Interaction of the Adipocyte Fatty Acid-binding Protein with the Hormone-sensitive Lipase

REGULATION BY FATTY ACIDS AND PHOSPHORYLATION

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Adipocyte fatty acid-binding protein (AFABP/ap2) forms a physical complex with the hormone-sensitive lipase (HSL) and AFABP/ap2-null mice exhibit reduced basal and hormone-stimulated lipolysis. To identify the determinants affecting the interaction fluorescence resonance energy transfer (FRET) imaging was used in conjunction with a mutagenesis strategy to evaluate the roles AFABP/ap2 fatty acid binding and HSL phosphorylation have in complex formation as well as determine the HSL binding site on AFABP/ap2. The nonfatty acid binding mutant of AFABP/ap2 (R126Q) failed to form a FRET-competent complex with HSL either under basal or forskolin-stimulated conditions, indicating that lipid binding is required for association. Once bound to HSL and on the surface of the lipid droplet, YFP-AFABP/ap2 (but not YFP-HSL) exhibited energy transfer between the fusion protein and BODIPY-C12-labeled triacylglycerol. Serine to alanine mutations at the two PKA phosphorylation sites of HSL (659 and 660), or at the AMPK phosphorylation sites of the lipid droplet surface, potentially providing access to the substrate (10). As such, lipolysis is a multicomponent highly regulated process linking a number of hydrolytic events leading toward fatty acid release from the droplet surface.

Adipocyte fatty acid-binding protein (AFABP)2, also known as ap2, is the predominant member of the multigene family of intracellular lipid-binding proteins found in adipose tissue (1). The fatty acid-binding proteins (FABPs) are low molecular weight (15 kDa) abundant, ubiquitously expressed proteins that bind and sequester hydrophobic ligands such as free fatty acids and/or closely related compounds facilitating their solubilization and trafficking between various aqueous compartments of the cell. Whereas the members of the multigene family share only 20–70% sequence identity, they fold into a structurally conserved β-barrel that produces an internal water-filled cavity that functions as the fatty acid binding site (1).

Lipolysis in adipocytes (defined as the regulated release of fatty acids from the cell) is facilitated via a series of regulatory phosphorylation events linking receptor-mediated increases in cAMP to the phosphorylation of several key proteins including perilipin A and the hormone-sensitive lipase (HSL) resulting in an increase in triacylglycerol, diacylglycerol, and monoacylglycerol hydrolysis and efflux of fatty acid from the adipocyte (2). Perilipin A phosphorylation results in a dynamic restructuring of the lipid droplet surface, potentially providing access to the lipid droplets for lipases (3, 4). Recent structure-function studies have indicated that diacylglycerol is the predominant glyceride substrate for HSL in vivo and is provided by the action of the upstream adipose triglyceride lipase (5). HSL function is under multidimensional regulation by hormones and catecholamines, and several investigators have shown that protein kinase A-dependent phosphorylation of Ser659 and Ser660 is required for both the translocation of HSL to the lipid droplet and increased hydrolytic activity (6, 7, 8). In addition, AMPK-dependent phosphorylation of HSL (Ser265) also results in activation of the lipase and is necessary for translocation (7, 9). Concomitant with HSL phosphorylation, the phosphorylation of perilipin facilitates the translocation of HSL from the cytosol to the lipid droplet and access to the substrate (10). As such, lipolysis is a multicomponent highly regulated process linking a number of hydrolytic events leading toward fatty acid release from the adipocyte.

AFABP/ap2-null mice exhibit decreased basal and hormone-stimulated lipolysis both in situ and in vivo, but demonstrate rates of fatty acid influx identical to those of wild-type mice, suggesting that the protein facilitates diffusion of fatty acids from the site of lipid hydrolysis (droplet surface) to the portal membrane as part of lipid efflux from the cell (11). Importantly, AFABP/ap2-null mice accumulate intracellular FFA, suggesting that lipid release (lipolysis) from adipocytes is attenuated while intracellular lipid hydrolysis has not been for-
mally addressed (11). An additional complexity to the system is that AFABP/aP2 forms a physical association with the hormone-sensitive lipase. Using a combination of yeast two-hybrid analysis, GST pull-down and immunoprecipitation analysis of full-length HSL and truncated derivatives, the AFABP/aP2 binding domain on HSL has been mapped to a region on the N terminus demarcated by amino acids 190–200 (12). This region on HSL is distinct from the catalytic region or the regulatory sites of phosphorylation that reside in the C-terminal domain leading to the consideration that the N-terminal domain is a regulatory docking domain (13). In vitro analysis of AFABP/aP2 interaction with HSL, using titration microcalorimetry indicates that the reaction is dependent upon fatty acids, although it was not clear from this study if the fatty acids were bound to AFABP/aP2 or to a fatty acid binding region on HSL that represents a site for product inhibition (14). In vitro, AFABP/aP2 stimulates HSL cholesteryl ester hydrolase activity, presumably by relieving product inhibition. However, such activation did not require physical association, because other FABPs such as those from liver and intestine that do not associate also activate the lipase (14).

Because the in vitro studies of AFABP/aP2 interaction with HSL utilize purified proteins devoid of regulatory modifications (phosphorylation) known to affect HSL structure and function, we have turned our attention to a cellular system to assess regulatory properties of the association. A previous report defined an experimental cell system utilizing 293 HEK cells stably transfected with the fatty acid transport protein 1 and perilipin A termed C8PA (15). Such cells transport and metabolize serum fatty acids avidly, preferentially synthesize triacylglycerol, form well-defined lipid droplets and are responsive to lipolytic stimulation. C8PA cells have been transiently transfected with plasmids expressing fluorescent fusion proteins (YFP-AFABP/aP2 and CFP-HSL) thereby allowing for not only identification of subcellular distribution but also close physical interactions via fluorescence resonance energy transfer (15). Moreover, the cells can be labeled using BODIPY-conjugated fatty acids, thereby providing for a convenient mechanism to identify lipid droplets and association between droplet-associated proteins and the stored triacylglycerol. In this report we extend our previous observations to an analysis of the AFABP/aP2 protein by utilizing mutant forms of AFABP/aP2 and HSL and FRET as an experimental tool for evaluating protein-protein association in C8PA cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Zeocin, Geneticin, Lipofectamine, and tissue culture reagents were obtained from Invitrogen (Carlsbad CA). BODIPY D 3835 (4,4-difluoro-5-(2-thienyl)-4-bora-3a, 4adiaza-s-indacene-3-dodecanoic acid; referred to as BODIPY-C12) was obtained from Molecular Probes, Inc., Eugene, OR. pEYFP-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. Pure oleic acid was purchased from NuChek Prep (Elysian, MN). Cholesteryl [1-14C]oleate was purchased from Amer sham Biosciences. Restriction and DNA-modifying enzymes were obtained from Promega. All other reagents were purchased from Sigma-Aldrich. DNA sequencing and synthesis of oligonucleotides used for PCR were carried out by the University of Minnesota Microchemical Facility.

**Cell Culture**—The cells used in this work (C8PA lipocytes) were HEK-293 fibroblasts stably expressing fatty acid transport protein 1 (FATP1) and perilipin A (15). The cells were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum; stable expression of FATP1 was maintained with 400 μg/ml genetin and stable expression of perilipin was maintained with 300 μg/ml Zeocin.

**Cloning and Mutagenesis**—The R126Q AFABP/aP2 nonfatty acid binding mutant was subcloned into the A206k pEYFP-C1 expression vector. Mutations in the HSL phosphorylation sites were introduced into wild-type HSL in the A206k pEYFP-C1 expression vector using the QuikChange™ site-directed mutagenesis technique of Stratagene Cloning Systems, La Jolla, CA as previously described (15). The mutational primer for HSL S565A was 5’-CTATGCGCAAGTGTGCTGAG-GCACCCC-3’ while the mutational primer for HSL S659A/ S660A was 5’-GGTTTCCACCCACGGCGGAGCAGGA-MGTGTCTCCTCAC-3’. Wild-type HSL was subcloned in frame into pEYFP-C1 to spectrally enable FRET analysis between the HSL portion of the AFABP:HSL complex and the BODIPY-labeled triacylglycerol droplet. All mutations were confirmed by DNA sequencing.

**Live Cell Imaging and FRET**—For FRET analysis, C8PA cells were grown on polylysine-coated 12-mm coverslips placed into 12-well dishes. At ~70% confluence, wells were transfected with expression plasmids specific for CFP-HSL and/or YFP-FABP. 24-h later, cells were lipid-loaded for 48 h 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 captured after 4 h. For triacylglycerol labeling, BODIPOY-C12 in Me3SO is added to monolayers (final concentrations 10 μg/ml) 60 min following forskolin addition and maintained on the monolayers for an additional 30 min until the cells were washed and images collected. During microscopy, cells were kept in media at 37 °C until imaging at room temperature.

Digital images were collected using a Roper CoolSnap HQ 12 bit monochrome camera and captured to a Pentium IV 2.6 GHz personal computer using Image Pro Plus AMS version 6.0 software (Media Cybernetics, Silver Springs, MD 20910) for microscope automation and image analysis on a Nikon Eclipse E800 photomicroscope equipped with Ludl Mac 5000 controls of shutters and excitation and emission filter wheels. Images were captured with a 40×, 0.75 n.a. plan fluor or a 60×, 1.40 n.a. plan apo immersion objective. Cells with relatively equal levels of expression of both vectors were selected for imaging. For detection of CFP-HSL, cells were viewed with an excitation filter of 436/20 nm, a dichroic beam splitter of 455 nm, and an emission filter of 480/40 nm. YFP- AFABP/aP2 was detected by using 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 obtained from the Chroma Technology (Brattleboro, VT). Images were acquired using 2 × 2 binning mode and 100–
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150-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.

Real Time FRET—Measurements were performed using the Spectral DV Micro-Imager system in Dual View mode from Optical Insight (Santa Fe, NM) that allows monitoring of donor and acceptor pair fluorescence emissions simultaneously. The ratio between YFP and CFP emission is then calculated as an indicator of FRET efficiency. The Spectral DV Micro-Imager is directly attached to the microscope and is equipped with a 505-nm long-pass dichroic filter used to separate the CFP signal from the YFP signal, a CFP emission filter (480 ± 30 nm) and an YFP emission filter (535 ± 40 nm). The excitation filter was 436 ± 20 nm. The fluorescence images of YFP and BODIPY were separated by a dichroic mirror (550 nm), and projected into the two detection arms with bandpass filters (535 ± 40 nm for YFP and 580 ± 40 nm for BODIPY (Chroma Technology, Brattleboro, VT).

Images were captured with a fast high resolution digital camera (CoolSNAP-HQ 12bit Cooled Monochrome Digital Camera, Roper Scientific, Tucson, AZ) and acquired in a PC using the ImagePro Plus AMS (Media Cybernetics). For all experiments only cells with nonsaturating levels of fluorescence are used. The images were then background-subtracted and shading-corrected using the “Correct Background” tool in ImagePro, which performs the shading correction as Corrected Image = (Max value of Shade Image) × (Acquired Image Background)/(Shade Image Background). The background image was a 10-frame average of the camera bias, taken with the identical situation as for imaging, but with the excitation light blocked. The shade image was collected from a 10-frame average of images of a calibration slide (Chroma Technology, Brattleboro, VT). Shading correction was necessary to obtain uniform values across the CCD chip.

The CFP-HSL image was then thresholded, changing the intensities of all pixels outside of the cell to zero. Thresholding was based on 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-AFABP/aP2 images were multiplied by the binary image, ensuring that the same pixels were analyzed in all three images as described in detail. Emission appearing in the FRET image because of emission from CFP-HSL or direct excitation of YFP-AFABP/aP2 was removed by subtracting a fraction of the CFP-HSL and YFP-AFABP/aP2 images from the FRET image. This fraction depended on the filter set and exposure condition used and was determined as described (17). Corrected FRET (FRETc) was calculated on a pixel-by-pixel basis for the entire image using Equation 1,

\[
\text{FRETc} = \text{FRET} - (0.50 \times \text{CFP-HSL}) - (0.02 \times \text{YFP-AFABP/aP2})
\]

(Eq. 1)

where FRET, CFP-HSL, and YFP-AFABP/aP2 correspond to background-subtracted images of cells co-expressing CFP-HSL and YFP-AFABP/aP2 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 image to remove high-frequency noise (18). The data were analyzed for statistical significance by using a paired Student’s t test.

Images were contrast stretched, pseudocolored, and formatted for display using Adobe Photoshop CS2 software (Adobe Systems, Mountain View, CA). Each live cell imaging and FRET experiment was done at least three independent times on different passages of cells. For each co-transfection and FRET analysis, monolayers were scanned to identify representative cells expressing comparable amounts of fusion proteins. Once identified, typically five separate cell fields were evaluated for localization of the proteins and FRET between them. As such, although a single image for each FRET experiment is shown, it represents a finding evaluated 15 times.

HSL Activity Assay—The HSL mutant constructs were assayed for cholesteryl esterase activity as previously described (13). Activity assays using p-nitrophenyl butyrate as a substrate were carried out analogously to that for cholesteryl hydrolase activity. Briefly, His-HSL and fatty acid free His-AFABP (purified as previously described (15)) were incubated in a 1:1 molar ratio in the presence or absence of 10 \(\mu\)M oleate and 2 \(\mu\)M pNPB for 60 min at 37 °C in triplicate. The reaction was stopped and the absorbance at 400 nm of the supernatant containing p-nitrophenol was measured. The experiment was carried out three times and each experiment individually analyzed using Microsoft Excel.

HSL Affinity-purified Antibody—An HSL peptide corresponding to amino acids 594–611 (NESEPSDPSEMSSQMETLG) was used to produce a rabbit polyclonal antibody toward HSL (Bioworld, Dublin, OH). Coupling of the peptide to Affi-gel 15 (Bio-Rad), and subsequent affinity chromatography with antisera were used for purification of the antibody.

RESULTS

Previous studies using C8PA lipocytes (293 HEK cells stably transfected with FATP1 and perilipin A) have demonstrated that physical association of CFP-HSL and YFP-AFABP/aP2 occurs in the cytoplasm under basal conditions and is increased markedly in response to forskolin treatment (Fig. 1). In adipocytes, lipolytic stimulation leads to the translocation of HSL to the droplet surface and is dependent upon phosphorylation of perilipin A and HSL (3, 4, 7). The C8PA lipocyte system mirrors such regulation and 20 \(\mu\)M forskolin treatment leads to not only translocation of HSL, but also an increase in FFA release (15). As shown in Fig. 1, YFP-AFABP/aP2 association with CFP-HSL is increased in response to forskolin and is largely found associated with the lipid droplets (panels F and H).

To assess the spatial relationship between the different aspects of the HSL-AFABP/aP2 complex to the triacylglycerol droplet, studies were carried out using fusion proteins of both AFABP/aP2 and HSL that allow for FRET analysis between the
YFP fusions (but not CFP fusions) and BODIPY-C12-labeled triacylglycerol. By assessing direct fluorescence of each reporter, as well as by energy transfer analysis, the location and spatial proximity of the AFABP/aP2-HSL complex to the lipid can be evaluated. As shown in Fig. 2, once bound on the droplet surface, the YFP-AFABP/aP2-HSL complex exhibits a FRET response with BODIPY-C12-labeled triacylglycerol while AFABP/aP2 YFP-HSL does not (compare panels C and F), implying that the AFABP/aP2 fusion partner is in closer proximity to the lipid droplet than is the corresponding HSL fusion. The FRET signal between YFP-AFABP/aP2 and BODIPY-C12-labeled triacylglycerol required the presence of HSL and did not occur in the absence of forskolin stimulation (results not shown). Control transfection using pYFP-AFABP/aP2 and pCFP-HSL carried out on the same day and measured at the same time revealed that CFP-HSL translocated to the droplet surface and exhibited energy transfer with YFP-AFABP/aP2 identically as shown in Fig. 1. It should be noted that the fluorescent HSL fusion exhibits catalytic activity comparable to HSL alone (15) indicating that the presence of the YFP does not block access to substrates such as triacylglycerol.

Previous studies using titration microcalorimetry have demonstrated that in vitro, fatty acids are required to enable complex formation between AFABP/aP2 and HSL (14). AFABP/aP2 is a fatty acid-binding protein with a well-characterized binding site defined, in part, by a hydrogen-bonding network between the fatty acid carboxylate and the side chains of Arg106, Arg126, and Tyr128 (19). Previous work in this laboratory demonstrated that an R126Q mutation in AFABP/aP2 resulted in a 30–50-fold reduction in the binding affinity for long chain fatty acids (19). Taking this into account, we evaluated the ability of R126Q AFABP/aP2 to form a complex with HSL. To that end, the R126Q mutant AFABP/aP2 cDNA was cloned as a YFP fusion and transiently cotransfected into the C8PA cells along with CFP-HSL. As shown in Fig. 3 CFP-HSL and YFP-AFABP/aP2 R126Q were located in the cytoplasm of the cell under basal conditions and the CFP-HSL translocated to the droplet surface in response to forskolin stimulation. However, in contrast to native AFABP/aP2 (Fig. 1), there was no energy transfer
between AFABP/aP2 R126Q and HSL under either basal or stimulated conditions. Control transfection using pYFP-AFABP/aP2 and pCFP-HSL revealed that CFP-HSL translocated to the droplet surface and exhibited energy transfer with YFP-AFABP/aP2 identically as shown in Fig. 1. These results are consistent with the microcalorimetry data and indicate that if AFABP is unable to bind fatty acids it does not form a physical association with HSL under conditions of lipolytic stimulation. Previous studies have shown that apoAFABP/aP2 stimulates the catalytic activity of HSL 2–3-fold, presumably by relieving product inhibition and that activation did not require complex formation. These findings indicate that activation of HSL is unlinked mechanistically to complex formation and that the form of AFABP/aP2 that does interact with HSL is the holoprotein.

The finding that the holoprotein form of AFABP/aP2 binds to HSL suggests that the interaction may be regulatory. If so, it would seem likely that holoAFABP/aP2 would interact with the catalytically competent form of HSL. Because maximal catalytic competence and acquisition of lipid binding is achieved following regulatory phosphorylation (7, 9, 20), we evaluated complex formation between AFABP/aP2 and HSL using HSL mutants with substitutions at the major PKA and AMPK phosphorylation sites.

Work from a variety of laboratories has demonstrated that phosphorylation of HSL by PKA at Ser^{659} and Ser^{660} is required for full activation and for translocation of the protein to the droplet surface and that translocation does not occur when these two serine residues are mutated simultaneously to alanine (7, 20). Mutation of these serine residues to alanine resulted in comparable basal catalytic activity (Fig. 4). Consistent with this, when pCFP-HSL S659A/S660A was transfected into C8PA cells there was no evidence of translocation of the mutant HSL to regions coincident with lipid droplet association under forskolin-stimulated conditions (Fig. 5, panels B and J). When the pCFP-HSL S659A/S660A was transiently transfected into C8PA cells with pYFP-AFABP/aP2 there was no evidence of FRET either in the presence or absence of forskolin stimulation. This suggests that the fatty acid bound AFABP/aP2 associates only with the activated phosphorylated form of HSL.

A second critical phosphorylation site in HSL is Ser^{565} and is subject to phosphorylation by the AMP-activated protein kinase (10). To assess the influence of Ser^{565} phosphorylation on association with AFABP/aP2, the pCFP-HSL S565A mutant was transfected either in the presence or absence of YFP-AFABP/aP2 and energy transfer evaluated. As shown in Fig. 6 (panel B) the S565A HSL mutant was unable to translocate to the droplet surface, consistent with other published reports (7), and in addition did not form a complex with AFABP/aP2 under either basal or forskolin-stimulated conditions (panels E and F). Overall, the results of the PKA and AMPK series of mutants indicates that the holoAFABP/aP2 only associates with the activated phosphorylated HSL. Parallel control transfection using YFP-AFABP/aP2 and CFP-HSL demonstrated that CFP-HSL translocated to the droplet surface and exhibited energy transfer with YFP-AFABP/aP2 identically as shown in Fig. 1 (results not shown).

While the binding site on HSL that associates with AFABP/aP2 has been defined by the region surrounding amino acids

![Graph](https://via.placeholder.com/150)
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190–200 using a series of truncations and point mutants coupled to GST pull-down studies (12), the binding site on AFABP/aP2 that interacts with HSL has remained elusive. The results shown in Figs. 2, 5, and 6 indicate that complex formation requires AFABP/aP2 to be fatty acid-bound and HSL to be phosphorylated. A region on AFABP/aP2 that has received considerable attention as important for conveying the presence of a bound fatty acid onto the protein structure is the helix \( \alpha_1 \)-helix \( \alpha_2 \) domain that forms the lid of the fatty acid binding cavity. Liou and Storch (16) have carried out a series of mutations of the helical region and identified Lys\(^{21} \) and Lys\(^{31} \) as important residues affecting the ability of AFABP/aP2 to interact with biological membranes. To evaluate if the same residues affected interaction with HSL, two mutants were created in the YFP-AFABP/aP2 framework: K21I and K31I, and each evaluated for binding with CFP-HSL using FRET analysis following co-transfection into C8PA lipocytes. As shown in Fig. 7, YFP-AFABP/aP2-K21I failed to form a complex with CFP-HSL under basal or forskolin-treated conditions (panels \( E \) and \( F \)), implying that this residue contributes to the HSL binding site. In contrast, mutation of Lys\(^{31} \) had no effect on either AFABP/aP2 localization or FRET with HSL (Fig. 7, panels \( K \) and \( L \)). This implies that residue Lys\(^{31} \) contributes to the HSL binding site and defines the helical domain, particularly helix \( \alpha_1 \), as central to HSL binding. K21I retains essentially all of its fatty acid binding activity (16) and as such, loss of interaction with HSL is not related to an inability to bind fatty acids.

The conclusion that the ligand-bound form of AFABP/aP2 interacts with the phosphorylated, activated HSL suggests that the association is regulatory. Fatty acids inhibit HSL activity (21) and apo-FABP rescues the enzyme from product inhibition, although such activation does not require physical association. In contrast, holo (ligand-bound) AFABP/aP2 may deliver FFA to the HSL providing a mechanism for feedback inhibition. Alternatively, holo-AFABP/aP2 may inhibit the intrinsic activity of HSL. To address this latter possibility, holo-AFABP/aP2 (95% FFA occupancy) was incubated with purified HSL and the hydrolysis of \( p \)-nitrophenyl butyrate (pNPB) evaluated. This substrate was chosen since AFABP/aP2 does not bind short chain FFA such as butyric acid thereby negating any potential effects of AFABP/aP2 by binding product FFA or delivering such lipid to HSL mediating inhibition. As shown in Fig. 8, holo-AFABP/aP2 had no effect on pNPB hydrolysis consistent with the model for AFABP/aP2 binding to the N-terminal docking domain while lipase activity resides in the C-terminal catalytic domain.

**DISCUSSION**

Obesity-linked insulin resistance leads to an up-regulation in adipose basal lipolytic rate resulting in an increase in fatty acids in the portal vein that suppresses insulin inhibition of hepatic glucose output (22). As such, understanding the molecular components of adipocyte lipolysis is central to the etiology of type 2 diabetes.

The studies described herein define three important considerations previously not understood concerning the biochemical parameters for AFABP/aP2 interaction with the hormone sensitive lipase; the requirement for fatty acids to be bound to AFABP/aP2 the dependence upon phosphorylation of HSL promoting a suitable conformational change that allows AFABP/aP2 interaction, and the mapping of the HSL binding site on AFABP/aP2 to a region surrounding helix \( \alpha_1 \).

Previous studies using titration microcalorimetry have shown that fatty acids are required for AFABP/aP2-HSL interaction (14). However, because AFABP/aP2 is a fatty acid-binding protein and HSL is purified in detergent, it has been difficult to discern the role of fatty acids in facilitating complex formation. Using C8PA lipocytes and fluorescence resonance energy transfer we eliminated the in vitro considerations and show in Fig. 3 that the apo-AFABP/aP2 does not bind to HSL. As a result of binding product fatty acids, apo-AFABP/aP2 activates HSL catalytic activity 3-fold. The activation of HSL by AFABP/aP2 does not require physical association since fatty acid-binding proteins that do not associate, intestinal FABP and liver FABP, also activate (14). In studies using ADIFAB-injected cultured adipocytes Kleinfeld et al. (28) have determined that the intracellular free fatty acid concentration in the adipocyte is quite low, in the nanomolar range. As such, the ability of holo-AFABP/aP2 to bind HSL on the droplet surface is relegated to only a minor amount of the total protein. It is likely that under conditions of obesity or inflammation to have basal lipolysis...
Elevated to such an extent that a greater fraction of AFABP/aP2 is in the holoprotein form. Once bound to HSL on the surface of the lipid droplet AFABP/aP2 is in close proximity to the lipid as revealed by FRET between YFP-AFABP/aP2 and BODIPY-C12-labeled lipid. Although we cannot rule out the possibility that the energy transfer measured is from BODIPY-C12 fatty acid bound to YFP-AFABP/aP2, we feel this is unlikely because there exists no FRET in the cytoplasm and the energy transfer occurs only on the droplet surface. Because YFP-HSL is catalytically active using either pNPB or cholesterol ester substrates, the presence of the fluorescent fusion is not likely to affect triacylglycerol association. One possibility is that the orientation of the C-terminal catalytic domain is associated with the droplet surface while the N terminus is directed away from the lipid surface thereby distancing the YFP-HSL fusion from the BODIPY-C12 beyond the effective range for energy transfer. The Förster constant (Ro) measured for the YFP-BODIPY-C12 pair was 52 Å, implying that the maximum distance measurable would be \( 1.5 \text{Ro} \approx 80 \text{ Å} \).

The requirement for HSL to be phosphorylated to interact with AFABP/aP2 suggests that a productive complex occurs only when HSL is activated and competent to carry out lipid hydrolysis. Complex formation was observed in the cytoplasm (minor) and on the droplet surface (major). Serine to alanine mutations at key phosphorylation sites in HSL rendered the protein incompetent for AFABP/aP2 binding yet still catalytically active in vitro. Interestingly, in the presence of forskolin, HSL was not able to translocate to the surface of the lipid droplet. A small amount of HSL did organize into punctate structures as has been reported by others (7). The definition of these structures is not known but may be linked to degradation of the fusion proteins. Importantly, there was no FRET under basal conditions either, where the punctate structures did not appear and where native HSL does form a complex with AFABP/aP2.

The finding that YFP-AFABP/aP2 K21I does not exhibit energy transfer with CFP-HSL while YFP-AFABP/aP2 K31I does implies that a region on or about helix \( \alpha 1 \) forms the HSL binding site. The side chain of Lys21 is found within a cluster of charged residues demarcated by Asp17, Asp18, Lys21, and Arg30. Asp17, Asp18, and Lys21 reside on helix \( \alpha 1 \) while Arg30 is found on helix \( \alpha 2 \). The side chain of Asp18 forms an ion pair with that of Lys21 while the side chain of Asp17 forms a salt bridge to that of Arg30. The two ion pairs form a quartet of charges on the surface of the protein that bridge the two helices (Fig. 9). As such, the inability of K21I to form a complex with HSL may suggest that corresponding acidic residues on HSL contribute to the binding site or alternatively, disruption of the salt bridge affects the helical domain sufficiently to disrupt HSL (but not fatty acid) interaction. Indeed, one of the key HSL residues crit-

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**Figure 7.** Energy transfer between YFP-AFABP/aP2 K21I or YFP-AFABP/aP2 K31I and CFP-HSL. C8PA lipocytes were co-transfected with pCFP-HSL and pYFP-AFABP/aP2 K21I or pCFP-HSL and pYFP-AFABP/aP2 K31I and loaded with oleate-BSA (3:1). After 48 h, the expression of CFP-HSL and YFP-AFABP-aP2 K21I and CFP-HSL and YFP AFABP-aP2 K31I in the absence or presence of 20 μM forskolin was determined. Energy transfer from CFP-HSL to YFP-AFABP-K21I or CFP-HSL to YFP-AFABP/aP2 K31I was determined under basal and forskolin-stimulated conditions.

**Figure 8.** Effect of holo-AFABP on HSL activity using pNPB as a substrate. Fatty acid free His-AFABP was incubated with or without 10 μM oleate and then added to His-HSL in a 1:1 molar ratio to measure esterase activity with pNPB as the substrate. The results are expressed as nmol of p-nitrophenol produced per minute. The graph shows the mean ± S.D. for one of three independent experiments.
ical for AFABP/aP2 interaction is Glu\textsuperscript{193} located in the N-terminal docking domain (12). Importantly, Xu et al. (22) reported that Lys\textsuperscript{21} is one of the few residues whose main chain atoms vary in position with fatty acid binding suggesting that fatty acid binding alters the helical domain. In addition, Licata and Bernlohr (24) used computational methods to assess the accessible surface area for each of the AFABP/aP2 side chains in both the apo and holo forms and identified Lys\textsuperscript{21} as significantly decreased surface accessibility in the holoprotein form. These observations could provide a structural explanation for why fatty acid binding is required for interaction with HSL.

The identification of a large supramolecular complex on the lipid droplet is beginning to be defined. Under conditions where net triacylglycerol hydrolysis is stimulated, HSL also forms a complex with perilipin A (10). In addition, perilipin A associates with CGI-58 (25). CGI-58 is an α/β hydrolase fold-containing protein that resembles a lipase, but lacks lipase activity since the active site serine is missing. Perilipin A has been shown to interact with CGI-58 under basal conditions and dissociates under stimulated conditions (26). CGI-58 in turn interacts with the adipose triglyceride lipase under stimulated conditions resulting in increased hydrolytic activity (27). As such, a dynamic complex involving multiple hydrolytic enzymes, co-factors and structural proteins exist on the surface of the droplet. Interestingly, Kraemer and co-workers (29) have shown that dimerization of HSL involves determinants that involve the N-terminal domain. Moreover, dimerization increases the specific activity of HSL ~40-fold, suggesting that if AFABP/aP2 binding inhibits HSL dimerization, it would be an effective mechanism to regulate HSL activity and diacylglycerol hydrolysis.

The results presented herein can be framed within the context of the AFABP/aP2 knock-out animals and their attenuated lipolysis phenotype. Whereas regulated fatty acid release from adipose tissue in vivo is markedly reduced in the AFABP/aP2-null mice (30), the rate of intracellular triacylglycerol hydrolysis has not been assessed. Indeed, free unesterified fatty acids accumulate intracellularly in adipose tissue from the AFABP/aP2 knock-out mice suggesting that lipid hydrolysis is not decreased, but may in fact be increased. These results suggest that AFABP/aP2 plays two roles in lipolysis. Firstly, AFABP/aP2 facilitates FFA efflux by trafficking FFA from the lipid droplet to the membrane in a reaction that is independent of physical association with HSL. Because fatty acids need to be trafficked from the site of hydrolysis (lipid droplet) to the plasma membrane, the loss of AFABP/aP2 may explain reduced FFA release. Secondly, AFABP/aP2 with a bound fatty acid serves a regulatory role by associating with the activated, phosphorylated HSL on the surface of the lipid droplet. If AFABP/aP2 interaction with HSL results in reduced lipid hydrolysis via either delivering a fatty acid for feedback inhibition and/or altering complex formation, the loss of the binding protein may result in increased lipid hydrolysis. An additional complexity for the system is the minor FABP expressed in adipocytes; the epithelial FABP (EFABP or ma1). EFABP forms a complex with HSL as revealed by titration microcalorimetry and contains the orthologous residues Lys\textsuperscript{23} and Arg\textsuperscript{32} as well as acidic amino acids Glu\textsuperscript{19} and Glu\textsuperscript{20} that are postulated to form the quartet of charges on the helix α1-helix α2 domain. As such, it is likely that EFABP physically interacts with HSL was well. Distinguishing between these possibilities will be difficult as AFABP/aP2 knock-out mice exhibit an increase in perilipin A and EFABP expression while decreasing HSL as revealed by titration microcalorimetry and contains the orthologous residues Lys\textsuperscript{23} and Arg\textsuperscript{32} as well as acidic amino acids Glu\textsuperscript{19} and Glu\textsuperscript{20} that are postulated to form the quartet of charges on the helix α1-helix α2 domain. As such, it is likely that EFABP physically interacts with HSL was well. Distinguishing between these possibilities will be difficult as AFABP/aP2 knock-out mice exhibit an increase in perilipin A and EFABP expression while decreasing HSL expression confounding evaluation of the results.

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