Physical Association between the Adipocyte Fatty Acid-binding Protein and Hormone-sensitive Lipase

A FLUORESCENCE RESONANCE ENERGY TRANSFER ANALYSIS

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Previous in vitro studies have established that hormone sensitive lipase (HSL) and adipocyte fatty acid-binding protein (AFABP) form a physical complex that presumably positions the FABP to accept a product fatty acid generated during catalysis. To assess AFABP-HSL interaction within a cellular context, we have used lipocytes derived from 293 cells (C8PA cells) and examined physical association using fluorescence resonance energy transfer. Transfection of C8PA cells with cyan fluorescent protein (CFP)-HSL, yellow fluorescent protein (YFP)-adipocyte FABP, or YFP-liver FABP revealed that under basal conditions each protein was cytoplasmic. In the presence of 20 μM forskolin, CFP-HSL translocated to the triacylglycerol droplet, coincident with BODIPY-FA labeled depots. Fluorescence resonance energy transfer analysis demonstrated that CFP-HSL associated with YFP-adipocyte FABP in both basal and forskolin-treated cells. In contrast, little if any fluorescence resonance energy transfer could be detected between CFP-HSL and YFP-liver FABP. These results suggest that a pre-lipolysis complex containing at least AFABP and HSL exists and that the complex translates to the surface of the lipid droplet.

Lipolysis in adipocytes is a complex biochemical process brought about by a combination of hormonal and metabolic determinants (1–6). Generally described, adipocyte lipolysis involves hormonal activation of protein kinase A that results in the phosphorylation of at least two key proteins, perilipin A, a droplet-associated protein implicated in controlling access of hydrolytic enzymes to the triacylglycerol, and the hormone-sensitive lipase (HSL),¹ believed to be the primary lipase in fat cells responsible for hydrolysis of stored lipid (7). Phosphorylation of perilipin A results in the dynamic restructuring of the lipid droplet, thereby allowing access of the phosphorylated hormone-sensitive lipase to the triacylglycerol substrate (8, 9). Although these two proteins are appreciated as central to lipid mobilization, other droplet-associated proteins, including other lipases or accessory factors, may also participate in the process and add additional levels of complexity and control (10–13).

Recently another participant in the lipolysis process has been identified as the intracellular fatty acid binding protein (FABP). The adipocyte FABP forms a physical complex with the hormone-sensitive lipase, activating the enzyme by sequestering fatty acids and relieving product inhibition (14). Association between the two proteins has been evaluated in vitro using a combination of yeast two-hybrid analysis, glutathione S-transferase pull downs, and co-immune precipitation as well as deletion and point mutation analysis (14, 15). The results of these studies indicated that the adipocyte FABP forms a complex with the amino-terminal domain of HSL (referred to as a docking domain) in a region bounded by amino acids 190–200 of the lipase (16). Docking of AFABP onto the amino-terminal domain of HSL juxtaposes the fatty acid-binding protein adjacent to the catalytic carboxyl-terminal region so as to accept a product fatty acid during triacylglycerol hydrolysis.

To explore the specificity of HSL-FABP interaction, isothermal titration microcalorimetry has recently been used (17). Using isothermal titration microcalorimetry, both the adipocyte and keratinocyte FABPs were shown to associate with HSL in a 1:1 stoichiometry and an affinity in the low nanomolar range (17). In contrast, the intestinal and liver FABP forms, proteins that exhibit the same α-carbon fold as does AFABP but have grossly different amino acid sequences, did not associate to any measurable extent. Moreover, the physical association of adipocyte FABP with HSL required the presence of fatty acids, but due to the experimental design, it was not clear if such fatty acids associate with the FABP or the lipase.

The in vitro analysis of FABP association with HSL using purified components does not address many of the regulatory features linked to lipolysis in vivo. For example, it is not clear if the FABP and HSL form a basal complex that co-translocates to the surface of the droplet or if HSL translocates to the droplet, at which time the FABP docks onto the enzyme. Also, it is not clear what role hormone-stimulated phosphorylation of HSL has to play in FABP association; all studies to date have been carried out using non-phosphorylated proteins. To address the first of these questions, we have turned our attention...
to the use of lipocytes derived from 293 cells overexpressing a combination of fatty acid transport protein 1 and perilipin A asting as a simple vehicle for assessing FABP-HSL interaction within the cellular context. Because of the high endogenous level of FABP in adipocytes, the surrogate cell line lacking HSL or FABP but having lipid droplets affords the opportunity to study protein-protein interaction as well as translocation in a hormonally sensitive system. From perilipin studies carried out by Londos et al. (7) as well as studies in the Greenberg laboratory (18) with cells expressing combinations of the fatty acid transport protein 1 and perilipin A, it is evident that cells expressing perilipin A exhibit increased hormone-stimulated lipolysis. Additionally, Hatch et al. (19) found that 293 fibroblasts expressing FATP1 exhibit increased oleate and linoelate influx and that such lipid is preferentially channelled into triacylglycerol. To that end, we stably co-express perilipin A and FATP1 in 293 cells producing lipocytes that accumulate triacylglycerol droplets in defined structures. Into such cells we have transiently expressed fluorescent fusion proteins of CFP-HSL and YFP-FABP and evaluated not only translocation of HSL in response to forskolin stimulation but also physical association between the lipase and FABP by FRET under basal and lipolytic conditions. In this report we find that the adipocyte FABP, but not liver FABP, forms a complex with HSL under basal conditions and that the pre-lipolysis complex co-translocates to the surface of the lipid droplet in the presence of forskolin.

EXPERIMENTAL PROCEDURES

Materials—Fatty acids were obtained from Nu-Chek Prep, Inc., Ely-
sian, MN. pCDNA3.1/Zeo, Zeocin, Geneticin, Lipofectamine, and tissue culture reagents were obtained from Invitrogen. BODIPY D 3835 (4,4-
difluoro-5-(3-octadecyl-5-carboxyfluorescein)-5-ethyl-6-carboxyfluorescein) and Alexa Fluor 488 were obtained from Molecular Probes, Inc., Eu-
gene, OR. pEYPF-C1 and pECFP-C1 were obtained from Clontech Laboratories, Inc., Palo Alto, CA. Gold Seal glass coverslips for imaging were purchased from Thomas Scientific, Swedesboro, NJ. The non-
esterified fatty acid (NEFA) assay system was purchased from WAKO Chemicals, Richmond, VA. Cholesterol [1-14C]olate was purchased from Amersham Biosciences. All other reagents were purchased from Sigma-Aldrich.

Cloning and Cell Biology—An A206K mutation was introduced into each of the green fluorescent protein derivatives to reduce intrinsic dimerization (20) using the QuikChangeTM site-directed mutagenesis technique of Stratagene Cloning Systems, La Jolla, CA. HSL was then subcloned in-frame into the A206K pECPF-C1 expression vector, whereas the FRET pair (CFP-LFP were subcloned into pCDNA3.1/Zeo). Expression of the fusion proteins was confirmed by both the expression of fluorescence in 293 cells and by Western blot analysis. Perilipin A was subcloned into pCDNA3.1/Zeo and expression in 293 cells con-
formed by Western blot analysis. All cloning was verified by DNA sequencing.

Generation of C8PA Lipocytes—Perilipin A, subcloned into pCDNA3.1/Zeo, was linearized and introduced into 293 cells stably expressing FATP1 (C8 cells) by electroporation according to the manu-
facturer's instructions (BTX division of Genetronics, Inc., San Diego, CA). Dilutions of the electroporated cells were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum onto surfaces pretreated with 25 μg/ml of poly-l-lysine to enhance attachment and incubated at 37 °C, 5% CO2. After 48 h, selection was initiated with the addition of 300 μg/ml Zeocin for expression of perilipin A and 400 μg/ml of Geneticin for expression of FATP1. Individual lines were developed and analyzed immunochemically for perilipin A expression using a goat anti-perlipin A antibody (9) and/or FATP1 expression using an anti-FATP1 antibody (19). A stable cell line expressing perilipin A and FATP1 (referred to as C8PA) was selected and maintained on 300 μg/ml Zeocin and 400 μg/ml Geneticin during the course of all studies. C8PA cells for all experiments were plated onto surfaces pre-
treated with 25 μg/ml of poly-l-lysine.

Immunocytochemistry—C8PA cells plated on 13-mm glass coverslips and incubated in 300 μM oleic acid, 100 μM fatty acid-free BSA were fixed in 3% paraformaldehyde for 15 min at 25 °C. The cells were rinsed in phosphate-buffered saline (PBS, pH 7.2) containing 0.01% digitonin and incubated for 2 h at 25 °C in blocking buffer containing 0.1% phosphate, 5% goat serum, 5% glycerol, 1.0% cold water fish gelatin, and 0.01% digitonin. The cells were subsequently incubated overnight at 4 °C in a 1:500 dilution of goat-anti-perlipin antibody (9) in PBS containing 0.01% digitonin. After washing in PBS with 0.01% digitonin, the coverslips were incubated for 1 h at 25 °C in a 1:400 dilution of Alexa Fluor 488 (2 mg/ml) donkey-anti-goat IgG in PBS with 0.01% digitonin. After washing, the coverslips were incubated at 25 °C in Dulbecco's modified Eagle's medium with BODIPY D3835 (10 μM), then washed with PBS, mounted, and visualized for perilipin or fat droplets by confocal microscopy.

Confocal Microscopy—Preparations were viewed using a Bio-Rad MRC-1024 confocal microscope attached to a Nikon Diaphot inverted microscope (Bio-Rad) equipped with a 15-milliwatt krypton/argon laser. Excitation filters allowing 488 and 568 nm were used sequentially to visualize the Alexa 488 and BODIPY probes, respectively. The samples were viewed using either a 20X, 0.75 n.a. plan apo or a 40X, 1.0 n.a. plan apo objective. Digital images were collected using plasmashift version 3.2 software (Bio-Rad). Stored digital images were analyzed using Image Pro Plus Version 4.5 software (Media Cybernetics, Silver Spring, MD 20910).

Lipolysis in C8PA Cells—For in situ lipolysis, C8PA cells were loaded with lipid to maximize droplet formation by a 48-h incubation with 300 μM oleic acid complexed to 100 μM fatty acid-free BSA. After loading conditions, the cells were washed 2 times with saline and incubated either with or without 20 μM forskolin in Krebs-Ringers Hepes buffer (pH 7.4) with 2% BSA and 5% glucose for 4 h (18). Media was withdrawn at 0, 1, 2, 3, and 4 h and assayed for non-
esterified fatty acids.

Fluorescence Resonance Energy Transfer—For FRET analysis C8PA cells were plated onto 13-mm coverslips placed into 12-well dishes. At ~70% confluence wells were transfected with expression plasmid specific for CFP-HSL and/or YFP-FABP. 24 h later cells were lipid-loaded with 300 μM oleic acid, 100 μM BSA to maximize droplet formation. Lipolytic conditions were initiated by the addition of 20 μM forskolin, and digital images for FRET were captured after 4 h.

During microscopy cells were kept in media plus 10% fetal bovine serum at room temperature. Digital images were collected using a Bio-Rad MRC-1024 confocal microscope attached to a Pentium IV 2.6 GHz personal computer using Image Pro Plus version 4.5 software (Media Cybernetics) for microscope automation and image analysis on a Nikon Eclipse E800 photomicroscope. Images were captured with a 40X, 0.75 n.a. plan fluor or a 60X, 1.40 n.a. plan apo objective. Cells with relatively equal levels of expression were selected for imaging. For detection of CFP-HSL, cells were viewed with an excitation filter of 406/20 nm, a dichroic beam splitter of 455 nm, and an emission filter of 480/40 nm. YFP-FABP was detected by using a filter set with an excitation filter of 500/25 nm, a dichroic beam splitter of 515 nm, and an emission filter of 535/30 nm. The filters for FRET were an excitation filter of 436/20 nm, a dichroic beam splitter of 455 nm, and an emission filter of 535/50 nm. Filters were selected from the Chroma Technology (Brattleboro, VT). Images were acquired using 2 × 2 bin-
ing mode and 100–250-ms integration times on the camera. The exposure times were equal within each series of images and were chosen so that all pixel intensities were within the linear range of the camera. Images were first background-subtracted and registered to ensure accurate pixel alignment. The CFP-HSL image because it had the largest signal-to-noise ratio, providing the clearest distinction between the cell and background. The thresholded CFP-HSL image was used to generate a binary image with all values within the cell = 1 and all outside = 0. The FRET and YFP-FABP images were multiplied by the binary image, ensuring that the same pixels were analyzed in all three images as described in detail (21). Emission appearing in the FRET image because of emission from CFP-HSL or direct excitation of YFP-
FABP was removed by subtracting a fraction of the CFP-HSL and YFP-FABP images from the FRET image. This fraction depended on the filter set and exposure condition used and was determined as described (21). Corrected FRET was calculated on a pixel-by-pixel basis for the entire image using: corrected FRET = FRET (0.50 × CFP-HSL) − YFP-FABP. YFP-FABP correspond to background-subtracted images of cells co-expressing CFP-HSL and YFP-FABP acquired through the FRET, CFP, and YFP channels, respectively. 0.50 and 0.02 are the fractions of bleed-through of CFP and YFP fluorescence, respectively, through the FRET filter channel. Controls were performed in which images were obtained in different orders. The order in which images were obtained had no effect. For presentation, a low pass filter kernel was applied to the corrected FRET
substrate was emulsified by adding 1 ml of 0.1M potassium phosphate
tained when cells were treated with 1 mM dibutyryl-cAMP
levels albeit in the absence of HSL. Similar results were ob-
acid release from cultured adipocytes (24), it does demonstrate
Although this 2-fold stimulation is significantly less than fatty
C8PA lipocytes in a process that was linear for at least 4 h.
forskolin stimulated a 2-fold increase in fatty acid release from
assays. Substrate preparation and assay conditions were as previously
evaluated fatty acid release from the cells. As shown in Fig. 1

RESULTS

Previously we have reported the development of fibroblastic
cells stably expressing fatty acid transport protein 1 (19). Such
cells, termed C8 cells, exhibit increased transport of long chain
fatty acids and accumulate triacylglycerols. However, triacyl-
glycerol accumulated by C8 cells was disorganized and
appeared rather diffusely distributed within the cell based on
oil red O staining. Greenberg and co-workers (18) as well as the
Londos laboratory (7) have reported that perilipin A, the key
droplet-associated protein in fat cells, organizes the lipid into
well defined structures and provides hormonal competence. To
that end we stably transected C8 cells with perilipin A (PA) to
produce a cell line termed C8PA that not only transported fatty
acids and synthesized triacylglycerol but organized said lipid
into well defined droplets. Fig. 1A shows the oil red O staining
of C8PA cells after a 48 h “load” in which FA-BSA (3:1) was
incubated in the medium to provide substrate for FATP1. We
refer to such lipid filled C8PA cells as lipocytes.

Adipose tissue in vivo as well as cultured adipocytes exhibits
CAMP-dependent activation of lipolysis and increases free fatty
acid release from cells (1). To assess the lipolytic capacity of
C8PA cells, we incubated cells with 20 mM forskolin for 4 h and
evaluated fatty acid release from the cells. As shown in Fig. 1B,
forskolin stimulated a 2-fold increase in fatty acid release from
C8PA lipocytes in a process that was linear for at least 4 h.
Although this 2-fold stimulation is significantly less than fatty
acid release from cultured adipocytes (24), it does demonstrate
that the cultured lipocytes are lipolytically competent at low
levels albeit in the absence of HSL. Similar results were ob-
tained when cells were treated with 1 ml dibutyryl-cAMP
(results not shown), but the magnitude of the increase was not
as robust. Therefore, forskolin stimulation was adopted as
standard conditions.

Lipolytic stimulation of cultured adipocytes results in a dra-
matic restructuring of the droplet surface and a reorganization
of the local droplet environment (8, 9). To assess the organiza-
tional structure of the lipid droplets in C8PA lipocytes, the cells
were incubated for 2 h with BODIPY-oleate, and the fluores-
cent fatty acid was accumulated into droplets. As shown in Fig.
2, under basal conditions, BODIPY-oleate accumulated into
small droplets organized into a central cluster. When visual-
ized with transmitted light differential interference contrast
(Fig. 2A), the droplets appear as a central tight cluster of
droplets, whereas by fluorescence (Fig. 2C), the clustered
nature of the organization of smaller droplets was clear. After
forskolin stimulation, the clustered droplets were dramatically
restructured, as revealed by both differential interference con-
trast (Fig. 2B) and BODIPY-oleate fluorescence (Fig. 2D).
Moreover, consistent with previous studies (9) perilipin A was
found on the droplets coincident with the fluorescent lipid
localization (Figs. 2, E and F).

Using the C8PA lipocyte cell model, we addressed the location
and translocation of adipocyte FABP and HSL by immuno-
ofluorescence using fluorescent fusion proteins. Previous
studies show that fusion proteins with adipocyte FABP are
fully active for fatty acid binding as well as association with
HSL (14, 16). To verify that CFP-HSL retains activity, we
transiently transfected CFP-HSL and HSL into 293 cells. The
cells were harvested and assayed for neutral cholesteryl-oleate
activity. Control 293 cells transfected with vector alone exhibit
no activity, verifying that these cells have little endogenous
neutral cholesteryl esterase activity (Fig. 3). CFP-HSL and
native HSL have significantly increased activity over vector-
transfected cells and show similar levels of activity. CFP-HSL,
therefore, maintains its neutral cholesteryl esterase activity as
compared with HSL.

Transient transfection of C8PA cells with CFP-HSL revealed
that the fusion protein was broadly distributed within the
cytoplasm but excluded from organelles and nuclei (Fig. 4A).
When C8PA cells were stimulated with forskolin, some but not
all CFP-HSL was reorganized and coalesced into a structure
coincident with the droplet surface Fig. 4B). To demonstrate that the structure CFP-HSL translocated to is the droplet surface, the fluorescence of BODIPY-oleate staining of the droplets was compared with that of CFP-HSL. As shown in Fig. 4, E and F, CFP-HSL translocation resulted in association with BODIPY-FA-labeled droplets. As such, the C8PA lipocyte system reproduces that exhibited by true adipocytes. It should be noted that these studies do not address the molecular organization of HSL on the droplet or even if the enzyme is lipid-associated. Moreover, it is not clear if HSL is directly on the surface of the lipid droplet or with another protein or macromolecule that is itself lipid-associated. However, for simplicity, we refer to such physical relocation as movement of HSL to the droplet surface.

When YFP-adipocyte FABP was transiently transfected into C8PA cells, it too was found cytoplasmically but also was found within the nucleus (Fig. 4C) as shown previously (25, 26). When YFP-AFABP C8PA cells were incubated with forskolin, there was no dramatic translocation of YFP-adipocyte FABP observed as there was for CFP-HSL, although there did appear to be some heterogeneity in the distribution throughout the cells (Fig. 4D).

To evaluate the physical association between CFP-HSL and YFP-FABP, fluorescence resonance energy transfer was utilized. Transient transfection of C8PA cells with CFP-HSL plus YFP-adipocyte FABP or CFP-HSL plus YFP-liver FABP was carried out, fluorescence in either the CFP and YFP channel was digitally captured, and FRET was calculated. As shown previously for individual transfection (Fig. 4), under basal conditions both CFP-HSL and YFP-adipocyte FABP were found cytoplasmically (Fig. 5, A and C). Calculating the true energy transfer from CFP emission to YFP excitation revealed that CFP-HSL and YFP-adipocyte FABP were physically associated (artificially colored magenta, panel 5E). This result is consistent with the isothermal titration microcalorimetry experiments previously reported (17) that indicated that adipocyte FABP and HSL formed a complex in the basal, non-phosphorylated state. When co-transfected C8PA cells were incubated with
forskolin, there was little change in YFP-adipocyte FABP localization (Fig. 5D), whereas CFP-HSL clearly translocated to the droplet surface (Fig. 5B). Analyzing FRET in the presence of forskolin (Fig. 5F) demonstrated physical association between adipocyte FABP and HSL on the droplet surface. However, a defined FRET signal remained in the cytoplasm, suggesting that not all physically associated protein co-translocated or possibly that acquisition to the droplet surface was limiting.

To demonstrate that the FRET calculated between HSL and adipocyte FABP was specific, parallel studies were carried out using co-transfection of CFP-HSL and YFP-liver FABP. Titration microcalorimetry had shown previously that despite essentially identical folding motifs for the two proteins (17), liver FABP does not associate to any measurable extent with HSL and that the affinity between HSL and liver FABP was at least 10-fold lower than between HSL and adipocyte FABP (17). Fig. 6 shows the co-transfection of CFP-HSL and YFP-liver FABP into C8PA lipocytes. As with co-transfection with YFP-adipocyte FABP, CFP-HSL was found cytoplasmically under basal conditions but translocated to the surface of the droplet after forskolin stimulation (Fig. 6, A and B). Similar to YFP-adipocyte FABP, YFP-liver FABP was cytoplasmic under both basal- and forskolin-stimulated conditions (Fig. 6, C and D). However, in contrast to FRET between CFP-HSL and YFP-adipocyte FABP, little if any FRET could be detected between CFP-HSL and YFP-liver FABP under either basal conditions or in the presence of 20 μM forskolin (Fig. 6, E and F). There is a very slight signal measured between the two proteins; however, there is no suggestion that any FRET was droplet-associated. These results are in complete agreement with the titration microcalorimetry and suggest that the specificity of association between HSL and adipocyte FABP measured in vitro is replicated in the lipocyte cell system.

**DISCUSSION**

The present investigation was undertaken with two objectives; to assess the physical interaction between fatty acid-binding protein and the hormone-sensitive lipase within the cellular context and to evaluate the relationship between physical association and HSL translocation. Before this work, the characterization of the physical interaction between AFABP and HSL had been done at the level of the yeast two-hybrid interaction of in vitro translation products, glutathione S-transferase fusion proteins and biophysical methods (14). FABPs as a class activate the enzymatic activity of HSL, presumably by binding fatty acids and preventing product inhibition from occurring (16). In this report that foundation was built upon in order to assess the interaction between FABPs and HSL in lipocytes using fluorescence resonance energy transfer.

To develop a cell culture system suitable for analysis of proteins in adipose lipid metabolism, the C8PA cell line was created. This cell line stably expresses the murine fatty acid transport protein 1 as well as perilipin A. Such cells transport fatty acids from serum or from that provided as a complex with serum albumin and incorporate such internalized lipid into droplets. Previously we had reported on the development of a stable cell line expressing FATP1 (19). FATP1 expressing cells accumulated triacylglycerol; however, the lipid was organized in contrast to FRET between CFP-HSL and YFP-adipocyte FABP.
FRET Analysis of C8PA lipocytes co-transfected with CFP-HSL and YFP-liver FABP. C8PA lipocytes were co-transfected with CFP-HSL and YFP-LFABP and loaded with oleate-BSA (3:1) for 48 h. After 48 h the expression of CFP-HSL (panels A and B) and YFP-LFABP (panels C and D) in the absence (A and C) or presence (B and D) of 20 μM forskolin was determined. Energy transfer from CFP-HSL to YFP-LFABP was determined under basal (E) and forskolin-stimulated (F) conditions. The scale bar represents 5 μm.

into irregular structures. Introduction of perilipin A into such cells dramatically organized the internalized lipid into regular clusters of small droplets. C8PA cells were responsive to both forskolin and dibutyryl cAMP to increase free fatty acid release, albeit to not the same extent as do true adipocytes or cultured fat cells. Interestingly, the addition of forskolin to C8PA cells results in a disruption of the triacylglycerol droplet surface. These results point out that the specificity of binding measured in vitro is mirrored by the in situ FRET analysis.

AFABP null mice exhibit reduced lipolysis and have lower serum free fatty acid levels when maintained on a low fat diet (31, 32). When placed onto a high fat diet, AFABP null mice are protected from diet-induced insulin resistance (31). In contrast, mice overexpressing EFABP into fat exhibit increased lipolysis and potentiated characteristics of the metabolic syndrome, including impaired glucose and insulin tolerance tests (15, 33). Placing those metabolic characteristics within the framework of FRET analysis suggests that the FABPs participate in a lipid shuttle, facilitating the intracellular diffusion of fatty acids, some destined for efflux, some for recycling. This study suggests that fatty acid binding is facilitated by the physical association of AFABP with HSL. What is unknown is the role of HSL phosphorylation in AFABP binding (if any) or the role for fatty acid binding by AFABP to association with HSL. The system described herein can now be extended to evaluate such
factors in both the cytoplasm and on the droplet surface using a series of AFABP and HSL mutants. Such studies are currently under way.

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