ANALYSIS OF LIPOLYTIC PROTEIN TRAFFICKING AND INTERACTIONS IN ADIPOCYTES
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Running title: Analysis of lipolytic trafficking

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This work examined the colocalization, trafficking and interactions of key proteins involved in lipolysis during brief protein kinase A (PKA) activation. Double label immunofluorescence analysis of 3T3-L1 adipocytes indicated that PKA activation increases the translocation of hormone sensitive lipase (HSL) to perilipin A (Plin) - containing droplets and increases the colocalization of adipose tissue triglyceride lipase (Atgl) with its coactivator, Abhd5. Imaging of live 3T3-L1 preadipocytes transfected with Agquorea Victoria-based fluorescent reporters demonstrated that HSL rapidly and specifically translocates to lipid droplets (LDs) containing Plin, and that this translocation is partially dependent on Plin phosphorylation. HSL closely, if not directly, interacts with Plin, as indicated by fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) experiments. In contrast, tagged Atgl did not support FRET or BiFC with Plin, although it did modestly translocate to LDs upon stimulation. Abhd5 strongly interacted with Plin in the basal state, as indicated by FRET and BiFC. PKA activation rapidly (within minutes) decreased FRET between Abhd5 and Plin, and this decrease depended upon Plin phosphorylation. Together, these results indicate that Plin mediates hormone-stimulated lipolysis via direct and indirect mechanisms. Plin indirectly controls Atgl activity by regulating accessibility to its coactivator, Abhd5. In contrast, Plin directly regulates the access of HSL to substrate via close, if not direct, interactions. The differential interactions of HSL and Atgl with Plin and Abhd5 also explain the findings that following stimulation, HSL and Atgl are differentially enriched at specific LDs.

A central function of adipocytes is the storage and mobilization of energy in the form of triglyceride. A considerable amount is known about the enzymatic basis for lipogenesis and lipolysis, however, our understanding of how these processes are organized and regulated within cells is incomplete. Until recently, cellular triglyceride was considered to be stored in droplets lacking biological structure or organization. Growing evidence, however, suggests that lipid droplets are specialized, heterogeneous organelles that perform distinct roles in lipid biosynthesis, transport and mobilization (1-3). A large number of proteins have been found to change their associations with lipid droplets in response to lipolytic stimulation. How these proteins are coordinated to control lipid storage and utilization remains largely unknown.
It is well established that activation of protein kinase A (PKA) is the major signaling mechanism by which hormones and neurotransmitters stimulate lipolysis in adipocytes (4,5). Most work regarding hormone stimulated lipolysis has focused on perilipin (Plin), a protein that binds to the surface of certain lipid droplets (LDs), and hormone sensitive lipase (HSL), which translocates to lipid during lipolytic activation.

Plin is the major target for protein kinase A (PKA)-mediated phosphorylation in adipocytes (6,7), appears to be essential for hormone stimulated lipolysis (8), and exerts both positive and negative effects on lipolytic rate (9-11). Plin was originally viewed as a physical barrier that passively regulated access of lipases to store triglyceride, however, more recent data indicates that Plin may function more as a LD scaffold that directs the trafficking of lipolytic proteins to a specialized subcellular domain (11). Nonetheless, the temporal and spatial relationships between Plin and other proteins in the lipolytic pathway during PKA activation remain ill-defined.

PKA activation promotes the translocation of HSL to lipid, which correlates with the magnitude of stimulated lipolysis (7,12). Work from several labs indicates that Plin and HSL synergize to trigger PKA-mediated lipolysis (11,13) and our recent work indicates that HSL specifically translocates to a subset of LDs containing Plin (11). It is presently unclear whether Plin and HSL interact directly, whether the interaction takes place on certain LDs and not others, or whether the interaction occurs on a timescale that is compatible with the initiation of lipolysis.

The complexity of regulated lipolysis has recently expanded with the identification of adipose triglyceride lipase (Atgl) (14,15) and discovery that Abhd5, a Plin-interacting protein (16,17), is a key coactivator of Atgl (18). In contrast to HSL, relatively little is known about Atgl. Nonetheless, genetic deletion and knockdown experiments demonstrate that Atgl plays a critical role in basal and stimulated lipolysis (15,19). Presently it is not known whether Atgl translocates to lipid in response to PKA activation, or if it is targeted to specific LDs. As mentioned, Abhd5 is a coactivator of Atgl that binds to Plin with high affinity. Furthermore, chronic (1 hour) lipolytic stimulation leads to the dissociation of Abhd5 from Plin (16,17). It is not known presently whether stimulation alters the interaction of Abhd5 and Plin on at timescale (i.e., minutes) that is relevant to the initiation of lipolysis, or whether the dissociation of Abhd5 from Plin is triggered by Plin phosphorylation. Nonetheless, these observations raise the possibility that PKA phosphorylation of Plin activates Atgl indirectly through its direct interactions with Abhd5.

The above data strongly suggest that lipolysis involves the dynamic trafficking, yet relatively little is known about temporal and spatial relationships among scaffolds, lipases and co-activators during stimulated lipolysis. Below we describe a series of experiments that explore, for the first time, the trafficking of lipolytic proteins using double-label immunocytochemistry of endogenous proteins and high resolution imaging of fluorescence reporters in living cells. The results of these experiments support the hypothesis that Plin acts as a scaffold that lies at the heart of PKA-regulated lipolysis. In vivo protein interaction assays indicate that Plin controls the association of HSL with specific LD surfaces via close, if not direct, interactions. In contrast, Plin does not directly interact with Atgl. Nonetheless, Plin is likely to control Atgl activity indirectly by rapidly releasing Abhd5 in a manner dependent on Plin phosphorylation. Because of the differential association of Atgl and HSL with Plin, these lipases can be targeted to distinct subcellular substrates following lipolytic activation.

**EXPERIMENTAL PROCEDURES**

**Cell culture and transfection**

3T3-L1 adipocytes were generated by growing preadipocytes on 25 mm glass coverslips until confluent, then incubating cells in differentiation medium (DMEM/high
glucose/10% fetal bovine serum/1% penicillin & streptomycin supplemented with 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine or IBMX, and 1 μg/ml insulin) for 3 days. The differentiation medium was replaced with growth medium supplemented with only insulin (1 μg/ml) and cultured for 4 more days.

For fluorescent reporter assays, 3T3-L1 preadipocytes were plated on glass coverslips and cultured in DMEM containing 10% fetal bovine serum overnight. Cultures were transfected at about 60% confluency using Lipofectamine or Lipofectamine LTX (Gibco), as recommended by the manufacturer. Transfection efficiency was 10-20%. Following transfection, cells were incubated 1-2 days in growth medium supplemented with 100 μM oleic acid complexed to BSA to facilitate formation of lipid droplets.

**Immunostaining**

All primary antibodies used were generated in rabbits. Therefore, to examine colocalization of two proteins in the same cell by immunofluorescence histochemistry we employed the procedure of Negoescu et al (20). The key to this approach is the application of saturating levels of a monovalent secondary antibody when detecting the first antigen. Briefly, fixed cells were permeabilized with PBS containing 5% normal goat serum and 0.01% saponin (Permeabilization Buffer) for 15 minutes, then were incubated for 50 minutes with primary antibodies against the first antigen (rabbit anti-HSL at 1:500 dilution, rabbit anti-Abhd5, 1:500, or affinity purified rabbit anti-Atgl 1:300). The slides were washed 4 times with PBS over a 20-minute period, then incubated for 1 hour with 7.5 μg/ml Cy3-conjugated goat anti-rabbit Fab fragment (Jackson ImmunoResearch, West Grove, PA). The slides were washed as before, then incubated for 45 minutes with primary antibodies against the second antigen (rabbit anti-Plin at 1:1000 dilution or rabbit anti-Abhd5 1:500). The slides were again washed and incubated for 45 minutes with 2 μg/ml Alexa 488-conjugated goat anti-rabbit (Fab)2 antibodies (Molecular Probes, Eugene, OR). Washed slides were post-fixed with 1% paraformaldehyde for 15 minutes. 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 eliminated fluorescent signals in the corresponding channel. Antibodies for HSL and Plin were as described (11). Antibodies for Abhd5 were a gift from Dr. D. Brasaemle (16). Rabbit polyclonal antibodies for Atgl were generated against the peptide CNTVAFPDPDALRMAPAS coupled to keyhole limpet hemacyanin. Antibodies were then affinity purified to the peptide. Atgl immunoreactivity was present in adipocytes, but not preadipocytes, and was eliminated by siRNA to Atgl (21).

**Construction of fluorescent reporter proteins**

EYFP was fused to the C terminus of Plin (Plin-EYFP) by cloning Plin into the Kpnl site of EYFP-N1 (Clontech). Plin lacking PKA sites (Δ6Plin) was copied by PCR from a Δ6Plin plasmid template (8) and substituted for wild type Plin to form Δ6Plin-EYFP. ECFP was fused to the N terminus of HSL (11,22), Atgl and Abhd5 using the HindIII site of ECFP-C1. Atgl and Abhd5 were generated by PCR using plasmid (14) or tissue cDNA. All constructs were verified by DNA sequencing.

Bimolecular fluorescence complementation (BiFC) constructs were created with split fragments of EYFP, as detailed by Hu et al (23). The N-terminal fragment, designated Yn, contained amino acids 1-158, while the C-terminal fragment, designated Yc, contained amino acids 155-239. For HSL, Atgl and Abhd5, the Yn fragment was cloned on the N-terminus using the AgeI and HindIII sites of the full length constructs described above to form constructs designated Yn-X, where X= the protein tested for interactions. The Yn fragment contained the linker sequence GGGGSGGGGS on its C-
terminus. The Yc fragment was generated by PCR from a template supplied by Dr. T. Kerppola and was cloned into the AgeI/NotI of the Plin-EYFP construct to form Plin-Yc. This fragment included the amino acid linker KQKVMNH at the N-terminus of Yc. For Atlg-Yc, Atgl was amplified by PCR and cloned into the HindIII/KpnI site of the Plin-Yc. Plin-Yn was generated by cloning the Yn fragment into the AgeI/NotI sites of Plin-Yc.

Microscopy
Images were acquired with the Olympus IX-81 microscope equipped with automated filter controls and a spinning disc confocal unit. Images were captured using a 60X 1.2 NA apochromatic water immersion lens and a Hamamatsu ORCA cooled CCD camera. The following Chroma (Rockingham, VT) filter sets were used for the indicated fluorophores: Alexa 488, 410001; Cy3 and nile red, 41002; EYFP, 31044; ECFP, 41028; EYFP FRET, exciter from 41028 and the dichroic/emitter from 31044. Microscope control and data acquisition was executed using IPLabs (Scanalytics, BD Biosciences) software.

Immunofluorescence images of adipocytes were collected by placing a spacer ring, made of Parafilm, between a clean coverslip and the coverslip containing cells. The coverslips containing cells were inverted so that the top of the cells apposed the objective lens. This orientation allowed best optical resolution of large and small LDs. Optical sections were taken at 0.4 μm z-axis intervals beginning at the surface of the lipid droplets.

For live cell imaging, coverslips containing transfected 3T3-L1 cells were placed in a Leiden chamber containing Krebs Ringer media (Sigma-Aldrich) containing 1 mM calcium chloride and 1% bovine serum albumin, and buffered with 10 mM HEPES (pH 7.4). Experiments were conducted at room temperature (22°-26°C). All cells imaged had similar levels of reporter gene expression. Images were captured at a rate of 1 per minute in confocal mode. Four baseline images were captured over 3 minutes, then cells were stimulated with a 10X concentration of forskolin and IBMX in buffer to yield a final concentration of 2μM and 200 μM, respectively, and data were collected for 9 additional minutes.

Image Analysis
Double-label immunofluorescence. Alexa 488 and Cy3 signals were merged and converted to 8 bit RGB images. Colocalization was performed using ImagePro Plus software (Media Cybernetics), with the analyst blind to the experimental conditions. The cell perimeter was outlined and Pearson’s regression coefficient determined for 2 adjacent confocal slices of each cell, which were averaged to yield one value per cell, which were averaged for presentation.

Movement and colocalization of fluorescent fusion proteins. The aim of this experiment was to examine the translocation and colocalization of lipolytic proteins relative to cellular LDs. For this purpose, the subcellular region containing the largest area of LDs was outlined and fluorescence intensity values of individual pixels in the ECFP and EYFP channels were indexed and imported into Graphpad software. Linear regression analyses were performed on indexed intensity values of the same LD region before stimulation and 8 minutes post stimulation. The extent of colocalization was evaluated by Pearson’s r², while the magnitude of translocation to LDs containing Plin-EYFP was assessed by the ratio of the regression slopes before and after stimulation.

Fluorescence resonance energy transfer (FRET). The experimental setup was identical to the translocation analysis just described. FRET was performed using the three filter method (24) and net FRET calculated using the FRET extension of IPLabs software. FRET signals were localized exclusively to LDs containing Plin-EYFP. To quantify the net FRET signals, the subcellular domain containing Plin-EYFP LDs was defined for each frame using the autosegmentation tool of IPLabs, and the calculated net FRET values of those pixels was summed for each frame. Values for each experiment were normalized to the maximal FRET values obtained for each cell. FRET constants were determined for each experimental day using 3T3-L1 cells transfected with single
fluorescent constructs. For a given experimental day, all acquisition parameters were equivalent for all cells and all channels. The adequacy of the FRET constants in eliminating bleed-through was verified with independent singly-transfected cells. FRET signals were only observed at LDs containing ECFP, and photobleaching of the acceptor eliminated the calculated net FRET signals.

**Bimolecular fluorescence complementation (BiFC).**

3T3-L1 preadipocytes were grown on 18 mm coverslips and were transfected with 500 ng each of the complementary Yn and Yc fusion constructs, along with 130ng of an ECFP tracer to identify transfected cells. Cells were cultured for 24 hours at 32°C in growth media supplemented with oleic acid, then fixed in PBS containing 1% paraformaldehyde. Fixed cells were examined using a 40X 0.9NA air objective by an observer that was blind to transfection conditions. Transfected cells were identified by ECFP fluorescence and cells scored as to the presence or absence of EYFP fluorescence. The rate of false positives arising from cellular autofluorescence (i.e., background) was 2%.

**Statistical analyses**

Differences in colocalization coefficients, slope ratios and total fluorescence were evaluated by one-way analysis of variance, and group means compared by Bonferroni’s test for multiple comparisons. BiFC efficiency was assessed by chi square.

**RESULTS**

**Double-label immunohistochemistry of endogenous Plin, Atgl, HSL and Abhd5 in 3T3-L1 adipocyte**

We first investigated the subcellular colocalization of endogenous lipolytic proteins in differentiated 3T3-L1 adipocytes under basal conditions, and after brief (10 min) lipolytic stimulation with forskolin and IBMX. Figure 1 shows double-labeled immunofluorescence micrographs of representative adipocytes (left), and summarizes the colocalization analysis of >6 cells/group (right). As expected from previous work, Plin was targeted exclusively to LDs. In general, the surface of small LDs stained more intensely than larger LDs, and high resolution confocal images of the surface of larger droplets indicated relatively sparse and discontinuous Plin staining. Abhd5 was mostly localized to lipid droplets, with some cytosolic staining. Double-label immunocytochemistry showed that Plin and Abhd5 proteins were highly colocalized on LDs, as expected from biochemical data showing that these proteins directly interact (16,17). In the unstimulated state, HSL was largely cytosolic, while Atgl was found in the cytosol and on lipid droplets. Double-label immunofluorescence indicated that HSL was weakly colocalized with Plin in unstimulated cells, whereas nearly half of Atgl fluorescence colocalized with Plin. Interestingly, Atgl and Plin appeared to be more strongly colocalized in the basal state than were Atgl and its co-activator, Abhd5.

Following brief stimulation with forskolin and IBMX, HSL was targeted to small- and medium-sized lipid droplets, where it was significantly more colocalized with Plin than under basal conditions. Unlike HSL, stimulation did not alter the overall colocalization of Atgl and Plin. However, Atgl and Abhd5 became highly colocalized following acute stimulation, with much of the increase in colocalization occurring on very small structures. The increased colocalization of Atgl and Abhd5 occurred at the same time there was a small, but significant, decrease in the colocalization of Abhd5 with Plin.

HSL and Atgl were partially colocalized in the cytoplasm before stimulation and on LDs after stimulation, and stimulation did not alter the overall degree of colocalization. As expected by the differential colocalization with Plin and Abhd5, numerous LDs were differentially-enriched with one lipase after stimulation.
The results of double-label immunofluorescence are consistent with the hypothesis that HSL is specifically recruited to Plin-containing LDs. It appears that stimulation decreases the association of Abhd5 with Plin, while increasing its association with Atgl. Overall, it appears that stimulation increases the translocation of HSL to Plin-containing droplets and the interaction of Abhd5 with Atgl. The colocalization of Atgl and Abhd5 occurs at subcellular sites that may or may not contain Plin, and as a consequence of the differential association with Plin, Atgl and HSL exhibit a distinct subcellular distribution following stimulation.

**Movement and interactions of fluorescently-tagged proteins**

Inferences about trafficking based on static immunofluorescence data rely on comparisons of different cells at different times. Furthermore, while changes in average colocalization strongly suggest trafficking, it does not provide information about the magnitude or timing of protein translocation, or whether translocation might occur in the absence of net changes in colocalization. To address these key questions, we generated a panel of fluorescent reporters for monitoring lipolytic protein trafficking and interactions in individual living cells. This approach not only allowed high spatial and temporal resolution of subcellular movement, but also permitted assessment of specific protein-protein interactions using the complementary techniques of FRET and BiFC.

**Stimulation triggers rapid translocation of ECFP-HSL to Plin-EYFP-containing LDs**

ECFP-HSL was largely cytosolic and rapidly translocated to Plin-EYFP containing LDs upon stimulation. Shown in Figure 2 are confocal slices of a 3T3-L1 preadipocyte expressing ECFP-HSL before (Panel A) and 8 minutes following stimulation (B) (video of translocation is provided in supplementary material, Video 1). Following stimulation, the pattern of ECFP-HSL was nearly identical to Plin-EYFP (compare Panels B and C). Plin-EYFP remained highly targeted to lipid droplets prior to and throughout stimulation. In over 50 independent experiments, we have not observed dissociation of Plin-EYFP from lipid droplets during acute (< 15 min.) stimulation.

Quantification of the ECFP-HSL fluorescence at the lipid droplets showed that pronounced translocation that began within 2 minutes of stimulation and peaked by 7 minutes (panel D). The magnitude and specificity of translocation was assessed by correlating, pixel by pixel, the fluorescence intensities of ECFP-HSL and Plin-EYFP before (panel E) and after (panel F) stimulation. In the basal state ECFP-HSL and Plin-EYFP were weakly correlated, and the slope of the relation was shallow, indicating low levels of HSL per unit Plin. Following 8 minutes of stimulation, ECFP-HSL was highly colocalized at Plin-containing droplets, as reflected in the increase in the slope and regression coefficient. Analysis of 10 experiments indicated that stimulation increased translocation to Plin-containing LDs by 3.6 ± 0.6 fold (p <.001) and improved the colocalization (r²) from 0.46 ± .07 to 0.76 ± .03 (p < .003).

As expected from the high degree of colocalization, the concentration of Plin-EYFP was highly predictive of the amount of ECFP-HSL that would translocate to individual lipid droplets. As previously reported, the abundance of Plin-EYFP was greatest at small LDs, and its concentration was far less on LDs with diameters greater than 5 um (11). It was therefore of interest to examine translocation in cells containing a range of LD sizes, with and without abundant Plin. Shown in Figure 3 is a representative cell containing small and moderately sized LDs, identified by post-staining with nile red staining (upper left panel). Plin-EYFP was strongly targeted to small LDs that formed a mat-like structure, while larger LDs contained little, if any, Plin-EYFP. Upon stimulation with forskolin/IBMX, ECFP-HSL rapidly translocated to small droplets containing Plin-EYFP (5.2-fold increase in regression slope), with little translocation to larger LDs (for video, supplementary material Video 2). We did not observe stimulation-dependent translocation...
of ECFP-HSL in the absence of Plin expression (supplementary material, Figure S1). Taken together, these data indicate that HSL specifically translocates to Plin containing LDs, and that large LDs, which do not contain Plin, are not targeted by HSL.

**Translocation of ECFP-HSL is partially dependent on Plin phosphorylation**

Plin is a major target for phosphorylation by PKA, and the dependency of HSL translocation on Plin suggested that PKA-mediated phosphorylation of Plin might be involved. This hypothesis was examined by monitoring the stimulation-dependent translocation of ECFP-HSL to Δ6Plin-EYFP, in which all potential PKA phosphorylation sites were mutated to alanine (8). Stimulation provoked the immediate translocation of ECFP-HSL to Δ6Plin-EYFP-containing LDs (Supplementary Video 3). The time course of translocation was indistinguishable from native Plin. Nonetheless, the magnitude of translocation, as assessed by the ratio of regression slopes, was significantly less than wild type Plin (2.2 ± 0.2 fold versus 3.6 ± 0.6 fold for wild type, p <.04, n = 10), indicating that HSL translocation is partially dependent on Plin phosphorylation.

**Translocation promotes FRET between ECFP-HSL and Plin-EYFP that is independent of Plin phosphorylation**

The use of ECFP and EYFP fusions allowed us to investigate the close interactions of the lipolytic partners using fluorescence resonance energy transfer (FRET). FRET between the ECFP donor and EYFP acceptor only occurs if proteins are within the Forster distance of 2-10 nm, indicating close or direct contact (25). The Forster distance is more than 1 order of magnitude less than the optical resolution of the microscope (~250 nm), and thus allows analysis of changes in protein interactions in the absence of changes in microscopic translocation.

As shown above, ECFP-HSL and Plin-EYFP were partially colocalized under basal conditions. Under these conditions, a small, but detectable FRET signal was found between the ECFP-HSL donor and Plin-EYFP acceptor. Stimulation triggered a 3- to 4-fold increase in the FRET signal that closely corresponded to the translocation of HSL to LDs (Figure 4; supplementary materials for Video 4). Parallel experiments indicated that stimulation-induced translocation of ECFP-HSL to Δ6Plin-EYFP also increased the FRET signal. Overall, FRET signal was proportional to the translocation of the ECFP-HSL donor to Plin-EYFP-containing droplets. Thus, stimulation did not increase the efficiency of FRET nor was stimulation-induced FRET dependent on Plin phosphorylation.

**Stimulation promotes translocation of ECFP-Atgl to LDs, but does not change its colocalization or interaction with Plin-EYFP**

In the absence of Plin, cells expressing Atgl were largely incapable of generating lipid droplets (not shown), however, Plin-EYFP co-expression allowed cells to form lipid droplets to an extent that was not significantly different from cells expressing Plin-EYFP alone. Shown in Figure 5 is a representative experiment (also supplementary Video 5). When co-expressed with Plin-EYFP, ECFP-Atgl was localized on Plin-containing lipid droplets (average r² = .65 ± .01, n=4). This colocalization was significantly greater than was the colocalization of HSL and Plin seen in the basal state, and approached the colocalization of HSL and Plin seen after stimulation (Panel A). Stimulation with forskolin/IBMX induced rapid translocation of ECFP-Atgl to Plin-EYFP-containing LDs, although the magnitude of the increase was less than that seen for ECFP-HSL (Panel B). The mean increase in fluorescence intensity at Plin-containing LDs was 2.6 ± .3 fold (p <.001, n = 4); however, stimulation did not significantly improve the colocalization between the Atgl and Plin reporters. Comparison of the ECFP-Atgl (Panel B) and EYFP-Plin (Panel C) images after stimulation showed that the highest degree of ECFP-Atgl fluorescence increased on extremely small LDs that contained moderate amounts of Plin EYFP. Numerous moderately-sized (~ 2-5 um) LDs that contained abundant Plin-EYFP attracted little, if any ECFP-Atgl. Unlike ECFP-
HSL, ECFP-Atgl did not significantly support FRET to Plin-EYFP acceptor (not shown).

**Stimulation rapidly decreases FRET between ECFP-Atgl and Plin-EYFPAbhd5 in a manner dependent on Plin phosphorylation**

ECFP-Abhd5 was largely cytoplasmic when expressed alone (not shown), but became highly colocalized with Plin-EYFP on cellular LDs when co-expressed ($r^2 > .90$) (Figure 6A). Stimulation for 10 minutes did not cause any discernable change in the subcellular distribution of ECFP-Abhd5, or its strong colocalization with Plin-EYFP.

As mentioned above, FRET allows investigation of changes in protein-protein interactions in the absence microscopic translocation. ECFP-Abhd5 donor strongly supported FRET to Plin-EYFP acceptor, indicating that these proteins strongly interact in the unstimulated state (Figure 6B; supplementary material Video 6). Stimulation lowered the FRET signal within 2 minutes, with a maximal reduction of more than 50% of the pre-stimulation signal ($p < .001$). ECFP-Abhd5 also served as a strong donor for FRET with phosphorylation-defective Plin-EYFP. In contrast to wild type Plin, stimulation was largely, if not completely, ineffective in reducing the FRET signal arising from the interaction of ECFP-Abhd5 with Δ6Plin-EYFP.

**Interactions between ECFP-Abhd5 and Atgl-EYFP**

Cells that co-expressed ECFP-Atgl and EYFP-Abhd5 did not accumulate appreciable lipid in the absence or presence of Plin (Figure 7, top) or Δ6Plin (not shown). No FRET signal was detected in cells lacking lipid. Occasionally, cells could be found that contained from 2-5 very small lipid droplets (Figure 7, bottom). Under these conditions, Abhd5 and Atgl were strongly colocalized at these lipid droplets where they closely interacted, as detected by FRET. Because cells co-expressing Atgl and Abhd5 had few or no lipid droplets, no attempt was made to investigate the effects of stimulation.

**Bimolecular fluorescence complementation (BiFC) between lipolytic proteins**

BiFC is a recently-developed method for investigating protein interactions in vivo (26). The method is based on the ability of non-fluorescent fragments of EYFP to form a fully-fluorescent protein when these fragments are held in close proximity by the interaction of fusion partners. The approach is conceptually similar to FRET in that the interaction of the fusion proteins generates a signal that would not otherwise exist. However, an advantage of BiFC is that the individual reporters do not generate any signal in the absence of an interaction between the fusion partners. In contrast to FRET, BiFC signals accumulate slowly (over hours), and once formed are essentially permanent. Thus, BiFC signals indicate protein interactions integrated over hours, with excellent signal to noise properties.

We confirmed that transfection of the test proteins fused to either N or C terminal fragments of EYFP did not yield a detectable signal above cellular autofluorescence (not shown). To assess the efficiency of BiFC, cells were cotransfected with ECFP to identify transfected cells, and the percentage of ECFP positive cells exhibiting EYFP fluorescence (i.e., BiFC) determined. BiFC was observed to varying degrees (Table 1). Interestingly, BiFC signals were observed only at lipid droplets for all positive pairs of interacting proteins (Figure 8). Of these, the interaction of Abhd5-Yn with Plin-Yc was the strongest, as it occurred in virtually all transfected cells and the fluorescence intensity per cell was about 4 times greater than any other positive combination. Interestingly, while the Yn-Abhd5/Plin-Yc combination produced efficient BiFC, Abhd5-Yc did not produce BiFC when paired with Plin-Yn, even though each was capable of BiFC with other partners (Table 1). This observation demonstrates the specificity of the BiFC interaction, and indicates that ability to form BiFC is sensitive to the orientation of the interacting partners.
BiFC also occurred between Yn-HSL and Plin-Yc, as anticipated by the colocalization and FRET experiments described above. In all cases, BiFC fluorescence was localized to lipid droplet structures, indicating that the affinity of Plin for lipid dominates the subcellular targeting of the complex. BiFC fluorescence was also observed between Δ6Plin-Yc and Yn-HSL (not shown), also consistent with data from the translocation and FRET experiments. Cells exhibiting HSL-Plin BiFC have HSL irreversibly targeted to lipid droplets, and it is noteworthy in this regard that these BiFC signals were localized to numerous, well-defined lipid droplets. This contrasts to similar experiments with Atgl where little lipid was observed, and suggests that although BiFC constitutively targets HSL to lipid droplets, the complex has low activity.

In contrast to HSL, Atgl did not form significant BiFC with Plin. Each of the BiFC pairs used to assess possible Atgl-Plin interactions was capable of producing BiFC with other partners (Table 1) but not with each other, strengthening the conclusion that Plin and Atgl do not interact in vivo.

As expected, BiFC was observed between Yn-Abhd5 and Atgl-Yc. Like FRET, BiFC signals were always associated with lipid droplets. Unlike HSL-Plin BiFC, the cells exhibiting Abhd5-Atgl BiFC had relatively few LDs that were very small, suggesting high lipolytic activity.

Somewhat unexpectedly, we observed BiFC between Plin-Yn and Plin-Yc. These results suggest that Plin may cluster or form higher-order oligomers, consistent with a scaffolding function. The Plin BiFC pairs appear to be functional in that they supported stimulation-induced translocation of HSL (not shown).

DISCUSSION

Lipolysis obviously occurs at the interface between hydrophobic triglyceride and the aqueous cytoplasma, however, where and how this takes place in cells remains poorly characterized. Until recently, cellular triglyceride was considered to be stored in droplets lacking biological structure or organization; however, growing evidence indicates that lipid droplets are specialized, heterogeneous organelles that perform distinct roles in lipid biosynthesis, transport and mobilization (1,2,27,28). Moreover, there is an emerging appreciation that hormone stimulated lipolysis involves the dynamic interactions of scaffold proteins, lipases and co-activators at specialized lipid droplets.

Previous work established that PKA-stimulated lipolysis involves the translocation of HSL to LDs containing Plin (7,12). While Plin has been often characterized as being a physical barrier that prevents attack by cellular lipases (3,29), we have suggested that Plin is best described as a scaffold that organizes the trafficking of lipolytic proteins at specialized LDs (11). The present work investigated the organization and trafficking of lipolytic proteins by 1) investigating the subcellular distribution of endogenous proteins under basal and stimulated conditions using double-label confocal microscopy, 2) by imaging the trafficking of fluorescently-tagged reporters in living cells and 3) by evaluating protein-protein interactions in living cells with FRET and BiFC.

Analysis of the subcellular distribution of endogenous lipolytic proteins confirmed that stimulation induces the translocation of HSL to Plin-containing LDs. While this general phenomenon of HSL translocation to lipid is well known, the present work demonstrates that HSL translocates specifically to droplets containing Plin, and that this likely involves direct interaction of HSL with Plin. As detailed previously (11) and confirmed here, the concentration of Plin at LD surfaces is inversely correlated with LD size, and most LDs with diameters greater than 5 um have little detectible Plin. Data from endogenous and fluorescently-tagged proteins demonstrate that HSL accumulates at droplets containing Plin after stimulation. This relation holds for individual droplets as well, where the Plin and HSL are colocalized in a discontinuous pattern after stimulation. The stimulation-induced
translocation of HSL to LDs requires Plin and is partially dependent upon Plin phosphorylation in 3T3-L1 cells.

Experiments using FRET and BiFC strongly indicate that HSL and Plin interact very closely, if not directly, in living cells, and that this interaction is rapidly promoted by PKA activation. Previous investigators have been unable to detect interactions between HSL and Plin by yeast two-hybrid or co-immunoprecipitation from detergent extracts (30). In contrast, the present experiments use techniques that address interactions at LD surfaces in living cells. In this regard, we have found that HSL interactions with Plin (including translocation, FRET and BiFC) only occur at structures containing lipid (Granneman, unpublished). These observations indicating that lipid is essential in stabilizing HSL-Plin interactions, and is consistent with recent experiments demonstrating that stimulation increases the ability to chemically cross-link Plin and HSL in situ (8).

Plin has been widely described as a barrier that physically prevents access of lipases to stored triglyceride. The facts that 1) Plin does not continuously cover LDs in the unstimulated state, 2) Plin does not dissociate from LDs on timescales relevant to initiation of lipolysis, 3) HSL translocation requires Plin, 4) HSL does not translocate to droplets lacking Plin, 5) HSL and Plin interact closely following stimulation and 6) Atgl is constitutively bound to Plin-containing LDs do not support the view that Plin functions as a lipase barrier at the LD surface. Rather, the data support the hypothesis that Plin functions as a scaffold that directs the movement lipolytic proteins. These data are fully compatible with recent observations in primary adipocytes of Plin knockout mice (31,32), and account for the observation that Plin and HSL synergistically promote PKA-dependent lipolysis (8,11,13).

The hypothesis that Plin acts as a scaffold for the PKA-mediated regulation of lipolysis is further supported by experiments examining its interactions with Abhd5. Abhd5 was first identified as a protein of unknown function that directly interacted with Plin, and that long term (1 hour) PKA activation led to its reversible dissociation from Plin-containing LDs (16). The biochemical function of Abhd5 was recently clarified by Zechner’s lab, which showed that Abhd5 is a co-activator of Atgl, a recently discovered lipase (18). Using double-label immunocytochemistry, we found that endogenous Abhd5 and Plin were highly colocalized in unstimulated fat cells. Brief (10 min.) stimulation moderately decreased the colocalization between Plin and Abhd5, and simultaneously increased the colocalization of endogenous Abhd5 with Atgl. Much of the increased colocalization occurred at small LDs and structures lacking Plin. While it is likely that complexes of Abhd5 and Atgl are active at Plin-containing droplets, our results suggest that the increase in the interaction of Abhd5 and Atgl that can be attributed to PKA activation occurs largely at sites lacking Plin. The data indicate that Plin acts as a sink for Abhd5 in the basal state and a donor of the co-activator following PKA activation. This scheme indicates that Plin regulates the activity of Atgl indirectly by sequestering or providing Abhd5. In this regard, the ability of Plin to sequester Abhd5 likely contributes to the well-known ability of Plin expression to promote lipid accumulation (9).

FRET analysis in living cells demonstrated that stimulation rapidly decreases the interaction of Plin and Abhd5, and that this regulation is dependent upon Plin phosphorylation. These dynamic data and the immunofluorescence data detailed above support the involvement of Atgl in PKA-dependent lipolysis. Double-label immunofluorescence indicates that there is considerable amount of Atgl is constitutively associated with LDs containing Plin and Abhd5 in the unstimulated state, in contrast to HSL. The stimulation-induced release of Abhd5 from Plin offers an efficient means of activating Atgl locally on Plin-containing droplets without significant translocation from cytosolic pools. It is also likely that complexes of Atgl and Abhd5 that are formed following stimulation target non-LD structures, like ER membranes. In this regard, it is clear from all experimental approaches taken
that HSL and Atgl can be partially targeted to different subcellular substrates because of differential interactions with Plin. Thus, HSL and Plin strongly interacted in BiFC and FRET experiments, but Atgl and Plin did not. Following stimulation, HSL and Atgl could be found differentially enriched on individual LDs, with HSL associating with Plin-containing LDs, and complexes of Abhd5 and Atgl targeting very small LDs, and perhaps other structures.

Double label immunofluorescence data indicate that PKA activation induces trafficking of Abhd5 to Atgl. We were able to confirm the direct interaction of Abhd5 with Atgl in vivo by FRET and BiFC. Unfortunately, we have been unable to monitor the dynamic trafficking among Plin, Abhd5 and Atgl because cells that co-express Abhd5 and Atgl did not accumulate enough lipid for analysis. We were somewhat surprised that expression of Plin or Δ6Plin did not permit significant lipid accumulation (although it did allow accumulation to occur when Atgl was expressed in the absence of Abhd5). The reason for this is not clear. On one hand, it could indicate that even when Abhd5 is bound to Plin it can activate Atgl. Alternatively, it is likely that Abhd5/Atgl complexes act at sites of triglyceride synthesis and early transport, thereby preventing the accumulation of triglyceride in relatively late LDs that contain Plin. Resolution of these questions will likely require development of techniques for reconstituting components in situ.

Based upon the present results and available data, we propose a general model for the initiation of PKA regulated lipolysis depicted in figure 9. In this scheme, Plin is an LD scaffold protein that regulates the trafficking of lipolytic effectors in response to PKA activation. In the basal state, Plin and Abhd5 are tightly bound. Some Atgl is localized to LDs containing Plin, however, Atgl is likely to be inactive since its co-activator is sequestered by Plin. PKA activation leads to Plin phosphorylation which has two parallel effects. First, Plin phosphorylation frees Abhd5 to activate Atgl on LDs with and without Plin. Second, PKA activation promotes the rapid translocation of HSL to Plin-containing LDs, in a manner that is partially dependent on Plin phosphorylation. Atgl and HSL appear to have complementary enzymatic activities (15,33), and the coordinated regulation of these lipases at Plin-containing LDs is essential for full, regulated lipolysis.

Needless to say, the present model lacks details that will be required for a full appreciation of hormone stimulated lipolysis. LDs contain an array of trafficking and metabolic proteins (1,2,27) and the dynamic interaction among these proteins during acute and chronic PKA activation remains largely unexplored. The present experiments take advantage of traditional immunological techniques as well as new “in vivo proteomic” assays to further investigate subcellular protein interactions, and suggest that this general approach will be useful in the further analysis of lipolytic trafficking.
REFERENCES
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Abbreviations: Atgl: adipose tissue triglyceride lipase; Abhd5: Abhydrolase domain containing 5; BiFC: Bimolecular fluorescence complementation; FRET: fluorescence resonance energy transfer; HSL: hormone-sensitive lipase; IBMX: 1-methyl-3-isobutylxanthine; LD: lipid droplet; PKA: protein kinase A; Plin: perilipin;
FIGURE LEGENDS

Figure 1. Left: Double-label immunofluorescence micrographs of lipolytic proteins in 3T3-L1 adipocytes. Cells were maintained under basal conditions, or were stimulated with forskolin/IBMX for 10 minutes. Left: Representative confocal images. R = red, G = green. Bar = 10 um. Right: Summary of colocalization of lipolytic proteins in 3T3-L1 adipocytes under basal conditions, and after 10 minutes of stimulation. Colocalization was performed as detailed in Methods. Values are means +/- SEM. *** p<.001 versus control.

Figure 2. Stimulation-induced translocation of ECFP-HSL to LDs containing Plin-EYFP in a live 3T3-L1 preadipocyte. A: ECFP-HSL fluorescence before stimulation. AFU: Arbitrary Fluorescence Units. B:: ECFP-HSL 8 minutes after stimulation with forskolin/IBMX. C: Plin-EYFP 8 minutes after stimulation (Plin-EYFP does not translocate in response to stimulation). D: Time course of stimulation-induced translocation of ECFP-HSL. Forskolin/IBMX was added at time= 0. E: Plot of ECFP-HSL and Plin-EYFP fluorescence at cellular LDs before stimulation. Slope = .077 ± .001 and \( r^2 = 0.56 \). F: Plot of ECFP-HSL and Plin-EYFP fluorescence at cellular LDs 8 minutes after stimulation. Slope = .370 ± .003 and \( r^2 = 0.77 \).

Figure 3. Live imaging of Plin-EYFP and ECFP-HSL in a 3T3-L1 preadipocyte with large and small LDs. Plin-EYFP was heavily concentrated on small, but not large LDs (labeled with Nile Red). Stimulation (8 minutes) provoked translocation of ECFP-HSL only to LDs containing Plin-EYFP. Bar = 10um

Figure 4. Effect of stimulation on net FRET between ECFP-HSL and Plin-EYFP. Net FRET was calculated using the three-filter technique and normalized to the maximal signal of each individual experiment. Filled circles, cells expressing wild-type Plin-EYFP; open circles, cells expressing \( \Delta 6 \)Plin-EYFP. Values are means +/- SEM., n = 5-6.

Figure 5. Stimulation-induced translocation of ECFP-Atgl to LDs containing Plin-EYFP in a live 3T3-L1 preadipocyte. A,B: ECFP-Atgl fluorescence before and 8 minutes after stimulation with forskolin/IBMX. C: Plin-EYFP fluorescence 8 minutes after stimulation. D: Time course of changes in ECFP-Atgl fluorescence at LDs. E, F: Correlation of ECFP-Atgl and Plin EYFP fluorescence before and after stimulation. AFU: Arbitrary Fluorescence Units. Stimulation increased ECFP-Atgl fluorescence at LDs, but did not improve colocalization with Plin-EYFP. Stimulation had no effect on Plin-EYFP fluorescence intensity.

Figure 6. Stimulation rapidly decreases the interaction of Plin-EYFP and ECFP-Abhd5 without inducing translocation. A: Fluorescence micrographs of Plin-EYFP and ECFP-Abhd5 before and after stimulation in 3T3-L1 preadipocytes. B: Effect of stimulation on net FRET signals generated by the interaction of Abhd5 with Plin-EYFP (n=9) and \( \Delta 6 \)Plin-EYFP (n=6). Values are means ± SEM.

Figure 7. Co-expression of ECFP-Atgl and EYFP-Abhd5 prevents lipid accumulation in 3T3-L1 preadipocytes. Top: Identical microscopic field showing a 3T3-L1 preadipocyte co-expressing ECFP-Atgl and EYFP-Abhd5 surrounded by nontransfected cells. The cotransfected cell (arrow) did not accumulate lipid, as indicated by Nile red staining, compared to adjacent nontransfected cells. Bottom: Rare cell containing 2-5 small LDs, exhibiting FRET between ECFP-Atgl and Abhd5-EYFP localized to LDs.
**Figure 8.** BiFC between lipolytic proteins in 3T3-L1 preadipocytes. Left: BiFC signals are shown in green, while the ECFP tracer is pseudo-colored red. Right: BiFC signals correspond to intracellular LDs stained with nile red.

**Figure 9.** Working model of lipolytic trafficking immediately following PKA activation. Top: Abhd5 and Plin are bound in the basal state, while HSL is largely cytosolic and Atgl is both cytosolic and partially bound to intracellular LDs. Bottom: Stimulation results in the phosphorylation-dependent dissociation of Abhd5 from Plin and its association with Atgl at LDs with and without Plin. Stimulation also triggers rapid translocation of HSL to Plin-containing LDs in a manner partially dependent on Plin phosphorylation. Once on LD surfaces, HSL and Plin closely interact in a manner that does not depend on Plin phosphorylation. TG, DG and MG: triglyceride, diglyceride and monoglyceride, respectively.
Table 1. Efficiency of BiFC between lipolytic proteins in 3T3-L1 preadipocytes.

<table>
<thead>
<tr>
<th>BiFC pair</th>
<th>Positive/ Transfected</th>
</tr>
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<tbody>
<tr>
<td>Yn-Abhd5 / Plin-Yc</td>
<td>106/106\textsuperscript{a}</td>
</tr>
<tr>
<td>Yn-Abhd5 / Atgl-Yc</td>
<td>95/100\textsuperscript{b}</td>
</tr>
<tr>
<td>Yn-HSL / Plin-Yc</td>
<td>85/105\textsuperscript{c}</td>
</tr>
<tr>
<td>Plin-Yn / Plin-Yc</td>
<td>64/100\textsuperscript{d}</td>
</tr>
<tr>
<td>Yn-Atgl / Plin-Yc</td>
<td>5/109\textsuperscript{e}</td>
</tr>
<tr>
<td>Plin-Yn / Atgl-Yc</td>
<td>1/101\textsuperscript{e}</td>
</tr>
</tbody>
</table>

Frequencies with different superscripts significantly differ (p < .025) by chi square statistic. Values of Yn-Atgl / Plin-Yc and Plin-Yn / Atgl-Yc are not significantly different from background.
Figure 1.
Figure 2.
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Figure 6.

(A) Images showing basal and stimulated conditions for Plin-EYFP and ECFP-Abhd5.

(B) Graph showing Net FRET over time for different conditions: △Plin-EYFP + ECFP Abhd5 and Plin-EYFP + ECFP-Abhd5.
Figure 8.
Figure 9.