin-A-EYFP and ECFP-HSL, the plasmids were mixed at a mass ratio of 1:2 prior to injection. The injection site was gently gathered to a gap width of 1.0 mm with Gerald bipolar forceps and seven 50 V, 20 ms square-wave pulses were delivered over 3 s immediately after injection. In experiments shown in Fig. 8, bottom, the animals were...
implanted at the time of adiporation with osmotic minipumps (Alzet) that delivered the 3-adrenergic receptor agonist CL-316,243 (CL) at a rate of 0.75 nmol/hr. Control and drug-treated tissues were harvested 3 days after adiporation. In parallel experiments, a group of adiporated mice received a single injection of 5 nmol of CL 15 min before harvesting (Fig. 8, top).

Three days after adiporation, SSWAT was dissected and immediately fixed with 4% paraformaldehyde in PBS. The region of electroporation was identified by fluorescence microscopy, dissected, and stored in PBS at 4 °C until imaged. Tissue containing adipocytes expressing fluorescent fusion proteins was placed in between two coverslips in a Leiden chamber and visualized without further processing with wide field or confocal fluorescence microscopy.

Microscopy and Image Analysis—Images (Figs. 1, 3, and 5) were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwoods, NJ) and a ×40 1.2 NA Apochromat water immersion differential interference contrast objective lens. Bodipy-493/503 and Alexa-488 were excited with Argon laser line at 488 nm, and Alexa-555 and Cy3 were excited with Argon laser line at 543 nm, using a dichroic beam splitter (HFT 488/543). All image acquisition was done in the multitrack mode with pinholes set to 1 Airy unit. Emissions from Bodipy-493/503 were collected by a band pass filter (BP 500–530 IR) for Alexa-488 and a long pass filter (LP 560) for Cy3. Under these conditions, no channel bleed-through was observed as assessed by cells stained with a single fluorescent probe. ECFP and EYFP (Figs. 7 and 8) were excited with argon laser lines at 458 nm and 514 nm, respectively, and images were acquired using a ×63 1.2 NA Apochromat water-immersion objective lens using a dichroic beam splitter (HFT 458/514). Emissions were collected by band pass filters BP 480–520 IR and BP 535–590 IR for ECFP and EYFP, respectively. Control single transfection experiments demonstrated that ECFP and EYFP signals were optically separated. Images in Fig. 2 were acquired using an Olympus IX81 microscope equipped with the Olympus spinning disc confocal system, a ×60 1.2 NA UPlan Apochromatic objective lens, and a Retiga 1300 cooled CCD camera. Confocal z-axis images were deconvolved using the point spread function algorithm and rendered in three dimensions with Microtome and VoxBlaster imaging software (Vaytek, Fairfield, IA). Images in Fig. 6B were acquired with the Olympus spinning disc confocal system, a ×60 1.2 NA water immersion lens, and a Hamamatsu Orca cooled CCD camera. EYFP and ECFP signals were isolated using Chroma (Rockingham, VT) filter sets 31044 and 41028, respectively. Quantitation of fluorescence intensities and colocalization were performed using the colocalization and line profile tools of Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD).
RESULTS

Plin Is Targeted to Peripheral but Not Central LSDs in 3T3-L1 Adipocytes Grown in Three-dimensional Matrix—According to the barrier/translocation model of lipolysis, Plin is expected to coat most, if not all, lipid droplet surfaces thereby denying access of cellular lipases under resting conditions, whereas lipolytic stimulation should promote the dissociation of Plin and its replacement by HSL at specific LSD surfaces. These predictions were first tested in 3T3-L1 grown in the three-dimensional matrix. After 2 weeks of culture in Matrigel, differentiated 3T3-L1 adipocytes developed a few large (20–30 μm) central LSDs. These cultures exhibit a much higher lipolytic response than those grown on plastic surfaces as measured by glycerol release into the medium (supplementary Fig. S2), consistent with their more differentiated phenotype. The central LSDs were strongly labeled by Alexa-488-bodipy (Fig. 1 panel A, green). Costaining of the cells with anti-Plin antibodies and Alexa-555-conjugated secondary antibodies followed by laser scanning confocal microscopic analysis showed that these central LSDs contained little Plin, if any, on their surfaces (Fig. 1, panel A, red). Instead, Plin was most concentrated on numerous small LSDs in the cell periphery. (Note that peripheral LSDs are largely photobleached because of low volumes of lipid.) Notable in this view is Cell 1, in which the confocal slice grazes the cell surface. Panel B illustrates the distribution of Plin in a separate confocal slice. Note that the borders of the central LSDs (examples 2–5) are defined by the small peripheral LSDs that surround it. There was no evidence of a continuous rim of immunofluorescence that would indicate uniform targeting of Plin to the central LSD. Although small areas of fluorescence suggestive of central LSD targeting could sometimes be seen in a given slice (e.g. cell 3 of panel B, opposite arrow, and cell 5), analysis of the three-dimensional projection demonstrates these are numerous small peripheral LSDs adjacent to the central LSD (panel C). Quantitation of Plin density using line profile analysis (panel D) shows preferential localization of Plin on droplets with diameters of less than 10 μm (panel E). Very similar results were obtained when analysis was carried out using spinning disk confocal microscopy (Fig. 2).

One possible explanation for the low levels of Plin on central LSDs is that the cells might have been partially activated. If this were the case, then HSL should decorate those areas of central LSDs from which Plin has dissociated. This possibility was ruled out by examining the distribution of HSL: immunofluorescence analysis of over 50 cells demonstrated that HSL was never found on the central LSDs under these basal culture conditions. (A representative field is shown in Fig. 3A.) Instead, the majority of HSL was found in a diffuse pattern in the cytosol. We also tested the possibility that other coat proteins might be found on the central LSDs. Staining of 2-week-old Matrigel cultures with anti-adipocyte differentiation-related protein (ADRP), however, showed little immunoreactivity (data not shown). When cultures grown on plastic (and thus less differentiated) were probed with anti-ADRP, some ADRP immunoreactivity could be detected, but it was localized to structures smaller and more distally distributed than Plin-containing LSDs (supplementary Fig. S3). To date, we have been unable to detect significant amounts of any known coat proteins on the surfaces of central LSDs. We conclude that 3T3-L1 adipocytes grown in three-dimensional culture contain at least two pools of lipid droplets: a set of peripherally
located, small LSDs coated with Plin, and the central core LSDs largely devoid of Plin. Moreover, the finding that Plin did not cover all LSD surfaces under non-stimulating condition makes it highly unlikely that the major role of Plin is to provide an obligatory barrier against cellular lipases.

Lipolytic Stimulation Causes HSL to Translocate to Peripheral LSDs Containing Phosphorylated Plin in 3T3-L1 Adipocytes and Mature Adipocytes Isolated from White Fat Tissue—A striking phenotype of Plin knock-out mice is the diminished response of adipocytes to lipolytic hormones (see Refs. 19 and 20, also see “Discussion”), raising the possibility that Plin acts as a positive regulator to facilitate hormone-stimulated lipolysis. We therefore examined whether Plin demarcates the site where lipolysis is to take place. According to this alternative model, stimulation leads to PKA activation, which in turn causes phosphorylation of both Plin and HSL. Phosphorylated Plin then recruits HSL to nearby droplet surface to initiate lipolysis.

Three-dimensional cultures of 3T3-L1 adipocytes were stimulated for 5–30 min at 37 °C with 10 μM forskolin and 1 mM IBMX to induce lipolysis. Cultures were fixed immediately and processed for immunofluorescence microscopy with antibodies against phospho-(Ser/Thr)-PKA substrate. Plin is the major substrate for PKA in adipocytes (21), and double label staining of stimulated cells with anti-Plin and anti-phospho-PKA substrate confirmed that Plin was the major PKA substrate in these cells (supplementary Fig. S4). Thus, anti-phospho-PKA substrate antibody served to localize the pool of phosphorylated Plin in stimulated cells. Comparison of the staining pattern with that of Alexa-488-Bodipy shows that phosphorylated Plin, like total immunoreactive Plin, was concentrated on peripheral lipid droplets with little found on the central LSDs (Fig. 3, C and D). Under this condition, the majority of the cells also displayed HSL staining on peripheral LSDs, indicating stimulus-induced translocation of HSL from the cytosol to the LSD surface took place (Fig. 3B, compare with Fig. 3A). Importantly, HSL was never found on the central LSDs. These data suggest that peripheral LSDs, but not central LSDs, are sites of active lipolysis.

To test if HSL is indeed recruited to the droplets containing phosphorylated Plin, we performed double immunofluorescence confocal

FIGURE 3. Immunolocalization of Plin, HSL, and phospho-PKA substrate in 3T3-L1 adipocytes grown in three-dimensional matrix. Differentiated 3T3-L1 adipocytes were grown in Matrigel for 14 days, and the cells were either treated with 100 nM PIA (control) or 10 μM forskolin and 1 mM IBMX (stimulated) for 5, 12, and 30 min. Cells were fixed and processed for immunofluorescence microscopy with the indicated antibodies. 0.25 μg/ml Bodipy was included in the secondary antibody to stain central lipid droplets. Images shown are single confocal slices recorded in multitracking mode. Note that there were no significant differences between the different stimulation times, and only representative fields from some treatments are shown. A, unstimulated cells, stained for HSL (red) and central LSDs (green). B, cells stimulated for 30 min, stained for HSL (red) and central LSDs (green). C, cells stimulated for 5 min, stained for phospho-PKA substrate (red) and central LSDs (green). D, cells stimulated for 12 min, stained for Plin (red) and central LSDs (green). Scale bar, 15 μm.
microscopy with anti-Plin and anti-HSL antibodies. In unstimulated cells, Plin resided predominantly on peripheral droplets (Fig. 4A, panel A) whereas HSL was distributed uniformly throughout the cytoplasm (panel B). Notably, the rimming pattern of Plin immunofluorescence on peripheral LSDs, indicated as fluorescence peaks in line scans (panel C), was not seen for HSL in unstimulated controls. Stimulation caused a dramatic redistribution of HSL to droplets that were positive for Plin (panels D–F) and phospho-PKA substrate (panels G–I). The colocalized HSL and Plin were always on peripheral droplets and never on central LSDs. Moreover, HSL and Plin remained colocalized during longer stimulation (up to 30 min, data not shown).

We noted that Plin immunofluorescence on lipid droplets was often clustered in a discontinuous and non-uniform pattern. To test if HSL is targeted to the same subdomain of the LSD where Plin resides, we stimulated a young (4-day-old) 3T3-L1 culture for 20 min and permeabilized the cells with streptolysin-O before fixation. The SLO proce-

FIGURE 4. A, localization of Plin and HSL in 3T3-L1 adipocytes by double label immunofluorescence. 14-day-old adipocytes in Matrigel were either stimulated for 12 min or kept in control conditions. The cells were fixed and processed for double immunofluorescence (see “Experimental Procedures”). Shown are single confocal slice images acquired using the multitracking mode. Top row, control unstimulated cells stained for Plin (A) or HSL (B), and representative line scan showing quantitation across peripheral LSDs (C). Colocalization coefficient (Pearson’s $r^2$) for the line scan was determined, and the number was included in the figure. In addition, analysis of the total cellular colocalization of fluorescence in A and B showed a Pearson’s $r^2$ of 0.09. Middle row, stimulated cells stained for Plin (D) or HSL (E), and representative line scan showing colocalization across peripheral LSDs (F). Coefficient of determination for total cellular colocalization of fluorescence in D and E was 0.46. Bottom row, stimulated cells stained for phospho-PKA substrate (G) or HSL (H), and representative line scan showing colocalization across peripheral LSDs (I). Coefficient of determination for total cellular colocalization of fluorescence in G and H was 0.69. Scale bar, 15 μm. B, localization of Plin and HSL in young 3T3-L1 adipocytes after SLO permeabilization. 4-day-old adipocytes in Matrigel were either stimulated for 20 min or kept in control conditions. The cells were permeabilized with SLO for 15 min before fixation and processing for double immunofluorescence with anti-Plin (green) and anti-HSL (red). Images were acquired with Olympus IX81 microscope and a ×40 0.85 NA UPlan Apochromatic objective lens. Scale bar, 20 μm.
increased PKA-stimulated lipolytic rate (Fig. 6). The presence of HSL had little effect on basal lipolytic rate, but markedly increased both the rate of basal and stimulated lipolysis (Fig. 6).

Panel

Mature Adipocytes in Vivo Contain a Novel Pool of Peripheral LSDs Coated with Plin-A—We next examined if the above observations could be extended to mature adipocytes in vivo. For these experiments, we employed a novel electroporation protocol (herein referred to as “adiporation”) that permits transfection of mature adipocytes in vivo with greater than 99% selectivity over other cells in the adipose tissue (12). Two fusion constructs, Plin-A-EYFP (12) and ECFP-HSL (16), were chosen because (1) they have been shown previously to be targeted correctly and functionally active, and (2) they could be resolved optically in co-transfection experiments.

The activities of these constructs were verified by reconstituting hormone-stimulated lipolysis in transfected 293T cells. 293T cells were transiently transfected with Plin-A-EYFP alone, ECFP-HSL alone, or a combination of Plin-A-EYFP and ECFP-HSL, and were fed overnight with oleic acid to promote lipid droplet formation. In the absence of HSL, the basal or stimulated rate of lipolysis (measured by glycerol release into the medium during a 2-h incubation period) was not appreciably affected by Plin expression (Fig. 6A, left panel). Expression of HSL increased both the rate of basal and stimulated lipolysis (Fig. 6A, right panel), indicating the construct was active. Co-expressing Plin in the presence of HSL had little effect on basal lipolytic rate, but markedly increased PKA-stimulated lipolytic rate (Fig. 6A, right panel), indicating that Plin is predominantly a positive regulator of PKA-mediated HSL activity in this system.

Expression of Plin-A-EYFP generated several small, sometimes irregularly shaped structures (Fig. 6B) in 293T cells. These structures were not observed in untransfected cells and stained positively for neutral lipids with Nile Red (not shown). Under basal conditions, ECFP-HSL was largely cytosolic and showed little colocalization to LSDs coated with Plin-A-EYFP. The subcellular distribution of Plin-A-EYFP was not altered after 30 min of stimulation with forskolin/IBMX; however, a significant fraction of cellular ECFP-HSL became strongly colocalized to LSDs containing Plin-A-EYFP.

To study targeting in vivo, Plin-A-EYFP and ECFP-HSL were introduced into supracapsular white adipose tissue by adiporation. Three days later, the fat pad was dissected and imaged with confocal microscopy. Plin-A-EYFP was targeted to numerous small lipid droplets (diameters <5 μm) that were interposed between the central lipid droplet and the cell surface (Fig. 7, panel A). The major lipid droplet, which had a diameter of greater than 50 μm, contained very little Plin-A. To address the possibility that these structures were induced by Plin overexpression, we compared the levels of Plin in electroporated cells to non-transfected control by immunocytochemistry after paraffin embedding (supplementary Fig. S5). The total level of Plin in control untransfected cells and tissue was harvested 3 days later. One group received no treatment (control, Fig. 7), a second group was stimulated with the lipolytic agonist CL 316,243 for 15 min prior to sacrifice, whereas the third group was infused continuously with CL for 3 days (Fig. 8).
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Under basal conditions, Plin-A-EYFP was tightly localized to peripheral lipid droplets (Fig. 7, panels A and B, E and F) whereas ECFP-HSL was found in a diffuse cytosolic pattern. In some cells, however, ECFP-HSL appeared to be pretargeted to peripheral LSDs that contained Plin-A-EYFP (Fig. 7, panels C and D). The degree of colocalization increased after fifteen minutes of stimulation such that ECFP-HSL was localized to LSDs containing Plin-A-EYFP in most cells (Fig. 8, top). Chronic stimulation reduced fat cell volume by greater than 50%, and resulted in extensive fragmentation of the central LSD and in extreme cases generated cells that contained only small LSDs (Fig. 8, bottom). In all cases, Plin-A-EYFP and ECFP-HSL were highly colocalized.

**DISCUSSION**

The current model of how lipolysis takes place largely comes from the work of Londos and co-workers (1, 4, 23, 24). According to this model, hormone-sensitive lipolysis is achieved through the action of HSL on stored triglyceride. Although lipolysis can be stimulated or inhibited by a variety of hormones, there is strong evidence that these distinct pathways converge at the level of PKA. Thus, lipolysis and antilipolysis are strongly predicted by the level of PKA activation, independent of the receptor activity (25–27). While HSL itself can be phosphorylated by PKA (28), there is strong evidence that the overall rate of lipolysis is governed by phosphorylation of coat proteins targeted to the LSD surface. What is presently unclear are the subcellular sites of lipase action and the exact role that lipid coat proteins play in hormone-stimulated lipid mobilization.

In adipocytes neutral lipids are stored in LSDs. While analysis of LSDs is still in its infancy, emerging evidence suggests they are dynamic and heterogeneous structures initially generated from the endoplasmic reticulum, the site of free fatty acid esterification (14, 29). In cell culture models, nascent lipid droplets are sequentially bound by several lipid coat proteins. With respect to lipolysis, it has been proposed that Plin acts as a physical barrier that “protects” triglycerides in the LSDs from attack by cytosolic HSL (2, 3). PKA activation is thought to alter the association of Plin-A with LSDs, thereby allowing HSL translocation to the stored triglyceride (7). A barrier model makes clear-cut predictions of the localization of Plin and HSL during basal and stimulated conditions. Importantly, simultaneous localization of Plin and HSL has not been previously investigated in adipocytes. This study therefore investigated the organization of these lipolytic molecules in three preparations with more appropriate morphologies than the widely used culture models: 3T3-L1 adipocytes grown in three-dimensional matrix, dissociated mature fat cells cultured overnight in three-dimensional matrix, and mature adipocytes in vivo using novel adaption and imaging techniques. Data from all three preparations indicate that a simple barrier/translocation model is insufficient to account for the subcellular localization of Plin and HSL under basal and stimulated conditions.

This conclusion is based on the following observations. (a) If Plin serves as a continuous barrier, then HSL should reside on lipid droplets that contain no Plin. This is not the case. Plin did not coat the core LSD of mature fat cells in vivo (Fig. 7) or 3T3-L1 (Figs. 1 and 2) or dissociated mature adipocytes (Fig. 5) grown in three-dimensional culture, yet we have never found HSL targeted to the core lipid droplet under basal or stimulated conditions. (b) In 3T3-L1 cells that contain mostly cytosolic HSL, HSL, and Plin colocalized to the same LSDs following stimulation, and remained colocalized for the duration of stimulation (up to 30 min) (Fig. 4). (c) In mature fat cells in vivo, Plin-A-EYFP, and ECFP-HSL colocalized to peripheral LSDs following acute stimulation (Fig. 8, top). Neither protein was found on the surface of the central LSD under basal or stimulated conditions. (d) Following chronic stimulation of fat cells in vivo (3 days CL), the core lipid droplet became fragmented into multiple internal LSDs. However, Plin-A-EYFP and ECFP-HSL remain highly colocalized (Fig. 8, bottom). (e) Plin enhanced HSL activity upon PKA activation in transfected 293T cells (Fig. 6A). (f) Plin-A-EYFP and ECFP-HSL were colocalized to the same structures in stimulated 293 cells (Fig. 6B).

The above results indicate Plin plays mainly a positive role by defining the sites of lipase action (HSL and possibly the newly discovered lipase, ATGL, Refs. 30 and 31), perhaps by modifying the surface of a specific set of LSDs. This proposal is consistent with the observation that hormone-stimulated lipolysis is severely impaired in mice lacking Plin (19, 20). (For a discussion of contradictory data showing hormone-stimulated lipolysis was unaffected in Plin knock-out mice, see Ref. 20.) It should be noted that these data do not discount the possibility that Plin also plays a negative role by suppressing basal lipolysis. (The fact that we do not see a suppression of HSL activity in Plin-transfected 293T cells (Fig. 6A) may be caused by the lack of other accessory proteins in this system.) There is ample evidence that unphosphorylated Plin supports the creation structures capable of accumulating lipid, and in this sense Plin is protective of stored triglyceride. It seems unlikely, however, that the pro-

**FIGURE 5.** Double immunofluorescence microscopic localization of HSL and Plin in adipocytes from dissociated EWAT. Dissociated adipocytes from adult mice were plated in Matrigel and cultured overnight (see “Experimental Procedures”). The cells were incubated in media containing 200 nM PIA (control) or 10 μM forskolin and 1 mM IBMX (stimulated) for 12 min. Shown are single confocal slice images acquired using the multitracking mode. A–C, control unstimulated culture; D–F, stimulated culture. A and D, Plin staining (green). B and E, HSL staining (red). C and F, representative line scans depicting quantitation across peripheral LSDs and the colocalization coefficients of the line scans. Colocalization of Plin and HSL total cellular fluorescence (Pearson’s r²) was 0.12 and 0.46 in the unstimulated and stimulated cells, respectively. Scale bar, 15 μm.
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Protective effect involves the formation of a continuous physical barrier around LSDs. Recent work indicates that Plin-containing LSDs contain numerous proteins such as caveolin 1 and Abhd5 (33, 34), and we have found that endogenous Plin does not exclude the association of ECFP fusions of Cav2b, Rab5, Abhd5 or EYFP-Plin to Plin containing LSDs in vivo or in vitro. Furthermore, close inspection indicates that Plin is discontinuously associated with individual LSDs following lipolytic activation (e.g. Fig. 4B), yet Plin and HSL remain colocalized. Rather than an exclusionary barrier, Plin may function as a scaffold that assembles a domain that is specialized for hormone-regulated lipolysis.

In situ imaging of adipocytes in adipose tissue with fluorescent fusion proteins indicates that the lipolytic machinery is organized in a special peripheral domain which we term “lipolytic domain.” This domain is specifically targeted by Plin-A-EYFP, is distinct from the central lipid core, and appears to be the site where hormone-induced lipolysis occurs. In some cells ECFP-HSL is enriched in this domain under non-stimulated conditions. Such pre-positioning may account for observations that are not readily accommodated by the barrier/translocation model, including the fact that up to 60% of HSL can be associated with lipid under non-stimulated conditions (35), that hormone-stimulated lipolysis occurs in permeabilized cells (8–10), and that bulk translocation of HSL is not a consistent feature of hormone-stimulated lipolysis (6, 7).

Following stimulation, Plin-A-EYFP and ECFP-HSL were highly colocalized, even under conditions in which the core LSD was highly fragmented into numerous internal LSDs that lacked Plin. These observations suggest that previous biochemical measures of Plin “dissociation” likely represent the generation of LSDs that are less buoyant (and therefore not recovered in the floating fractions) because of HSL-mediated loss of triglyceride.

An important concept that emerges from this work is that lipolysis

FIGURE 6. A, reconstitution of PKA-stimulated lipolysis in transfected 293T cells. Duplicate wells of 293T cells were transiently transfected with Plin-A-EYFP with or without ECFP-HSL. The cells were fed overnight with oleic acid, washed, and stimulated with forskolin and IBMX for 2 h at 37 °C. Glycerol release was measured and expressed as nmol per 10⁶ cells. Plin translocation of ECFP-HSL in transfected 293T cells. 293T cells were transiently transfected with Plin-A-EYFP and ECFP-HSL. The cells were fed overnight with oleic acid, washed, and stimulated with forskolin and IBMX for 30 min at 37 °C. Cells were fixed, and images were acquired using an Olympus spinning disc confocal microscope. Representative line scans across Plin-containing structures are shown.

2 J. Granneman and H.-P. H. Moore, unpublished data.
appears to be organized in a specialized subcellular domain that is distinct from the core LSD. This organization does not exist in model cells lacking core LSDs, but is readily observed in model cells grown in three-dimensions, and in true adipocytes in vitro and in vivo. It is not clear whether the Plin-coated lipid droplets seen in model cells, like the 3T3-L1 adipocytes, are the functional equivalent of the lipolytic domain. These cells may be more similar to newly developing young adipocytes in which the HSL translocation is observed, versus older adipocytes where it is not (7). By contrast, adiporation allows imaging and manipulation of fat cells in their native environment and offers a particularly effective approach to investigating the organization of lipolytic signaling that cannot be readily duplicated in model cell systems.

Proteomic studies suggest that lipid droplets are dynamic organelles with an abundance of metabolic and trafficking proteins in addition to Plin (13, 14). Moreover, Plin has been recently shown interact with proteins like Abhd5 and caveolin (33, 34, 36), raising the possibility that a major role of Plin is to influence the composition of LSDs by directing lipid/protein trafficking into and out of this organelle. Rather than a barrier, Plin may function as a scaffold that helps stabilize or create an organelle whose structure and composition is optimal for lipid storage and regulated lipolysis.
What might be the functions of this specialized lipolytic domain? We hypothesize that several critical functions might be achieved by restricting lipolysis to specialized subcellular domains. The high surface/volume ratio of small peripheral LSDs could increase the efficiency of lipolytic signaling. In addition, LSDs are thought to be surrounded by a phospholipid monolayer, and the expansion and contraction of LSDs (whose surface area changes with the square of its diameter) could require membrane trafficking that might be handled by rab proteins that are enriched in purified LSDs (13, 14). Furthermore, mobilized fatty acids are highly effective detergents, and we hypothesize that restricting lipolysis to specific detergent-insoluble domains limits the deleterious effects of mobilized free fatty acids. Consistent with this notion, caveolins (components of detergent insoluble microdomains) were found on lipid droplets in cultured cells (13, 14, 37, 38), and mice deficient in caveolin 1 show impaired response to lipolytic stimuli (33). Additionally, it is possible that transmembrane signaling molecules are differentially concentrated near the lipolytic machinery. For example, we have shown that β3-adrenergic receptors more effectively phosphorylate LSD Plin versus nuclear Creb, whereas the reverse is true for β1 receptors (39). Finally, it is possible the size of the lipolytic domain varies according to physiological demand. In this regard, very recent work from the Greenberg laboratory has shown that estrogen treatment of ovariectomized mice greatly up-regulates Plin expression, but not HSL, and that up-regulation correlates with enhancement of hormone-sensitive lipolysis (40).

In summary, our results indicate that mature adipocytes store their neutral lipids in functionally distinct pools. Plin is preferentially targeted to a novel set of peripheral LSDs that define the site of hormone-stimulated lipolysis. Significantly, the role of phosphorylated Plin is likely to facilitate the access of HSL to its neutral lipid substrates. Our studies indicate that a full understanding of lipolytic signaling will require development of in vivo approaches to complement the existing culture model systems. Given the importance of lipid handling by fat cells for systemic insulin sensitivity, such approaches are likely to generate new insights into fat cell physiology and pathophysiology that could lead to new points of therapeutic intervention. The current study represents a first step toward this goal.

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