PROTEIN-PROTEIN INTERACTIONS AND THE CHARACTERIZATION OF ADIPOCYTE FATTY ACID BINDING PROTEIN

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Dedication

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Abstract

Adipose tissue functions to not only store and provide an energy source in the form of triacylglycerol but to signal the nutritional status of those reserves through the secretion of adipokines and lipids. In obesity, this homeostatic regulation of whole body lipid metabolism is compromised resulting in disease states, such as type 2 diabetes and atherosclerosis. Adipocyte fatty acid binding protein, AFABP/aP2, functions to solubilize and traffic fatty acids, and other lipid metabolites, within the aqueous environment of the adipocyte. AFABP/aP2 knockout mice are resistance to diet and genetic induced obesity linked insulin resistance, while still being obese. The molecular mechanisms of this uncoupling of obesity and insulin resistance have not been elucidated. The identification and characterization of HSL and JAK2 as interacting partners of AFABP/aP2 are presented here. Fatty acid binding by AFABP/aP2 is necessary for the interactions, as indicated by the loss of interactions with the fatty acid binding mutant R126L/Y128F of AFABP/aP2. The phosphorylation state of HSL and JAK2 regulates their interaction with AFABP/aP2. An ExYK motif in AFABP/aP2, DDYMK, in the helix-turn-helix domain is the site of these interactions and a similar ExYK motif in HSL and JAK2 are important for these interactions. We propose that AFABP/aP2 is a fatty acid sensor, through lipid-regulated interaction, from AFABP/aP2's helix-turn-helix domain to an ExYK motif in HSL and JAK2. Furthermore, through its lipid-regulated interactions, AFABP/aP2 is a homeostatic regulator of whole body lipid metabolism, coordinating gene expression, signal transduction and metabolism.

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CHAPTER 1:

ADIPOCYTE LIPID METABOLISM AND ADIPOCYTE FATTY ACID BINDING PROTEIN

Portions of this chapter are taken directly from: **Biochemistry of Lipids, Lipoproteins and Membranes**, edition 5, Vance and Vance; Chapter 10: Lipid Metabolism in Adipose Tissue, Hertzel, AH, **Thompson, BR**, Wiczer, BM, Bernlohr, DA; Elsevier Press, in press.

1 Obesity and Insulin Resistance

Adipose tissue is involved in the passive storage of triacylglycerol and in whole body energy metabolism. Excess storage of triacylglycerol in adipose tissue, as a result of an imbalance between fatty acid uptake and lipolysis, results in obesity (1,2). Obesity is linked to insulin resistance, hypertension, hyperlipidemia and can lead to type II diabetes and atherosclerosis (3,4). Many factors and cell types have been implicated in obesity-linked diseases, not the least of which is lipid metabolism in the adipocyte.

Basal lipolysis is increased in obesity, resulting in increased plasma free fatty acid concentrations (1). This increase in plasma fatty acids contributes to obesity linked insulin resistance in at least two ways. First, the theory of lipotoxicity, or increased storage and metabolism of fatty acids in muscle and liver, can directly result in insulin resistance in these tissues (2). Second, free fatty acids can signal macrophages in adipose tissue to increase pro-inflammatory cytokines through a TLR-4 signaling mechanism (5,6). These pro-inflammatory cytokines, such as IL-6, TNF-alpha, and MCP-1, then affect adipocytes, resulting in insulin resistance and changes in lipid metabolism (7-10). This feed-forward mechanism results in a sub-acute chronic inflammatory status of adipose tissue in obesity and leads to whole body insulin resistance. Understanding lipid metabolism, specifically in the adipocyte is an important topic in light of these observations.

Lipid metabolism in the adipocyte is a complex, highly regulated process, Figure 1. Lipid metabolites regulate many of the enzymes involved in lipid metabolism but also

can serve a signaling function for transcriptional programming, signal transduction from the plasma membrane and intracellular signaling (2,11). Adipocyte fatty acid binding protein, AFABP/aP2, accommodates the diffusion and solubilization of lipid metabolites, such as fatty acids, and may play a role in these signaling functions (12-14). As such, a better understanding of the role AFABP/aP2 plays in lipid metabolism in the adipocyte and its regulatory function is necessary to combat disease states such as type II diabetes and atherosclerosis.

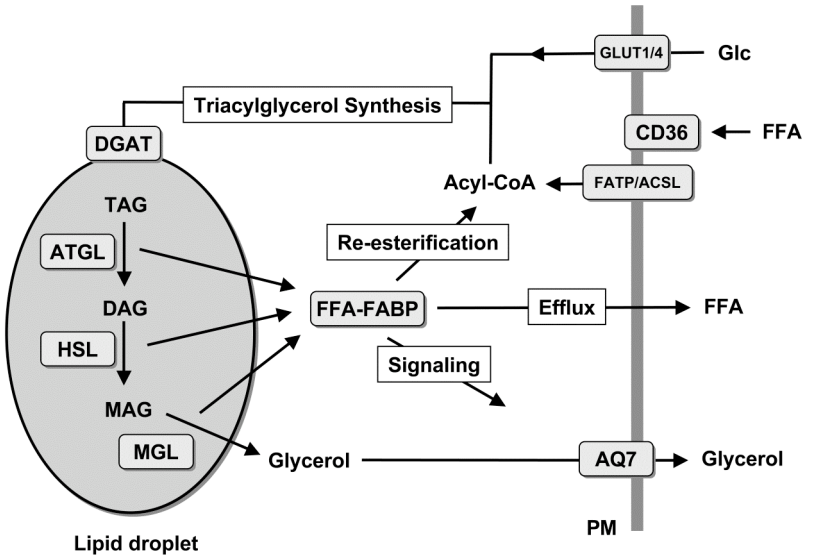

Fig. 1

Figure 1. Lipid cycling in adipocytes. Adipocytes transport glucose (Glc via GLUT 1 or 4) and fatty acids (FFA via CD36 /ACSL) into the cell in response to insulin stimulation leading to the synthesis of triacylglycerol, the terminal step of which is catalyzed by diacylglycerol acyltransferase (DGAT). Triacylglycerol synthesis is balanced by hydrolysis catalyzed by a series of triacylglycerol (adipose triacylglycerol lipase; ATGL), diacylglycerol (hormone sensitive lipase; HSL) and monoacylglycerol (MGL) lipases. Glycerol produced by complete TAG hydrolysis is exported from the adipocyte via an aquaporin protein (AQ7) while FFA are bound by intracellular fatty acid binding proteins (FABP) and subjected to re-esterification, efflux, or metabolized to signaling molecules. TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; ACSL, long chain acyl-CoA synthetase.

2 Adipocyte Lipid Metabolism

2.1 Lipids and Insulin

A primary function of adipose tissue is to serve as a storage site for the excess energy derived from food consumption. Energy stored in the form of triacylglycerol can be utilized by the organism to fulfill subsequent metabolic requirements during times of little or no consumption. In the case of white adipose tissue (WAT), these requirements entail efficient storage of large amounts of energy in a form that can be readily mobilized to supply the needs of peripheral organs and tissues (2,15,16). Lipids, particularly fatty acids, are exceptionally efficient fuel storage species. The highly reduced hydrocarbon tail of triacylglycerol can be readily oxidized to produce large quantities of NADH and $FADH_2$ and subsequently ATP. At the same time, the very hydrophobic nature of the hydrocarbon tail precludes concomitant storage of excess water that would increase the mass and spatial requirements of the organism. Also, the relatively straight, chain-like structure of the fatty acid permits dense packing of many molecules into each cell, maximizing the use of storage space available.

During feeding, pancreatic β cells secrete insulin in response to both elevated blood glucose levels and elevated blood lipid levels. Insulin is the most important physiological stimulus for energy storage. The effect of insulin directly counteracts the effects of glucagon and the catecholamines. The insulin receptor is found in many diverse cell and tissue types, not the least significant of which is adipose.

Insulin binding to the adipocyte insulin receptor simultaneously stimulates lipogenesis and inhibits lipolysis (15,16). Insulin action effectively clears fatty acids and glucose

from the blood both by increasing uptake and storage, and by decreasing mobilization of stored energy. The mechanisms by which these effects are accomplished are highly complex and involve an integrated series of regulated events (17). In regards to insulin-stimulated glucose uptake, the insulin receptor acts mainly through the phosphatidylinositol-3-kinase (PI3 kinase) pathway, facilitating the translocation of GLUT4 to the plasma membrane and stimulation of glucose transport, Figure 2.

2.2 Fatty acid uptake and acyl-CoA production

At the adipose tissue beds, fatty acids are liberated from triacylglycerol rich lipoproteins through the action of lipoprotein lipase. Released fatty acids are bound by albumin and are the donors of lipid for fatty acid uptake. The mechanism of fatty acid influx into adipocytes is complex, involving multiple steps, Figure 2. While not completely defined, several general concepts dominate the field. CD36 (also called FAT) might serve as a fatty acid receptor, accepting lipids from albumin and thereby producing a localized high concentration. CD36 is a 88 kDa highly glycosylated integral membrane protein located in caveolae, a region of the plasma membrane rich in sphingolipids containing saturated very long chain fatty acyl groups, and belongs to the family of class B scavenger receptors (16,18). CD36 is largely expressed in adipocytes, macrophages, heart, and skeletal muscle and deficiencies have been genetically linked to hyperlipidemia and hypertension (16,19-21). Several studies in skeletal muscle have shown the important role of CD36 in facilitating fatty acid influx, though it has not been as well studied in the context of adipocytes. Protonation of fatty acids due to the relative acidity of the plasma membrane, coupled with the low aqueous solubility of

fatty acids at neutral pH and high permeability of fatty acids in the membrane, creates a sufficient driving force for diffusion across the outer and inner leaflets of the membrane. The actual site of diffusion across the membrane is unknown but may be localized to caveolae.

For fatty acids on the inner membrane, a variety of acyl-CoA synthetases (ACS) catalyze the ATP- and CoA-dependent esterification, thereby providing a thermodynamic driving force for further diffusion. Using expression cloning, Lodish and co-workers identified the long chain acyl-CoA synthetase 1 (ACSL1) as well as a novel homologue of ACSL1 termed fatty acid transport protein 1 (FATP1) as facilitators of fatty acid influx (22). ACSL1, as well as other members of the acyl-CoA synthetase family, facilitate the esterification of fatty acids with coenzyme A (CoA) via an ATP-dependent mechanism forming fatty acyl-CoA . FATP1 is now known to belong to the larger FATP multigene family (FATP1-6) and possesses both long chain and very long chain acyl-CoA synthetase activity (23-25). Adipocytes predominately express ACSL1, FATP1 and FATP4. Over-expression of FATP1 and ACSL1 in fibroblastic cell lines results in increased fatty acid influx, which is not seen with over-expressed catalytically inactive forms (26,27). This finding suggests that esterification of long chain and very long chain fatty acids is coupled to fatty acid influx in a process termed vectoral acylation. Similar to translocation of GLUT4, ACSL1 and FATP1 translocate from intracellular vesicles to the plasma membrane in response to insulin stimulation (28). However, the magnitude of insulin-stimulated fatty acid influx is modest (2-3 fold) relative to hexose uptake. Since fatty acyl-CoAs are the biologically

active form of fatty acids, coupling fatty acid esterification with fatty acid influx into the adipocyte would allow fatty acids to be redirected and utilized for phospholipid synthesis, oxidation, or triacylglycerol synthesis. Indeed, triacylglycerol synthesis may occur on or around the plasma membrane and be functionally coupled to fatty acid influx (29).

2.3 Fatty acid and triacylglycerol biosynthesis

Adipocytes readily convert the products of glycolysis into fatty acids via the de novo biosynthetic pathway (15,16). Briefly, surplus citrate is transported from the mitochondrion and cleaved to produce cytosolic acetyl-CoA. This acetyl-CoA is acted upon by acetyl-CoA carboxylase producing malonyl-CoA. The next steps of the fatty acid biosynthetic pathway are carried out by the multifunctional fatty acid synthase, which utilizes NADPH to catalyze multiple condensations of malonyl-CoA with acetyl-CoA or the elongating lipid, eventually generating palmitate.

De novo fatty acid synthesis is, in part, negatively regulated by the AMP-activated protein kinase (AMPK) (30,31). When the adipocyte is in an energy deficit state due to the lack of available glucose or other cellular stresses, ATP levels decrease and AMP levels increase. This causes the AMP:ATP ratio in the adipocyte to increase and thus AMP binds and activates AMPK kinase, LKB. AMP also binds to AMPK, which allows LKB to recognize AMPK as a substrate and results in the phosphorylation and activation of AMPK. Active AMPK phosphorylates acetyl-CoA carboxylase, deactivating it and, hence, decreasing fatty acid synthesis (30-32).

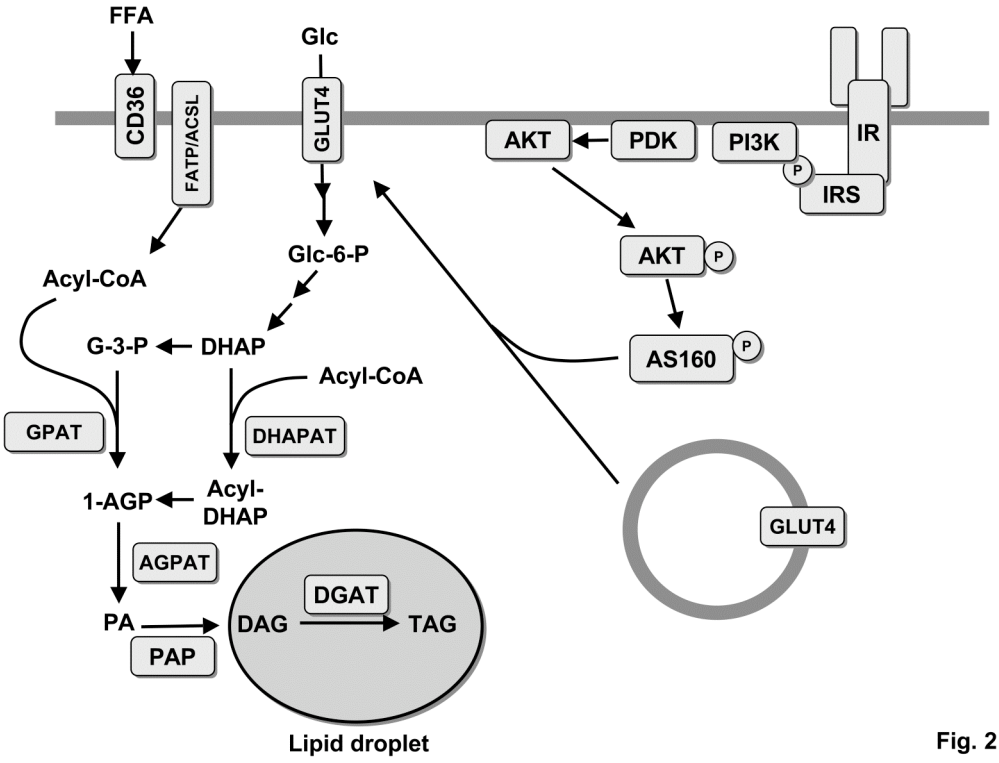

Fig. 2

Figure 2. Insulin-stimulated glucose uptake and triacylglycerol biosynthesis. Activation of the insulin receptor (IR) upon insulin binding results in the recruitment of IRS to the insulin receptor and subsequent tyrosine phosphorylation of IRS. This allows the subsequent recruitment and activation of phosphatidylinositol 3-phosphate kinase (PI3K), phosphatidyl inositol-dependent protein kinase (PDK), and AKT/PKB. PDK phosphorylates and activates AKT at the plasma membrane. AKT dissociates from the plasma membrane and phosphorylates AS160. Phosphorylated AS160 facilitates the translocation of GLUT4 containing vesicles to the plasma membrane, resulting in an increase in glucose transport. Glucose (Glc) transported into the adipocyte is phosphorylated (Glc-6-P) and converted to dihydroxyacetone phosphate (DHAP) via glycolysis and, subsequently, glycerol-3-phosphate (G-6-P). DHAP is converted to acyl-dihydroxyacetone phosphate (Acyl-DHAP) via DHAPAT and, subsequently, to 1-acyl-glycerolphosphate (1-AGP). 1-AGP is also directly produced from G-3-P via GPAT. AGPAT, then, adds a second acyl-CoA to 1-AGP to form phosphatidic acid (PA). PA is dephosphorylated by PAP to form diacylglycerol (DAG) and DGAT esterifies a third acyl-CoA with DAG to create triacylglycerol (TAG). IRS, insulin receptor substrate; AS160, AKT substrate of 160 kDa; DHAPAT, dihydroxyacetone phosphate:acyltransferase; GPAT, glycerolphosphate:acyltransferase; AGPAT, acyl-glycerolphosphate:acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol:acyltransferase.

Triacylglycerol synthesis combines two products of glycolysis, glycerolphosphate and dihydroxyacetone phosphate, with acyl-CoAs, Figure 2. In adipocytes, two pathways exist for the production of phosphatidic acid (33). In one, glycerol-3-phosphate is sequentially esterified with two acyl-CoAs to produce 1-acylglycerolphosphate and 1,2-diacylglycerolphosphate. In the second pathway, dihydroxyacetone phosphate is esterified with acyl-CoA to produce acyl-dihydroxyacetone phosphate. An acyl-dihydroxyacetone phosphate reductase subsequently produces 1-acylglycerolphosphate that leads to the production of 1,2-diacylglycerolphosphate. The resultant phosphatidic acid is dephosphorylated generating 1,2-diacylglycerol, and triacylglycerol is formed through the activity of diacylglycerol acyltransferase (DGAT).

The DGAT catalyzed reaction is crucial for it represents a branch point for hydrocarbon flow towards either the triacylglycerol or phospholipid pathways (34). However, this view has been modified through the production of DGAT null mice. DGAT deficient mice are viable, are resistant to diet induced obesity, and have reduced triacylglycerol synthesis, though they still have significant amounts of triacylglycerol (35). This implies that either an alternate triacylglycerol biosynthetic pathway is utilized or additional DGAT isoforms are present in adipose cells. To address this question, Farese and colleagues identified a second DGAT (DGAT2) with kinetic properties distinct from the original DGAT (now termed DGAT1) (35,36). DGAT2 null mice have lipopenia and skin barrier abnormalities. The DGAT2 null mice also have severely reduced triacylglycerol synthesis and triacylglycerol content in their tissues, suggesting

that DGAT2 is responsible for the majority of triacylglycerol biosynthesis (37). Moreover, additional DGAT-like sequences are present in the murine and human genome suggesting an unappreciated complexity in diacylglycerol metabolism.

2.4 Triacylglycerol mobilization

Lipolysis refers to the process by which triacylglycerol molecules are hydrolyzed to fatty acids and glycerol. During times of metabolic stress (i.e. during fasting or prolonged, strenuous exercise when the body's energy needs exceed the circulating nutrient levels), the triacylglycerol droplet within an adipocyte is degraded producing fatty acids to be used as an energy source by other tissues. Numerous stimuli are capable of eliciting the lipolytic response in adipocytes (38). A family of lipases, adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase catalyzes the hydrolysis of the triacylglycerol ester bonds producing glycerol and three moles of fatty acids (1,15,39).

The process of triacylglycerol hydrolysis is a complex phenomenon that involves at least three lipases, lipid droplet associated proteins and FABPs, although other adipocyte lipases (i.e. triacylglycerol hydrolase) may play a role in basal lipolysis, Figure 3. The data at this time support the model that three lipases are the major contributors to adipocyte lipolysis. Complete hydrolysis of triacylglycerol involves the hydrolysis of three ester bonds to liberate three fatty acids and a glycerol moiety. ATGL catalyzes hydrolysis of the first ester bond to release one fatty acid and results in diacylglycerol formation (40,41). HSL can catalyze the hydrolysis of triacylglycerol,

but has 10-fold greater activity towards diacylglycerol, hydrolyzing the second ester bond at either the *sn*-1 or *sn*-3 position (42,43). A third enzyme, monoacylglycerol lipase, catalyzes hydrolysis of the remaining ester to yield a third fatty acid and glycerol (Figure 1). Since adipocytes do not express glycerol kinase to any great extent (although they might when PPARγ is activated), they are generally unable to reuse glycerol. Therefore, glycerol is transported out of adipocytes via aquaporin 7 and must be shuttled back to the liver for oxidation or gluconeogenesis. Mono- and diacylglycerols can be re-esterified by the endoplasmic reticulum acyltransferases. During a lipolytic stimulus, re-esterification is minimized so that the net direction of these reactions is toward fatty acid efflux from the cell (44,45).

Free fatty acids are minimally soluble in the aqueous cytoplasm. The charged carboxylate group provides enough electrostatic hindrance to prevent association with the neutral triacylglycerols whereas the hydrocarbon tail reduces solubility in water. At sufficiently high concentrations fatty acids exert a detergent-like effect that would disrupt membranes and/or they could cluster together in micelles in the crowded cytoplasm. To alleviate this problem, the adipocyte and other lipid-metabolizing cell types have evolved intracellular fatty acid binding proteins (FABPs), a family of small, soluble, highly abundant proteins that bind and sequester fatty acids (12). Adipocytes express two FABPs, the adipocyte and epithelial FABPs. Once outside the adipocyte, fatty acids are immediately bound to serum albumin and carried in the bloodstream to the liver, muscle and other tissues for oxidation.

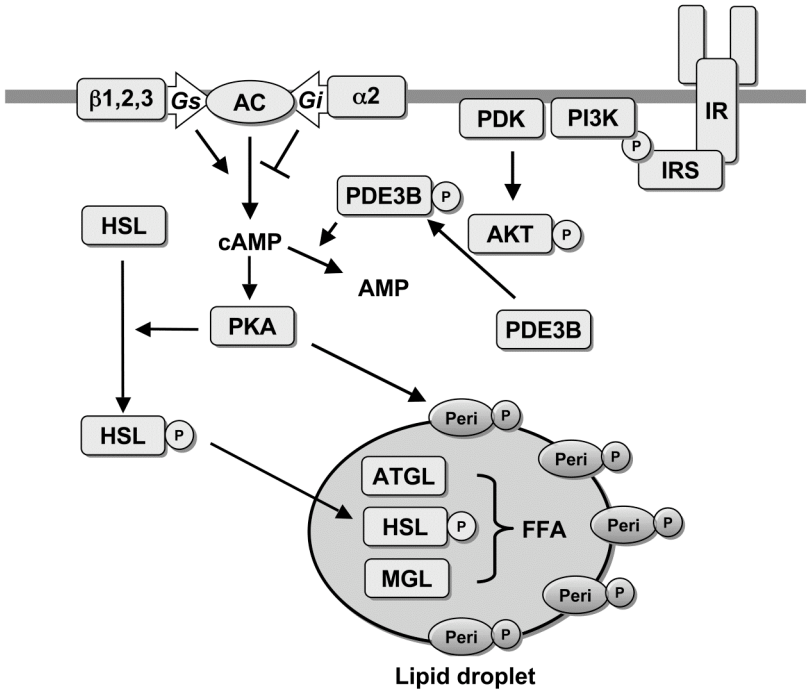

Fig. 3

Figure 3. Regulation of lipolysis via adrenoreceptor-coupled systems and insulin. Binding of lipolytic agonists to β-adrenoreceptors (β_1, β_2 and β_3) couples to G_s protein that in turn activates adenylyl cyclase (AC) thereby producing cAMP. cAMP activation of protein kinase A (PKA) results in phosphorylation and activation of hormone-sensitive lipase (HSL) and perilipin (peri) and subsequent translocation of HSL to the lipid droplet. Lipolytic activation stimulates the catalytic activity and access of regulatory lipases (ATGL, HSL and MGL) to the droplet surface resulting in generation of fatty acids (FFA). Ligand binding to α_2-adrenoreceptors results in coupling with G_i and a decrease in adenylyl cyclase activity. Insulin binds to its cell surface receptor (IR) and initiates a signaling cascade linking phosphorylation of insulin receptor substrate (IRS) to the activation of phosphatidylinositol 3-phosphate kinase (PI3K), phosphatidyl inositol-dependent protein kinase (PDK) and AKT/PKB. AKT phosphorylates and activates the cGMP-inhibited phosphodiesterase (PDE3B), increasing cAMP hydrolysis. The resulting decrease in PKA activity thus provides the basis for the anti-lipolytic effects of insulin.

HSL has long been thought of as the rate-limiting enzyme in lipolysis. This lipase is regulated through the stimulation of β-adrenergic receptors by catecholamines or more recently discovered, by natriuretic peptides, and the resulting activation of cAMP

dependent protein kinase (PKA) (39,46,47). HSL is an 84 kDa protein modeled to be organized into N-terminal (1-300) and C-terminal (300-767) domains. The regulatory and catalytic activity of the enzyme lies within the predicted α/β hydrolase fold of the C-terminal domain. Three residues, Asp703, His733, and Ser423, form the catalytic triad. Phosphorylation of Ser659 and Ser660 in the C-terminal domain results in a modest increase in specific activity of the enzyme and is correlated with a translocation of the protein from the cytoplasm to the surface of the lipid droplet (48). Regulation of HSL by phosphorylation/dephosphorylation makes it unique among lipases.

Adipocytes of HSL null mice retain 40% of the triacylglycerol lipase activity compared to wild type animals (43). In addition, they accumulate diacylglycerol, while showing no difference in triacylglycerol hydrolysis compared to wild type animals. These data led to the consideration that HSL is rate-limiting for hydrolysis of diacylglycerol rather than triacylglycerol and to the discovery of ATGL (41).

ATGL is a 54 kDa protein which contains a patatin-like domain in its N-terminal region, a domain commonly found in lipases. In addition, ATGL has a conserved glycine rich GXGXXG nucleotide binding motif, a GXSXG serine hydrolase motif and a DX(G/A) motif with a conserved aspartate residue. The serine and aspartate residues form a catalytic dyad that is necessary for lipase activity in this family of proteins (41). In vitro hydrolase activity assays showed that ATGL has triacylglycerol hydrolase activity but no diacylgylcerol, cholesteryl ester or retinyl ester hydrolase activity. ATGL knockout mice have defective lipolysis in adipose tissue resulting in increased

adipose tissue mass, confirming its importance as a major adipose tissue lipase (49). ATGL is located at the lipid droplet surface under basal and stimulated conditions making it likely that ATGL is responsible for the majority of basal lipolysis although other triglyceride lipases might also play a role (40,50).

The process of lipolysis involves structural proteins in addition to hydrolases. The most significant contribution is by the lipid droplet associated protein perilipin. Perilipin constitutively interacts with lipid droplets through three hydrophobic domains. Over expression of perilipin results in increased triacylglycerol deposition and inhibition of lipolysis, whereas perilipin null mice are lean under high fat conditions with increased basal lipolysis (51,52). It is thought that perilipin blocks access of lipases to their substrate under basal conditions. PKA stimulation results in the phosphorylation of perilipin at six serine residues. The three C-terminal phosphorylation sites are necessary for stimulated lipolysis in adipocytes (53). These serine residues have been implicated in HSL translocation to the lipid droplet and HSL interaction with perilipin at the droplet surface (54-56). Ablation of one of these, Ser517, results in decreased ATGL activity and decreased lipolysis under stimulated conditions (57). The mechanism by which perilipin affects ATGL activity is unknown, but it may be mediated through the cofactor CGI-58. 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 (58). Perilipin interacts with CGI-58 under basal conditions and dissociates under stimulated conditions. Recently it has been shown that CGI-58 interacts with ATGL under stimulated conditions resulting in increased activity (59).

The fatty acid binding protein (AFABP/aP2) facilitates the intracellular solubilization and diffusion of fatty acids produced by lipolysis. The N-terminal domain of HSL is a docking site for interaction with AFABP/aP2 (60,61). AFABP/aP2 stimulates the activity of HSL in vitro by relieving product inhibition by fatty acids, however, this activity seems to be independent of the interaction between AFABP/aP2 and HSL (62). The AFABP/aP2 may also shuttle fatty acids produced by ATGL or the monoacylglycerol lipase as well, but there is no evidence for physical association with these enzymes. Consistent with the model of AFABP/aP2 as a fatty acid shuttle facilitating efflux, AFABP/aP2 null mice exhibit reduced lipolysis while fatty acids accumulate intracellularly (63).

3 Lipid Mediated Signal Transduction

3.1 Fatty acids and acyl-CoAs

Fatty acids and acyl-CoAs are not only substrates for metabolic enzymes, but also serve as regulatory molecules influencing the activity of several metabolic enzymes. For example, the lipolytic activity of HSL is inhibited by non-esterified fatty acids, resulting in feedback inhibition of the enzyme. Thus, if there is a surplus of fatty acids, HSL activity is reduced resulting in a potential accumulation of diacyglycerol that in turn would lead to insulin resistance. Additionally, fatty acids inhibit de novo lipogenesis via regulation of fatty acid synthase as well as cholesterol synthesis through the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase.

Acyl-CoAs are also key regulators of enzymatic activity and particularly affect de novo lipogenesis (64). Acetyl-CoA carboxylase utilizes acetyl-CoA as a substrate to catalyze the formation of malonyl-CoA. This product is an essential substrate for lipogenesis via fatty acid synthase, as well as being an inhibitory factor for fatty acid oxidation. Acetyl-CoA carboxylase is inhibited by acyl-CoAs resulting in feedback inhibition of de novo lipogenesis, reduced malonyl-CoA production and concomitant activation of the oxidative pathways. Acyl-CoA, like fatty acids, also inhibits HSL, fatty acid synthase and 3-hydroxy-3-methylglutaryl-CoA reductase. Additionally, acyl-CoAs, as well as the acyl-CoA metabolite diacylglycerol, are key regulators of protein kinase C (PKC) which when activated in adipocytes results in serine phosphorylation of IRS-1 and the development of insulin resistance.

Fatty acids have also been implicated in the inflammatory response in adipose tissue, although the mechanisms are complex and likely to be indirect. Addition of supraphysiological levels of fatty acids, particularly palmitate but to a lesser extent oleate, to cultured adipocytes results in the activation of the c-Jun N-terminal kinase. This kinase is a critical regulator of the inflammatory response and regulates cell proliferation, survival, cell death, ER stress response, DNA repair and metabolism. Importantly, c-Jun N-terminal kinase is also activated by TNFa which results in serine phosphorylation of insulin receptor substrate, thereby reducing insulin signaling (65). However, with the discovery of vectoral acylation, it is now apparent that fatty acid influx results in esterification of the fatty acids at the plasma membrane and direct channeling of the products into the triacylglycerol biosynthetic pathway. Thus, the

actual molecular mechanism of c-Jun N-terminal kinase activation by fatty acids is unclear and is not likely to be due to an increase in intracellular fatty acids.

Fatty acids and its metabolites also function as ligands for transcription factors (66). Most notably, the PPAR receptors have been shown to directly bind fatty acids, although with lower affinity than some other potential ligands. However, fatty acids are relatively abundant in adipose tissue and may still be very important for endogenous PPAR activity. Additionally, nitroalkenes have also been suggested to be PPARγ ligands. Although present at much lower concentrations than free fatty acids, nitrated fatty acids have a much higher affinity for PPARγ. Besides fatty acids, acyl-CoAs have been implicated in transcriptional control. The crystal structure of bacterially expressed hepatocyte nuclear factor-4 reveals the presence of a tightly bound acyl-CoA, implying that such lipids are physiological regulators of protein structure and function.

3.2 Eicosanoids

While many investigators have focused on the study of PPAR receptors, the identity of a biologically relevant ligand has remained elusive. Extensive studies have demonstrated the ability of a variety of hydrophobic ligands to bind and activate PPARγ. Two classes of enzymes that might be involved in the generation of a natural ligand include the cyclooxygenase enzymes, which catalyze the rate-limiting step of prostanoid biosynthesis, as well as the lipoxygenase enzymes. Phospholipase A_2 may be important in cleaving a fatty acid from phospholipids generating the main source of arachidonic acid, the substrate for the cyclooxygenases.

The search for potential natural ligands for PPARγ has focused on polyunsaturated and oxidized fatty acids. Some arachidonic acid metabolites, in particular, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2 and 15S-hydroxyeicosatetraenoic acid, produced by 15-lipoxygenase, have been suggested as direct ligands of PPARγ. Other arachidonic acid metabolites derived from the lipoxygenase pathway, namely 8-S-hydroxyeicosatetraenoic acid and leukotriene B4 have been suggested as direct ligands for PPARs (67). Additionally, derivatives of linoleic acid, including 9- and 13-hydroxyoctadecadienoic acids, 13-oxooctadecadienoic acid, and nitroalkenes have been identified as potential natural ligands for PPARγ. While many of these molecules have been suggested as biologically relevant ligands, there also have been concerns whether the actual endogenous concentration of these are sufficient to support PPARγ activity. This factor is particularly important when considering that fatty acids themselves are weak activators of PPARγ, suggesting that within the cellular context there are a variety of biologically relevant ligands with differing affinities and abundances. Since different ligands produce subtly different structures when bound to PPARγ, the ability to bind to co-activator complexes varies among lipids. Hence, different ligands have the capacity to activate different cassettes of genes, implying that lipids have the ability to selectively regulate adipocyte metabolism.

Cylopentenone prostaglandins of the A- and J-type contain a reactive a,b-unsaturated carbonyl group in a cyclopentane ring which allows covalent modification of nucleophiles through Micheal addition reactions. 15-deoxy $\Delta^{12,14}$- prostaglandin J_2 and

prostaglandin A1 can covalently modify redox sensitive transcription factors, typically through a cysteine residue (68). For IKKa and b, covalent modification of a cysteine within the activation loop inhibits its activity. NF-kB can also be modified by 15-deoxy $\Delta^{12,14}$- prostaglandin J_2, resulting in decreased DNA binding and transcriptional activity. Increased activity through covalent modification occurs with PPARγ, Keap1, (increases Nrf2 activity), and p53. Transcription factors that have decreased activity include c-Jun (decrease AP-1 activity) and hypoxia inducible factor 1.

Other metabolites of arachidonic acid exhibit profound effects on lipid metabolism in adipose tissue. Prostaglandin E_1 and E_2 (PGE_1 and PGE_2) acutely inhibit catecholamine-induced lipolysis (69). PGE_2 exerts its action through binding to EP receptors, which contain seven transmembrane regions and are members of the G-protein coupled receptor family. The receptors mediating prostanoid-induced inhibition of lipolysis in adipocytes are thought to be of the EP3 sub-type, which couples to the inhibitory Gi subunit. Mechanistically, EP3 receptors have been associated with inhibition of adenylyl cyclase, thus reducing cAMP levels. Thus it is through this mechanism that only stimulated (and not basal) lipolysis is inhibited. Contrary to acute PGE treatment, prolonged treatment of adipocytes with PGE results in down-regulation of Gi subunits and increases lipolysis.

3.3 Diacylglycerol and ceramide

PKC is a family of serine/threonine kinases with varying tissue specific expression patterns and whose activity can affect a multitude of cellular functions. The family is

divided into three subgroups: conventional isozymes, novel isozymes and atypical isozymes. The first two are activated by diacylglycerol. Diacylglycerol activation of PKC leads to serine phosphorylation of IRS-1, resulting in reduced insulin stimulated tyrosine phosphorylation as well as increased degradation of insulin receptor substrate-1 (70). There is evidence for direct phosphorylation of insulin receptor substrate-1 by PKCθ, however, PKCθ also activates IkB kinase and c-Jun N-terminal kinase in adipocytes. Both of these protein kinases have been shown to phosphorylate a serine residue of IRS-1. In contrast to PKCθ, PKCδ leads to phosphorylation of p47, activating NADPH oxidase. These molecular mechanisms that result from increased diacylglycerol accumulation in adipocytes may at least in part, explain the insulin resistance associated with obesity.

Ceramide (N-acyl sphingosine) is a serine-based lipid containing saturated acyl chain typically of 16 and 24 carbons in length. By virtue of their hydrophobicity, ceramides function in a variety of signaling pathways, particularly those affecting membrane structure and permeability (71). Ceramide is an important signal effector molecule in stress pathways, such as those activated by TNFa, resulting in activation of protein kinases including c-Jun N-terminal kinase and the atypical PKCζ/l. TNFa, through increases in ceramide and subsequent activation of the atypical PKCζ/l, has been shown to decrease PPARγ in adipose tissue. Ceramide also activates protein phosphatase 2A, thereby dephosphorylating proteins such as PKB/AKT in the insulin signaling cascade and preventing efficient insulin signaling and/or promoting growth arrest.

3.4 Lipids as mediators of inflammation

Adipose tissue is composed not only of adipocytes, but also other cell types, including adipocyte progenitor cells, endothelial cells, and blood cells. More recently, several studies have indicated the presence of infiltrated macrophages in adipose tissue, with the degree of adiposity correlating with the quantity of macrophages. In the extremely obese individual, it is thought that up to 40% of the mass of adipose tissue is comprised of resident inflammatory cells. This correlation also extends to the degree of insulin resistance. Of the paracrine factors thought to play key roles in cellular regulation, fatty acids produced by adipocyte lipolysis have been considered central. Accordingly, a number of studies have focused on the local adipocyte fatty acid-macrophage signaling system (72).

Both adipocytes and macrophages secrete a number of paracrine and endocrine factors, some specific to only one cell type, while others are secreted by both types. The initiating factor is thought to be fatty acids effluxed from the adipocyte. The fatty acids liberated from the adipocyte are thought to bind to TLR4 receptors (classically studied with respect to binding a range of microbial products) and signal an inflammatory response in the macrophage. Such activated macrophages are thought to be the major source of locally produced proinflammatory cytokines, such as TNFα and interleukin-6, which can bind to receptors on adjacent adipocytes and regulate lipid metabolism and insulin action. Thus, a paracrine loop exists between adipocytes that release fatty acids and macrophages that secrete TNFα, interleukin-6, creating a feed-forward cycle that

potentiates inflammatory conditions in WAT. Additionally, the adipocyte expresses and secretes a diverse range of additional factors that have a direct impact on adipocyte metabolism, as well as on whole body energy homeostasis and insulin action.

Adipocytes have profound sensitivity to TNFα, interferons α, β, and γ, and interleukins 1, 6, and 11 (73,74). In general, such cytokines inhibit lipogenesis and triacylglycerol storage by adipocytes, activate lipolysis and antagonize insulin action. Additionally, many cytokines interfere with proliferation of preadipocytes in 3T3-L1 or 3T3-F442A cell lines and/or diminish adipogenesis in vivo. TNFα is expressed at high levels in adipose tissue as seen in several genetically defined rodent models of obesity and insulin resistance. In leptin receptor deficient mice, TNFα expression is elevated but expression of other cytokines such as interleukin-1 or interleukin-6 is not increased. Measurements of insulin receptor tyrosine kinase activity in obese leptin deficient rats show a reduced function, which could be restored by administering soluble TNFα receptor to sequester the secreted TNFα protein. TNFα interferes with insulin signaling by affecting the phosphorylation of the insulin receptor, thus contributing to the development of insulin resistance in these animals (8,75-77). In the 3T3-L1 adipocyte cell culture model, TNFα inhibits the expression of C/EBPα and PPARγ, thus leading to reduced expression of genes that are involved in triacylglycerol accumulation and metabolism (78,79). Interleukin 6 is secreted by WAT under basal conditions and its expression is increased by TNFα and by other conditions linked to wasting disorders (cancer, cachexia, HIV).

TNFα and the interferons decrease lipogenesis by down-regulating the mRNA levels of the key lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase; the interferons diminish mRNA levels of fatty acid synthase but not of acetyl-CoA carboxylase. TNFα, interleukin-1, and some interferons also increase lipolysis, although the mechanism is probably post-transcriptional, since Northern blot analysis shows that TNFα and the interferons decrease the level of perilipin mRNA (80-82).

3.5 Leptin and adiponectin

Adipose tissue secretes a large number of proteins, many of which affect overall whole body energy homeostasis (83). Leptin is synthesized and secreted from adipocytes and circulates at levels that are proportional to the body fat content. Consequently, leptin is capable of signalling the brain as to the degree of whole body adiposity. Although the brain expresses the highest number of leptin receptors, there are also leptin receptors ubiquitously expressed in peripheral tissues. Leptin receptors are members of the class 1 cytokine receptor family and are linked to JAK-STAT signaling systems (84). This signal affects overall energy homeostasis by affecting processes such as food intake, satiety and energy expenditure. Consistent with this, the lack of leptin in mice results in hyperphagia and severe obesity (85).

Adiponectin is a protein exclusively expressed and secreted from adipocytes. PPARγ ligands have been shown to upregulate adiponectin expression. Circulating levels of adiponectin correlate positively with insulin sensitivity and negatively with body mass index. Adiponectin exerts its affect by binding two receptors (86). The oligomeric state

of adiponectin determines which receptor is bound and activated (87). Signalling through the adiponectin receptors activates AMPK and results in suppression of inflammatory cascades such as TNFa, as well as suppressing macrophage foam cell formation (84). Several groups have generated adiponectin null mice with somewhat varying results. The phenotypes found range from severe insulin resistance on a high fat diet to no change in insulin sensitivity, but an increase in fatty acid oxidation in skeletal muscle. More recently, the lack of adiponectin was shown to have a defect in insulin-dependent suppression of hepatic glucose output, with minor effects on muscle glucose uptake or oxidation (88,89). Although the exact mechanism of action is still being elucidated, overall it is still clear that adiponectin functions as an important insulin sensitizer.

4 AFABP

4.1 FABP family

AFABP/aP2 is part of a multi-gene family of intracellular lipid binding proteins having a molecular mass around 15 kDa known as fatty acid binding proteins (FABPs) (90). Members of this family have been found throughout the animal kingdom, both in invertebrates and vertebrates. It is thought that individual genes have arisen from an ancestral gene through gene duplication around 900 million years ago (91-93). Since the discovery of the first FABP in 1972, the function of these proteins has remained elusive (94).

FABP's facilitate the solubilization and diffusion of fatty acids in the aqueous environments of the cell. As discussed above, intracellular fatty acids are derived from the breakdown of triacylglycerol and not from fatty acid uptake because of vectoral acylation. Therefore, the substrate pool for FABPs is determined by the amount of triacylglycerol and the rate of its hydrolysis. FABPs are expressed in many tissues and their levels of expression are relative to the fatty acid metabolism capacity in that tissue. Adipocytes, hepatocytes and cardiomyocytes have a high capacity of fatty acid metabolism and very high levels of FABPs, between 1-5% of total cytoplasmic proteins. Tissues with low lipid metabolism express low levels of FABPs. Chronically elevated plasma lipid levels can further increase the abundance of these proteins, suggesting a cellular response mechanism to deal with excess fatty acids.

Although, the amino acid sequence identities of FABP's range from 20-70%, all known structures, including AFABP/aP2, have the same three-dimensional motif. This motif is described as a 10-stranded anti-parallel beta-barrel, with an internal ligand-binding cavity (95,96). This similarity at the structural level results in similar, but not identical, binding specificity and affinity (high nM for AFBAP/aP2) for the FABPs. Most FABPs, including AFABp/aP2, bind one fatty acid in the cavity, although the liver isoform can accommodate two. AFABP/aP2 can bind medium to very-long chain fatty acids, leukotrienes, eicosanoids and other hydrophobic ligands. The wide tissue distribution of FABPs suggests that they may have similar but distinct functions in a tissue specific manner. The low sequence identity may confer specific protein-protein interactions for each member, facilitating tissue specific functions based on which

members are expressed. Indeed, it has been shown that liver FABP forms an interaction with PPARα, while AFABP/aP2 interacts with PPARγ.

Crystal structures of AFABP/aP2 in the apo-form and in many holo-forms have been elucidated. The holo structures determined that R126 and Y128 coordinate the carboxylate of the fatty acid deep within the binding cavity (97-100). Ligand binding assays confirmed that mutation of these residues decreases affinity 100-fold. The cavity is capped by the helix-turn-helix domain, essentially burying the fatty acid within AFABP/aP2. Additionally, a structural nuclear localization signal was identified in the helix-turn-helix domain, comprised of K21, K31, and R30. Mutation of the NLS resulted in nuclear exclusion of AFABP/aP2 (101,102). Nuclear export of AFABP/aP2, was shown to be CRM1 dependent, and a subsequent, structural nuclear export sequence was identified and comprised of a cluster of leucine residues (101,102). Considering that AFABP/aP2 is 15 kDa and should freely diffuse in and out of the nucleus, the importance of the NLS and NES remain unclear.

4.2 Expression

In adult mammals, AFABP/aP2 is predominately expressed in adipocytes and macrophages. AFABP/aP2's mRNA expression is regulated by fatty acids, insulin, dexamethasone, lipopolysaccharide, oxidized LDL and PPARγ agonists (103-107). Transcription factor binding sites for PPARγ and CEBPα have been mapped to the promoter region of AFABP/aP2. These two transcription factors are responsible for the

50-fold change in mRNA expression during the conversion from preadipocytes to adipocytes and for maintenance in the mature adipocyte (108-110). PPARγ activity is regulated by hydrophobic ligands such as fatty acids and eicosanoids, although the endogenous ligand is still unclear, suggesting that it is responsible for the increased AFABP/aP2 mRNA and protein observed in obesity.

4.3 Interactions

AFABP/aP2 directly interacts with PPARγ (111). Co-immunoprecipitation experiments revealed a weak interaction that was strengthened with the addition of PPARγ ligands. Considering that AFABP/aP2 binds many, if not all PPARγ ligands, it is unclear which protein the ligands are affecting. Significance of this interaction has been hard to elucidate. Depending on the cell type and ligand used to activate PPARγ, AFABP/aP2 activates and inhibits PPARγ activity (101,102,105,111-113). Confounding the interpretation further is the fact that AFABP/aP2 could be delivering ligand to PPARγ or sequestering ligand from PPARγ, independent of interaction. Together, the data for the interaction suggests there is a regulatory loop consisting of AFABP/aP2 sensing fatty acid levels in the cell, relaying that information to PPARγ, which in turn regulates the mRNA levels of AFABP/aP2 and other lipid metabolizing genes, thereby altering lipid metabolism in an appropriate manner.

AFABP/aP2 was identified as an interacting partner of HSL in a yeast 2 hybrid experiment (60). Subsequent experiments revealed that HSL residues 190-200 are necessary for the interaction (61). AFABP/aP2 stimulates the in vitro activity of HSL,

although this was determined to be independent of interaction, as FABP's that do not interact with HSL still activate HSL (62). Further studies determined that fatty acids are necessary for the interaction, although it is unclear which protein is binding these fatty acids (62). The significance of this interaction remains to be elucidated. Chapters 2 and 3 examine more details of this interaction in a cellular context.

4.4 Animal models

AFABP/aP2 has long been thought to solubilize and traffic fatty acids in the aqueous compartments of the cell. To this end, it has been proposed that it facilitates lipid metabolism through the delivery of substrates, such as fatty acids, to the endoplasmic reticulum for triacylgycerol synthesis, to the mitochondria and peroxisomes for oxidation, to other cellular compartments for the production of eicosinoids and leukotrienes, to transcription factors for activation of transcription and to the plasma membrane for efflux. The evidence for these proposed functions has been lacking.

In order to elucidate the function of AFABP/aP2, knockout mice were developed (114). Interpretation of AFABP/aP2 null mice is confounded by the compensatory upregulation of EFABP in adipocytes. EFABP is normally expressed at extremely low levels in adipocytes compared to AFABP/aP2, 1:99. Although EFABP is upregulated 40-fold in AFABP/aP2 null mice, this results in a 50% decrease in total FABP in adipocytes (115). AFABP/aP2 knockout mice exhibited reduced insulin resistance in the context of both diet induced and genetic obesity (114,116-118). This effect on

insulin sensitivity was not observed in lean mice. Isolated adipocytes from AFABP/aP2 null mice have decreased basal and stimulated lipolysis, while having increased intracellular concentrations of fatty acids (63). An in vivo approach of stable isotope labeling confirmed the lipolysis defect and determined AFABP/aP2 null mice preferentially utilize glucose over fatty acids (119). AFABP/aP2 null mice also exhibit increased de novo lipogenesis in adipocytes (115,120). On a high fat diet, AFABP/aP2 null mice have increased intracellular and plasma levels of the insulin sensitizing adipokine adiponectin, while having decreased pro-inflammatory cytokines such as TNFα (115). AFABP/aP2 null mice crossed into ApoE null mice showed protection against atherosclerosis independent of diet (118,121). In order to determine whether this phenotype was conferred by macrophages or as a consequence of whole body lipid metabolism changes, AFABP/aP2 knockout bone marrow was transplanted into ApoE null mice. Protection from atherosclerosis was seen in this study, confirming that this phenotype is determined by AFABP/aP2 expression in the macrophage.

As AFABP/aP2 had a significant role in atherosclerosis, further studies utilized peritoneal macrophages from AFABP/aP2 null mice (122,123). AFABP/aP2 null macrophages exhibit increased PPARy activity leading to increased LXR activity and the upregulation of ABCA1, resulting in increased cholesterol efflux. These macrophages also exhibit decreased IKK-NFkB pathway activity with LPS stimulation, resulting in decreased pro-inflammatory cytokine secretion. These two pathways have been proposed to be the mechanism for the protection against atherosclerosis, although the molecular mechanism for AFABP/aP2's role in these pathways is not clear.

Mice have been generated with a combined deficiency in AFABP/aP2 and EFABP (124,125). These mice gained significantly less weight when challenged with a high fat diet unlike AFABP/aP2 null mice. Despite this, the double knockout mice show phenotypes similar to the single knockout, increased insulin sensitivity in the context of diet induced and genetic obesity, and decreased atherosclerosis in an ApoE null background (123-126). Additionally, it has been reported that in humans a single point mutant in the promoter region results in decreased mRNA for AFABP/aP2 and increased insulin sensitivity (127). These studies emphasize the role of AFABP/aP2 in lipid metabolism and link AFABP/aP2 to other cellular processes such as transcriptional reprogramming and signal transduction.

High throughput screening has identified biphenyl azole inhibitors of AFABP/aP2. These compounds inhibit fatty acid binding and coordinate in the binding cavity in a similar manner as a fatty acid. Treatment of ApoE null mice decreased mean atherosclerotic lesion area on a western diet. Treatment of ob/ob mice increased glucose homeostasis and insulin sensitivity (128). Together, the data suggest that inhibition by pharmacologic means or genetic means results in a similar phenotype. Further, these studies suggest that fatty acid binding is necessary for these phenotypes.

5 Concluding Remarks

AFABP/aP2's role in lipid metabolism has clearly been demonstrated, including lipolysis, lipogenesis, and cholesterol homeostasis. In light of these observations, it is necessary to understand the molecular details of how AFABP/aP2 affects fatty acid

metabolism and cellular signaling networks. Is it through passive diffusion and delivery of fatty acids or regulated interaction based on the availability of fatty acids? To this end, the following chapters will show two protein-protein interaction partners of AFABP/aP2, HSL (Chapters 2 and 3) and JAK2 (Chapter 4). These interactions set up a hypothesis that AFABP/aP2 is a fatty acid sensor, affecting cellular processes through ligand dependent interactions with its helix-turn-helix domain and the interacting proteins ExYK motif.

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CHAPTER 2:

PHYSICAL ASSOCIATION BETWEEN THE ADIPOCYTE FATTY ACID BINDING PROTEIN AND HORMONE SENSITIVE LIPASE: A FLUORESCENCE RESONANCE ENERGY TRANSFER ANALYSIS

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Brian Thompson's contribution to this chapter was Figure 3, and preparation of the text and discussion.

ABSTRACT

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 CFP-HSL, 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. FRET analysis demonstrated that CFP-HSL associated with YFP-Adipocyte FABP in both basal and forskolin treated cells. In contrast, little if any FRET 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 translocates to the surface of the lipid droplet.

INTRODUCTION

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), 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). While 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, co-immune precipitation as well as deletion and point mutation analysis (14-16). 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 N-terminal domain of HSL juxtaposes the fatty acid binding protein adjacent to the catalytic C-terminal region so as to accept a product fatty acid during triacylglycerol hydrolysis.

To explore the specificity of HSL-FABP interaction, isothermal titration microcalorimetry (ITC) has recently been used (17). Using ITC, 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 over expressing a combination of fatty acid transport protein 1 and perilipin A as a simple vehicle for assessing FABP-HSL interaction within the cellular context. Due to 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 and colleagues (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, (19) found that 293 fibroblasts expressing FATP1 exhibit increased oleate and lignocerate influx and that such lipid is preferentially

channeled 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.

MATERIALS AND METHODS

Materials—Fatty acids were obtained from Nu-Chek Prep, Inc., Elysian, MN. pcDNA3.1/Zeo, 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,4a-diaza-*s*-indacene-3-dodecanoic acid) and Alexa Fluor 488 were 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. The non-esterified fatty acid (NEFA) assay system was purchased from WAKO Chemicals USA, Inc, Richmond, VA. Cholesteryl [1-^{14}C] oleate was purchased from Amersham Biosciences (Piscataway, NJ). All other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Cloning and Cell Biology—An A206K mutation was introduced into each of the GFP derivatives to reduce intrinsic dimerization (20) using the QuikChange™ Site-Directed Mutagenesis technique of Stratagene Cloning Systems, La Jolla, CA. HSL was then subcloned in frame into the A206K pECFP-C1 expression vector while AFABP and LFABP were subcloned into A206K pEYFP-C1. 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 confirmed 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 manufacturers instructions (BTX division of Genetronics, Inc., San Diego, CA). Dilutions of the electroporated cells were plated in DMEM containing 10% FBS onto surfaces pretreated with 25 μg/ml of poly-*L*-lysine to enhance attachment and incubated at 37°C, 5% CO_2. 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-perilipin 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 pretreated with 25 μg/ml of poly-*L*-lysine.

Immunocytochemistry—C8PA cells plated on 13mm 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 0.01 M 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-perilipin antibody (9) in PBS containing 0.01% digitonin. Following washing in PBS with 0.01% digitonin, the coverslips were incubated for 1 hr 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. Following washing, the coverslips were incubated at 25° C. in DMEM with BODIPY D3835 (10 μgs /mL), then washed with PBS, mounted and visualized for perilipin or fat droplets by confocal microscopy.

Confocal Microscopy—Preparations were viewed using a BioRad MRC-1024 confocal microscope attached to a Nikon Diaphot inverted microscope (BioRad Labs, Hercules, CA) equipped with a 15 mW Krypton/Argon laser. Excitation filters allowing 488 nm, 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 LaserSharp version 3.2 software (BioRad Labs, Hercules, CA). Stored digital images were analyzed using Image Pro Plus version 4.5 software (Media Cybernetics, Silver Springs, 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. Following loading with fatty acids, the cells were washed 2 times with phosphate-buffered 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 hours (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 approximately 70% confluence, wells were transfected with expression plasmids 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 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 Roper CoolSnap HQ 12 bit monochrome camera and captured to a Pentium IV 2.6 GHz personal computer using Image Pro Plus version 4.5 software (Media Cybernetics, Silver Springs, MD 20910) 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 436/20 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 obtained from the Chroma Technology (Brattleboro, VT). Images were acquired using 2x2 binning 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 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-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 (FRETC) was calculated on a pixel-by-pixel basis for the entire image using: FRETC = FRET – (0.50 x CFP-HSL) – (0.02 x YFP-FABP), where FRET, CFP-HSL, and 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 image to remove high-frequency noise (22). Images were contrast stretched, pseudocolored and formated for display using Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

HSL activity assay—Plasmids directing the expression of either HSL or CFP-HSL were transiently transfected into 293 cells, washed twice in phosphate buffered saline and harvested into 1mL of 50 mM Tris-HCL, 0.1 mM EDTA pH 7 with 20 μg/mL leupeptin, 2μg.mL antipain and 1μg/mL pepstatin. The extract was sonicated at 30 % power 3 times for 30 seconds each on ice using a Misonix Sonicator XL. The homogenate was centrifuged at 12,000 x g for 15 min and the supernatant used in the assays. Substrate preparation and assay conditions were as previously described (23). Briefly, for 4 mL of substrate 0.45 mM cholesteryl oleate, 1.4 mg phosphatidylcholine/ phosphatidylinositol (3:1, w/w) and 0.9 μCi/mL of cholesteryl ^{14}C oleate were mixed and dried under nitrogen. The dried substrate was emulsified by adding 1 mL 0.1 M potassium phosphate buffer pH 7.0 and sonicated 2 X 1minute in a Branson 2510 water bath sonicator. Addition of 3 mL buffer followed by sonicating 4 X 30 seconds emulsified the substrate. For reactions, 100 μL of substrate is mixed with 50 μL of enzyme at 37° C and the production of fatty acids measured after 30 minutes as described (23).

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 triacylglycerol. However, triacylglycerol accumulated by C8 cells was disorganized and appeared rather diffusely distributed within the cell based on oil red O staining. Greenberg and colleagues (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 transfected 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. Figure 1A shows the oil red O staining of C8PA cells following a 48 hour "load" in which BSA:FA (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 μM forskolin for four hours and evaluated fatty acid release from the cells. As shown in Figure 1B, forskolin stimulated a 2-fold increase in fatty acid release from C8PA lipocytes in a process that was linear for at least 4 hours. While 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 obtained when cells were treated with 1mM dibutyryl-cAMP (results not shown) but the magnitude of the increase was not as robust. Therefore, forskolin stimulation was adopted as standard conditions.

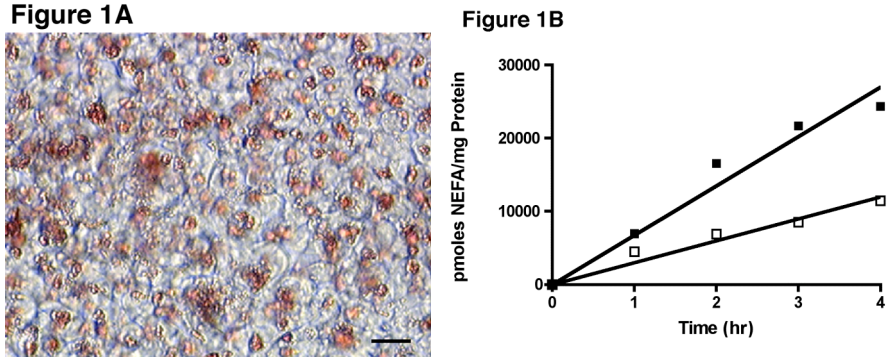

Figure 1. Organization of lipid droplets and lipolysis in C8PA lipocytes. Panel A; C8PA cells were incubated with oleate:BSA (3:1) for 48 hr and stained with oil red O to visualize the neutral lipid. The scale bar represents 10 microns. Panel B; lipid loaded C8PA cells were incubated either with or without 20 μM forskolin and the release of free fatty acids into the medium determined.

Lipolytic stimulation of cultured adipocytes results in a dramatic restructuring of the droplet surface and a reorganization of the local droplet environment (8,9). To assess the organizational structure of the lipid droplets in C8PA lipocytes, the cells were incubated for 2 hours with BODIPY-oleate and the fluorescent fatty acid accumulated into droplets. As shown in Figure 2, under basal conditions, BODIPY-oleate accumulated into small droplets organized into a central cluster. When visualized with transmitted light DIC (Figure 2A), the droplets appear as a central tight cluster of droplets while by fluorescence (Figure 2C), the clustered nature of the organization of

smaller droplets was clear. Following forskolin stimulation, the clustered droplets were dramatically restructured as revealed by both DIC (Figure 2B) and BODIPY-oleate fluorescence (Figure 2D). Moreover, consistent with previous studies (9) perilipin A was found on the droplets coincident with the fluorescent lipid localization (Figures 2E, 2F).

Using the C8PA lipocyte cell model, we addressed the location and translocation of adipocyte FABP and HSL by immunofluorescence using fluorescent fusion proteins. Previous studies have shown 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 to HSL.

Figure 2

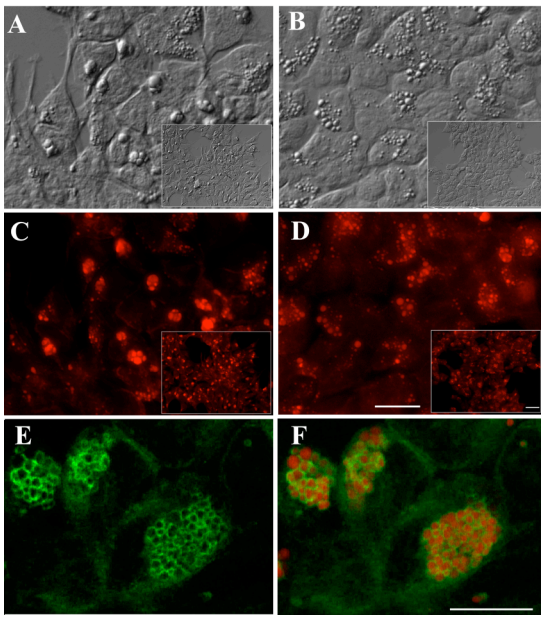

Figure 2. Fluorescence analysis of C8PA cells. BODIPY-oleate loaded C8PA cells under basal (A) or with 20 μM forskolin (B) as analyzed using DIC imaging. Fluorescent images of the BODIPY-oleate loaded C8PA cells under basal (C) conditions or with 20 μM forskolin (D). Immunofluorescence localization of perilipin A in C8PA cells (E) overlayed onto an image of the same field showing localization of the neutral lipid after incubation with BODIPY-oleate (F). The scale bar on panel D is 10 microns and represents the image size for panels A through D. The scale bar on the inset for panel D is 25 microns and represents the image size for each inset in panels A through D. The scale bar in panel F is 5 microns and represents the images in panels E and F.

Figure 3

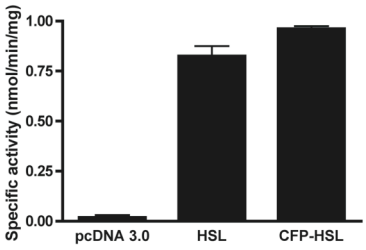

Figure 3. Activity of CFP-HSL. Expression plasmids encoding either HSL or CFP-HSL were transiently transfected into 293 cells and the cholesteryl esterase activity in extracts determined as described.

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 (Figure 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 Figure 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 to that of CFP-HSL. As shown in Figure 4E and 4F, 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 (Figure 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 was for CFP-HSL although there did appear to be some heterogeneity in the distribution throughout the cells (Figure 4D).

Figure 4

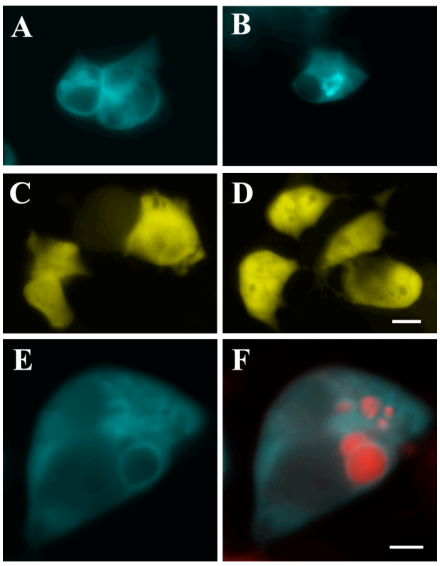

Figure 4. Fluorescent localization of CFP-HSL and YFP-AFABP in C8PA lipocytes. C8PA lipocytes were transiently transfected with expression plasmid for either CFP-HSL (panels A and B) or YFP-AFABP (panels C and D), loaded with oleate:BSA (3:1) for 48 hr and then and evaluated under basal (A and C) conditions or in the presence of 20 μM forskolin (B and D). For panels A through D, the scale bar represents 5 microns. Parallel cultures were incubated with BODIPY-oleate plus CFP-HSL, stimulated with 20 μM forskolin, and the localization of CFP-HSL (panel E) relative to neutral lipid droplets (panel F) determined. For panels E and F, the scale bar represents 2 microns.

To evaluate 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 channels digitally captured and FRET calculated. As shown previously for individual transfection (Figure 4), under basal conditions both CFP-HSL and YFP-adipocyte FABP were found cytoplasmically (Figure 5A, 5C). 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 (Figure 5B) while CFP-HSL clearly translocated to the droplet surface (Figure 5D). Analyzing FRET in the presence of forksolin (Figure 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.

Figure 5

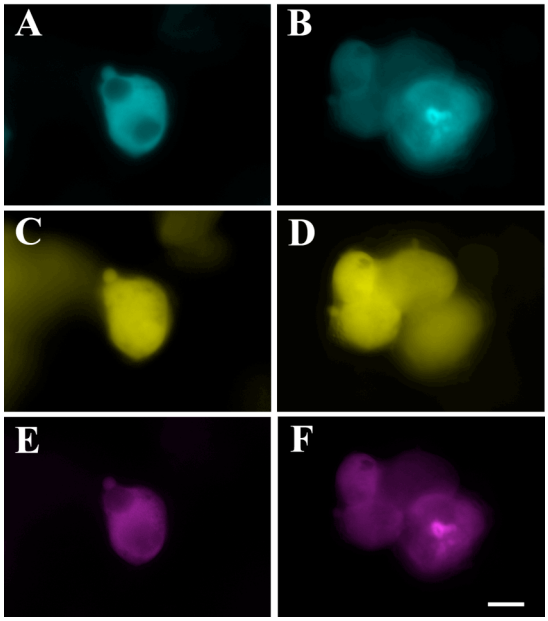

Figure 5. FRET analysis of C8PA lipocytes co-transfected with CFP-HSL and YFP-AFABP. C8PA lipocytes were co-transfected with CFP-HSL and YFP-AFABP and loaded with oleate:BSA (3:1) for 48 hr. After 48 hr the expression of CFP-HSL (panels A and B), and YFP-AFABP (panels C and D) in the absence (A and C) or presence (B and D) of 20 μM forskolin was determined. Energy transfer from CFP-HSL to YFP-AFABP was determined under basal (E) and forskolin-stimulated (F) conditions. The scale bar represents 5 microns.

Figure 6

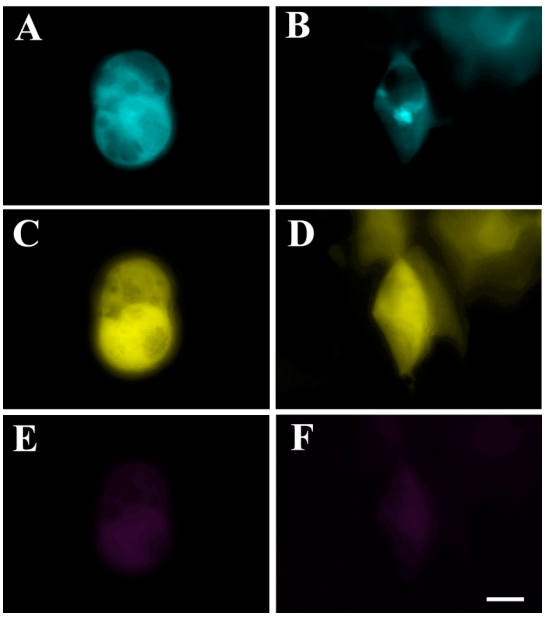

Figure 6. FRET analysis of C8PA lipocytes co-transfected with CFP-HSL and YFP-Liver FABP. C8PA lipocytes were co-transfected with CFP-HSL and YFP-LFABP and loaded with oleate:BSA (3:1) for 48 hr. After 48 hr the expression of CFP-HSL (panels A and B), YFP-LFABP (panels C and D) in the absence (A and 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 microns.

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). Figure 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 following forskolin

stimulation (Figure 6A, 6B). Similar to YFP-adipocyte FABP, YFP-liver FABP was cytoplasmic under both basal and forskolin stimulated conditions (Figure 6C, 6D). 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 (Figure 6E, 6F). 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. Prior to 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, GST-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 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 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, addition of forskolin to C8PA cells results in a disruption of the triacylglycerol droplet cluster resulting in a more disperse organization of smaller lipid droplets distributed within the cells (Figure 2). The perilipin A that is expressed is found encircling the droplets (Figure 2E, 2F) similar to that observed in 3T3-L1 adipocytes (27). An additional feature of C8PA cells is that they do not contain endogenous FABP or HSL. Cultured adipocytes, either primary or cell lines (3T3-L1), contain abundant FABP and HSL that would effectively compete with the fluorescent proteins in FRET analysis. Therefore, C8PA cells have the utility of being hormonally sensitive lipocytes without expressing either FABP or HSL.

Fluorescence resonance energy transfer has been used for cellular applications to assess protein localization, translocation, and association between fluorescently tagged proteins (28,29). In the context reported herein, a CFP-HSL fusion protein was

constructed that retained catalytic activity and had a fluorescent reporter in frame with the amino terminal of HSL. Since the site of interaction between AFABP and HSL had previously been mapped to amino acids 190-200 of HSL (16) we reasoned that a reporter on the amino terminus would be more likely to function in FRET analysis. Moreover, since the C-terminal domain of HSL contains the catalytic region, we were concerned that a CFP fusion protein at the C-terminus would potentially affect association with the triacylglycerol droplet. To that end, the in-frame CFP-HSL was constructed and found to have activity that is similar to, if not identical with native HSL. Using similar reasoning we created a YFP-AFABP since all other fusion proteins with the fatty acid binding protein (GST fusions) were active in fatty acid binding (14,16). Moreover, x-ray crystal analysis of AFABP (30) reveals that the amino terminus of the protein is ~180° from the site of ligand entry-exit suggesting that the YFP-AFABP (or YFP-LFABP) would be active.

Using transient transfection into C8PA lipocytes, CFP-HSL was found cytoplasmically under basal conditions and translocated to the surface of the droplet in response to forskolin. Not all CFP-HSL was found droplet associated suggesting that some other component was limiting, perhaps some aspect of surface accessibility. When YFP-AFABP was transiently transfected separately into C8PA cells, the fusion was also found cytoplasmically but its location was not altered to the same extent with foskolin treatment, as was CFP-HSL. Importantly, when CFP-HSL and YFP-AFABP were co-transfected into C8PA cells and analyzed for physical interaction via FRET, significant association was revealed cytoplasmically under basal conditions. Not all of the protein

appeared to be associated and the factors that govern association basally remain to be determined. However, *in vitro* assays suggest that fatty acid binding can influence the association raising the possibility that only AFABP with a bound lipid is capable of associating with HSL. When forskolin was added to the cells, CFP-HSL translocated to the droplet surface and was found associated with YFP-AFABP although cytoplasmic FRET could still be detected. This analysis does not indicate if the same molecules that associated basally in the cytoplasm translocated or if another population of molecules not associated basally did form a complex once docked onto the lipid droplet surface. However, the simple model is that CFP-HSL and YFP-AFABP form a complex under basal conditions and that complex coordinately translocates to the droplet surface. Since HSL is a dimer and the titration microcalorimetry indicates a 1:1 stoichiometry of binding between HSL and AFABP, the minimal complex is a $\alpha_2\beta_2$ heterotetramer. This analysis does not address the possibility of additional components in a translocation complex.

An important control is also revealed using FRET analysis. *In vitro* binding using isothermal titration microcalorimetry had indicated that liver FABP did not associate with HSL, or at least was reduced in affinity 10 to 20-fold (17). Using YFP-LFABP expressed into C8PA cells, a much-reduced FRET signal could be detected between CFP-HSL and YFP-LFABP and that very slight signal did not apparently translocate to the 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 over expressing EFABP into fat exhibit increased lipolysis and potentiated characteristics of the metabolic syndrome including impaired glucose and insulin tolerance tests (33,34). 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 underway.

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CHAPTER 3:

INTERACTION OF THE ADIPOCYTE FATTY ACID BINDING PROTEIN WITH THE HORMONE SENSITIVE LIPASE: REGULATION BY FATTY ACIDS AND PHOSPHORYLATION

This chapter is a reprint of the published manuscript of the same title with minor alterations. Smith, A. J., **Thompson, B. R.**, Sanders, M. A., and Bernlohr, D. A. (2007) *J. Biol. Chem.* **282**, 32424-32432

Brian Thompson's contribution to this chapter was Figure 4 and 8, and in the preparation of the text and discussion.

ABSTRACT

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 non fatty 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, YFPAFABP/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 (565), blocked FRET between HSL and AFABP/aP2. Substitution of isoleucine for lysine at position 21 of AFABP/aP2 (K21I), but not 31 (K31I), resulted in a non-HSL binding protein indicating that residues on helix αI of AFABP/aP2 define a component of the HSL binding site. These results indicate that the ligand-bound form of AFABP/aP2 interacts with the activated, phosphorylated HSL and that the association is likely to be regulatory – either delivering FA to inhibit HSL (facilitating feedback inhibition) or affecting multi-component complex formation on the droplet surface.

INTRODUCTION

AFABP, 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. While the members of the multigene family share only 20-70% sequence identify, 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 multi-dimensional regulation by hormones and catecholamines and several investigators have shown that protein kinase A dependent phosphorylation of either S659 and S660 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 (S565) 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 multi-component highly regulated process linking a number of hydrolytic events leading towards 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 plasma 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 formally 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 amino 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 since 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 fluorescent fusion proteins (EFYP-AFABP/aP2 and CFP-HSL) thereby allowing for not only identification of sub-cellular 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 (Carlesbad CA). BODIPY D 3835 (4,4difluoro-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. pYFP-C1 and pCFP-C1 were obtained from Clontech Laboratories, Inc., Palo Alto, CA. Gold Seal glass cover slips for imaging were purchased from Thomas Scientific, Swedesboro, NJ. Pure oleic acid was purchased from NuChek Prep (Elysian, MN). Cholesteryl [1-^{14}C] oleate was purchased from Amersham Biosciences. Restriction and DNA modifying enzymes were obtained from Promega. All other reagents were purchased from Sigma~~n~~-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 DMEM supplemented with 10% fetal bovine serum; stable expression of FATP1 was maintained with 400 μg/mL geneticin and stable expression of perilipin was maintained with 300 μg/mL Zeocin.

Cloning and mutagenesis: The R126Q AFABP/aP2 non-fatty acid binding mutant was sublconed into the A206K pYFP-C1 expression vector. Mutations in the HSL phosphorylation sites were introduced into wild type HSL in the A206K pCFP-C1 expression vector using the QuikChange^{TM} site-directed mutagenesis technique of Stratagene Cloning Systems, La Jolla, CA as previously described (15). The mutational

primer for HSL S565A was 5'CTATGCGCAGGAGTGTGGCTGAGGC AGCCC-3' while the mutational primer for HSL S659A, S660A was 5'GTTTCCACCCACGGCGCGCAGCCCAAGGTGTCCTCCAC-3'. Wild type HSL was subcloned in frame into pYFP-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 13 mm cover slips placed into 12 well dishes. At approximately 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 hours 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, BODOIPY-C12 in DMSO is added to monolayers (final concentrations 10 μg/mL) 60 minutes following forskolin addition and maintained on the monolayers for an additional 30 minutes until the cells were washed and images collected. During microscopy, cells were kept in media plus 10% FBS at 37^{o} 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 40X, 0.75 n.a. plan fluor or a 60X, 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 2x2 binning mode and 100 – 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) X (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-FABP/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: FRETC = FRET – (0.50 x CFP-HSL) – (0.02 x YFP-FABP/aP2), where FRET, CFP-HSL, and YFP-FABP correspond to background-subtracted images of cells co-expressing CFP-HSL and YFP-FABP/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 *t* test. Images were contrast stretched, pseudocolored and formated for display using Adobe Photoshop CS2 software (Adobe Systems, Mountain View, CA). Each live-cell imaging and FRET experiment was done at least 3 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 5 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 10uM oleate and 2uM pNPB for 60 minutes 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 (NSEPSDSPEMSQSMETLG) was used to produce a rabbit polyclonal antibody towards HSL (Bioworld, Dublin, OH). Coupling of the peptide to Affi-gel 15 (Bio Rad) and subsequent affinity chromatography with antiserum was 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 (Figure 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 20μM forskolin treatment leads to not only translocation of HSL, but also an increase in

FFA release (15). As shown in Figure 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 Figure 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 YFP-AFABP/aP2 and CFP-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 Figure 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.

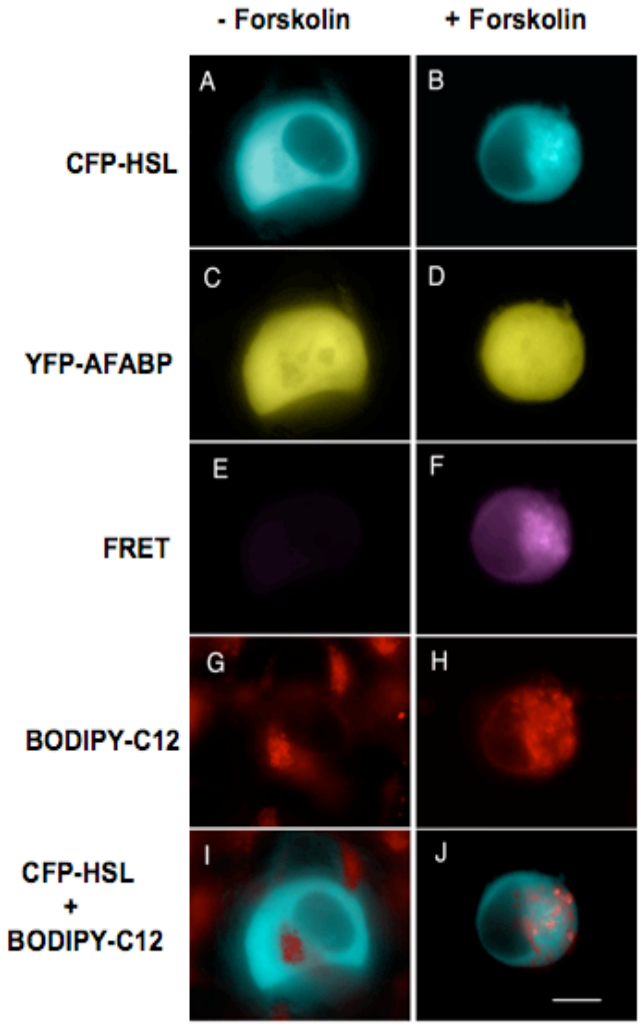

Figure 1. Translocation and FRET analysis of C8PA lipocytes co-transfected with CFP-HSL and YFP-AFABP.
C8PA lipocytes were co-transfected with pCFP-HSL and pYFP-AFABP/aP2 and loaded with oleate-BSA (3:1). After 48 h the expression of CFP-HSL and YFP-AFABP/aP2 in the absence or presence of 20 μM forskolin was determined and compared to the BODIPY-C12 labeling of triacylglycerol. Energy transfer from CFP-HSL to YFP-AFABP/aP2 was determined under basal and forskolin-stimulated conditions. The scale bar represents 5 microns.

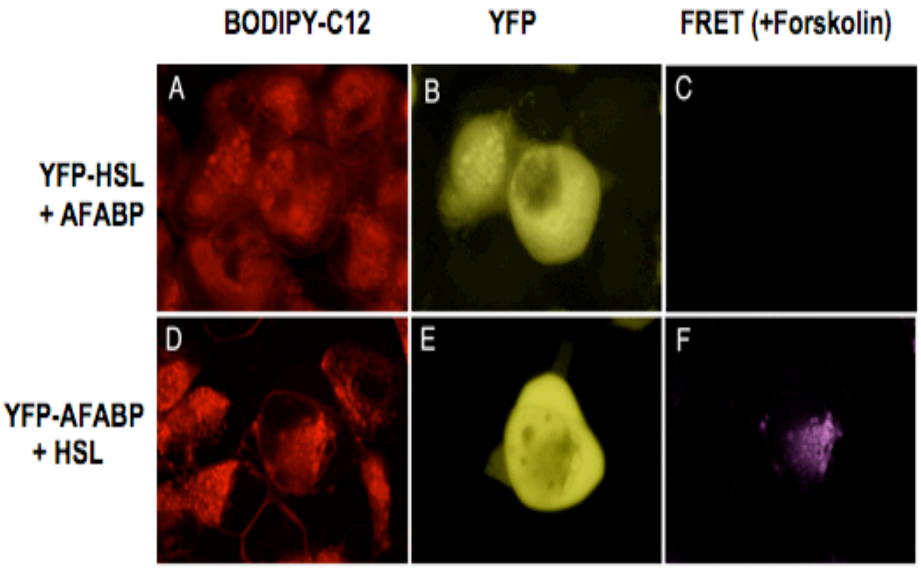

Figure 2. FRET analysis between YFP-AFABP/aP2 and BODIPY-C12 labeled-triacylglycerol. C8PA lipocytes were co-transfected with either pYFP-HSL and pAFABP/aP2 or pYFP-AFABP/aP2 and pHSL and loaded with oleate-BSA (3:1). After 48 h, oleate-loaded C8PA cells were treated with or without 20 μM forskolin. Representative images showing BODIPY-C12 labeled triacylglycerol, YFP-HSL or YFP-AFABP/aP2, and the FRET between BODIPY-C12 and YFP are shown.

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 R106, R126 and Y128 (19). Previous work in this laboratory demonstrated that an R126Q mutation in AFABP/aP2 resulted in a 30 to 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 an YFP fusion and transiently cotransfected into the C8PA cells along with CFP-HSL. As shown in Figure 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 (Figure 1), there was no energy transfer between AFABP/aP2 R126Q and HSL under either basal or stimulated conditions. Control transfection using YFP-AFABP/aP2 and CFP-HSL revealed that CFP-HSL translocated to the droplet surface and exhibited energy transfer with YFP-AFABP/aP2 identically as shown in Figure 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. Since 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 S659 and S660 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 (Figure 4). Consistent with this, when CFP-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 (Figure 5, panels B and J). When the CFP-HSL S659A, S660A was transiently transfected into C8PA cells with YFP-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.

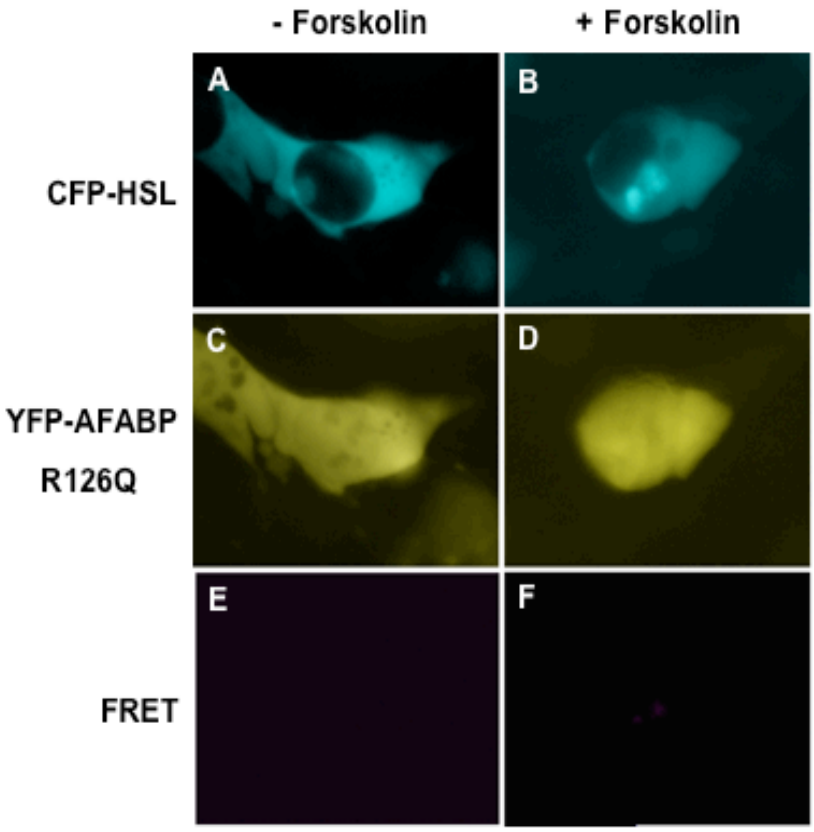

Figure 3. Fatty acid binding by AFABP/aP2 is required for association with HSL. C8PA lipocytes were co-transfected with pCFP-HSL and pYFP-AFABP R126Q and loaded with oleate-BSA (3:1). After 48 h the expression of CFP-HSL and YFP-AFABP R126Q in the absence or presence of 20 μM forskolin was determined. Energy transfer from CFP-HSL to YFP-AFABP R126Q was determined under basal and forskolin-stimulated conditions.

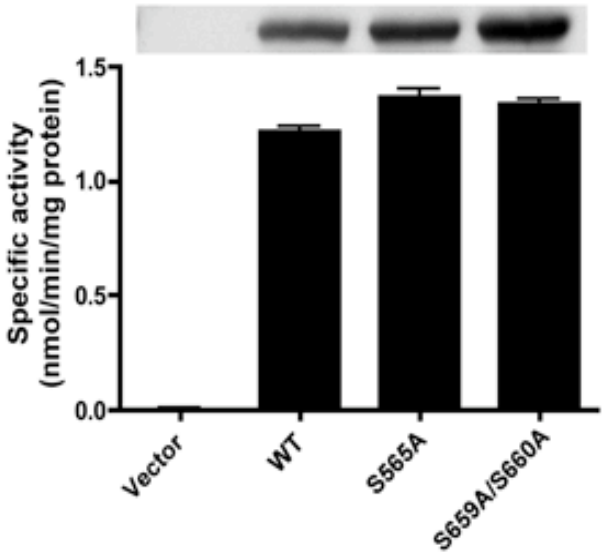

Figure 4. Activity of HSL isoforms with mutations at the PKA (S659 and S660) and AMPK (S565) phosphorylation sites.
Expression plasmids encoding pcDNA 3.0 (vector), pCFP-HSL, pCFP-HSL S659A, S660A, or the pCFP-HSL S565A were transiently transfected into C8PA cells and the cholesteryl esterase activity in extracts determined as described. Inset: western blot of cell extracts used for activity assays using affinity-purified anti-HSL antibodies.

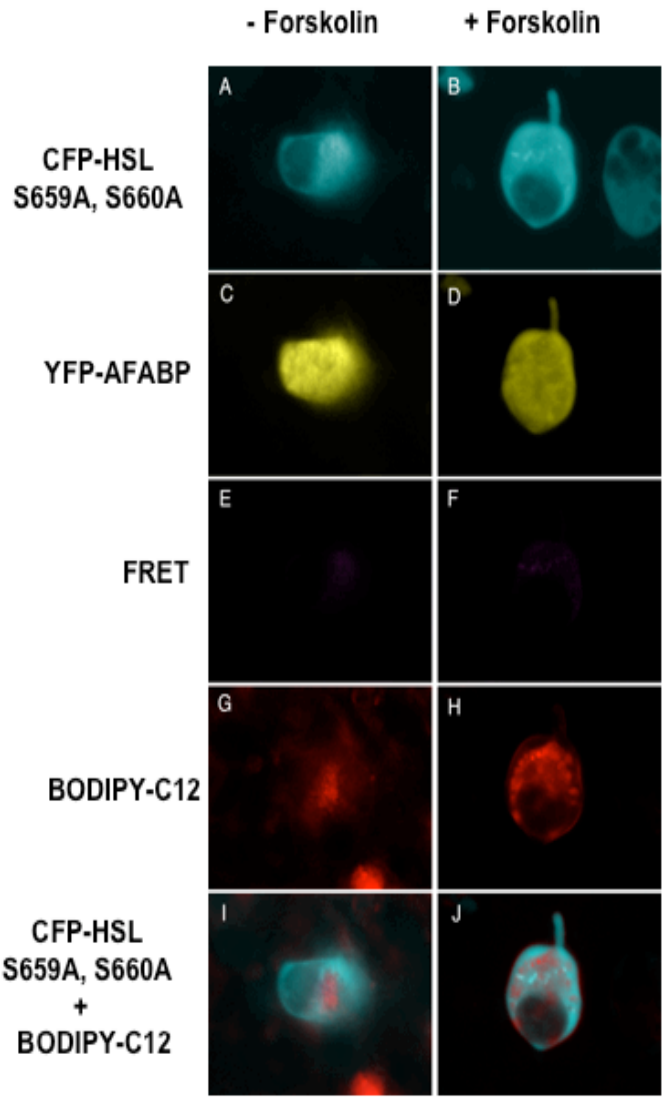

Figure 5. Translocation and FRET analysis between the CFP-HSL S659A, S660A and YFP-AFABP/aP2.
C8PA lipocytes were co-transfected with pCFP-HSL S659A, S660A and pYFP-AFABP/aP2 and loaded with oleate-BSA (3:1). After 48 h the expression of CFP-HSL S659A, S660A and YFP-AFABP/aP2 as well as energy transfer in the absence or presence of 20 µM forskolin was determined. BODIPY-C12 staining of the lipid droplets merged with CFP-HSL S659A, S660A is shown under basal and forskolin-stimulated conditions.

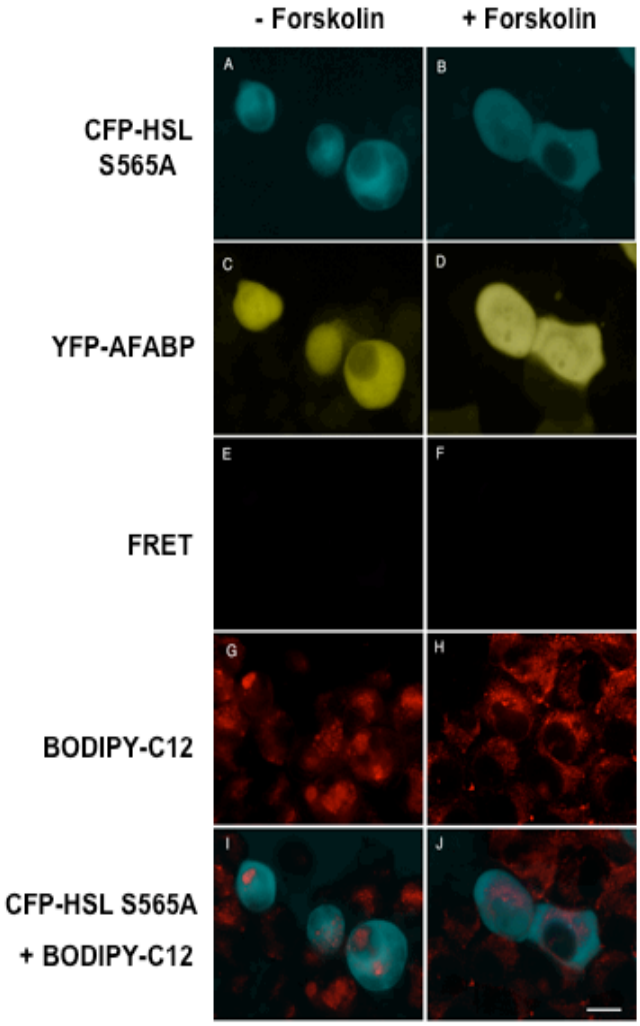

Figure 6. Energy transfer between CFP-HSL S565A and YFP-AFABP/aP2. C8PA lipocytes were co-transfected with pCFP-HSL S565A and pYFP-AFABP/aP2 and loaded with oleate-BSA (3:1). After 48 h the expression and translocation of CFP-HSL S565 in the absence and presence of 20 μM forskolin was assessed. Energy transfer from CFP-HSL S565 to YFP-AFABP was determined under basal and forskolin-stimulated conditions. BODIPY-C12 staining of the lipid droplets merged with CFP-HSL S565A shown under basal and forskolin-stimulated conditions.

A second critical phosphorylation site in HSL is S565 and is subject to phosphorylation by the AMP-activated protein kinase (10). To assess the influence of S565 phosphorylation on association with AFABP/aP2, the CFP-HSL S565A mutant was transfected either in the presence or absence of YFP-AFABP/aP2 and energy transfer evaluated. As shown in Figure 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 Figure 1 (results not shown).

While the binding site on HSL that associates with AFABP/aP2 has been defined by the region surrounding amino acids 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 Figures 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 α1-helix α2 domain that forms the lid of the fatty acid binding cavity. Storch and colleagues have carried out a series of mutations of the helical region and identified K21 and K31 as important residues affecting the ability of AFABP/aP2 to interact with

biological membranes (16). 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 Figure 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 K31 had no effect on either AFABP/aP2 localization or FRET with HSL (Figure 7, panels K and L). This implies that residue K21 contributes to the HSL binding site and defines the helical domain, particularly helix α1, 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 later 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 Figure 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.

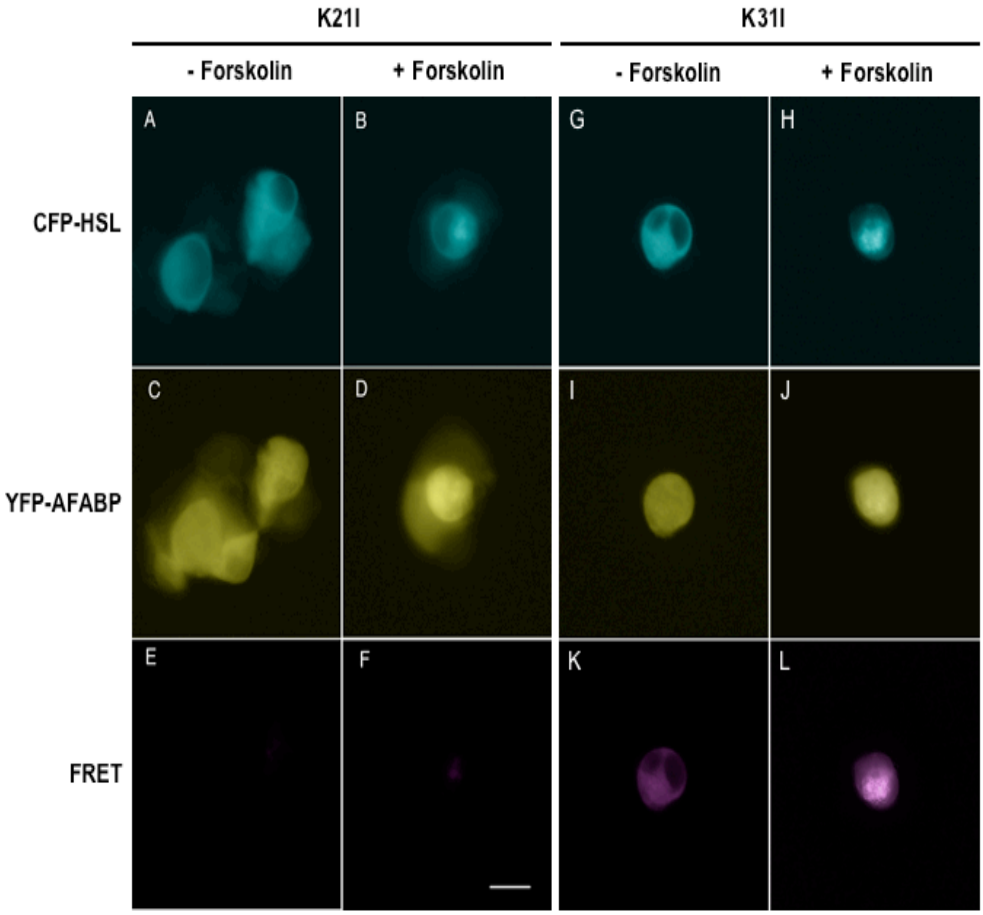

Figure 7. Energy transfer between YFP-AFABP/aP2 K21I or YFP-AFABP/aP2 K31I and CFP-HSL.
C8PA lipocytes were co-transfected with pCFP-HSL and pYFP-AFABP/aP2 K21I or pCFP-HSL and pYFP-AFABP/aP2 K31I and loaded with oleate-BSA (3:1). After 48 h the expression of CFP-HSL and YFP-AFABP-K21I and CFP-HSL and 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.

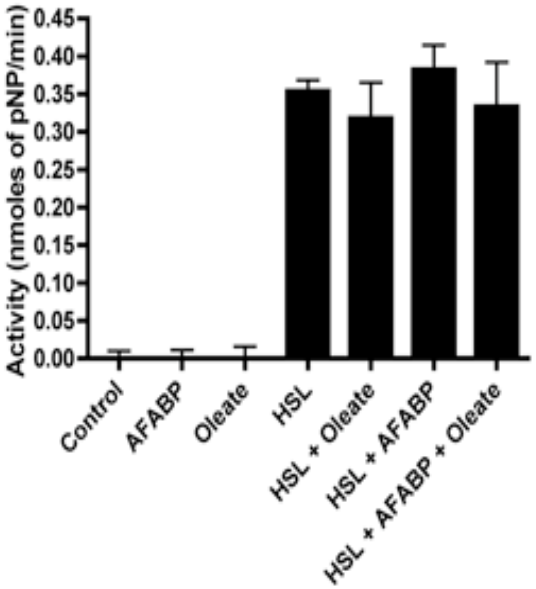

Figure 8. Effect of holo-AFABP on HSL activity using pNPB as a substrate.
Fatty acid free His-AFABP was incubated with or without 10 μM oleate and then added to His-HSL in a 1:1 molar ratio to measure esterase activity with pNPB as the substrate. The results are expressed as nmoles of *p*-nitrophenol produced per minute. The graph shows the mean +/- standard deviation for one of three independent experiments.

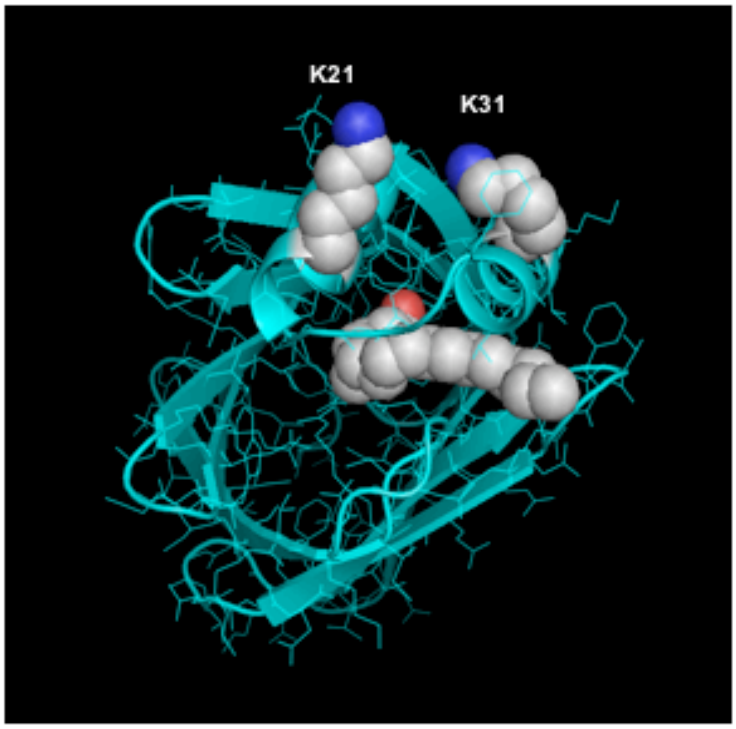

Figure 9. Ribbon diagram of AFABP/aP2 showing the positions of K21 and K31 (nitrogen atoms labeled blue) on helix α1 and helix α2 with bound oleate (carboxyl oxygen atoms in red). From PDB 1LIB.

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, 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 α1.

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 Figure 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 and colleagues have determined that the intracellular free fatty acid concentration in the adipocyte is quite low, in the nanomolar range (28). 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-labled 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 since there exists no FRET in the cytoplasm and the energy transfer occurs only on the droplet surface. Since YFP-HSL is catalytically active using either pNPB or cholesteryl 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 ~1.5Ro = ~80 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 α1 forms the HSL binding site. The side chain of K21 is found within a cluster of charged residues demarcated by D17, D18, K21 and R30. D17, D18 and K21 reside on helix α1 while R30 is found on helix α2. The side chain of D18 forms an ion pair with that of K21 while the side chain of D17 forms a salt bridge to that of R30. The two ion pairs form a quartet of charges on the surface of the protein that bridge the two helicies (Figure 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 critical for AFABP/aP2 interaction is E193 located in the N-terminal docking domain (12). Importantly, Xu et al reported that K21 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 (23). In addition, LiCata and colleagues (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 K21 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 colleagues have shown that dimerization of HSL involves determinants that involve the N-terminal domain (29). 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 knockout 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 knockout 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. Since 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 mal1). EFABP forms a complex with HSL as revealed by titration microcalorimetry and contains the orthologous residues K23 and R32 as well as acidic amino acids E19 and E20 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 knockout mice exhibit an increase in

perilipin A and EFABP expression while decreasing HSL expression confounding evaluation of the results (31).

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CHAPTER 4:

INTERACTION OF ADIPOCYTE FATTY ACID BINDING PROTEIN AND JAK2: AFABP/AP2 AS A REGULATOR OF JAK2 SIGNALING

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Thompson, B.R., Suttles, J., Carter-Su, C., Bernlohr, D.A., submitted to JBC.

Brian Thompson's contribution to this chapter was development of all the figures and writing the entire text.

ABSRTACT

Adipocyte fatty acid binding protein (AFABP/aP2) facilitates the intracellular solubilization and trafficking of lipids within the aqueous environment of the cell. Studies in the AFABP/aP2 knockout mouse suggest that the protein may have roles in cellular processes broader than lipid transport. We present here the finding that AFABP/aP2 interacts with JAK2 in a yeast two-hybrid system. This interaction was confirmed with co-immunoprecipitations in adipose tissue and 3T3-L1 adipocytes as well as in 293 cells over expressing JAK2 and AFABP/aP2. Mutational analysis of AFABP/aP2 revealed that fatty acid binding activity (R126L/Y128F) is necessary for the interaction and that the helix-turn-helix motif (D18K) is important for the interaction. Mutational analysis of JAK2 (Y1007F, Y1008F) revealed that AFABP/aP2 associates with the basal, unphosphorylated form of the protein. IL-6 stimulated phosphorylation of Stat3 and induction of SOC3 mRNA expression were potentiated in a time and dose dependent manner in peritoneal macrophage cell lines derived from AFABP/aP2-EFABP/mal1 double knockout mice relative to cells from wild type animals. These results suggest that ligand-bound AFABP/aP2 binds to and attenuates JAK2 signaling and establishes a new role for AFABP/aP2 as a fatty acid sensor.

INTRODUCTION

Obesity is a key component of the metabolic syndrome linking insulin resistance, dyslipidemia and hypertension. This clustering of metabolic states is recognized as a

major risk factor for the development of type 2 diabetes and atherosclerosis (1,2). Adipocyte fatty acid binding protein, AFABP/aP2, knockout animals are resistant to diet induced insulin resistance and atherosclerosis whereas transgenic over expression models demonstrate potentiated characteristics (3-8). The molecular mechanisms that underlie this phenotype have yet to be elucidated but may be due to indirect effects and the availability of lipid ligands for PPARγ or direct effects on AFABP/aP2 interaction with cellular proteins (9-11).

AFABP/aP2 is a member of the cytoplasmic fatty acid binding protein multigene family (12). FABPs are low molecular mass 15 kDa cytoplasmic proteins that bind fatty acids with high affinity in a 1:1 complex within an interior ligand-binding domain (12). They function to solubilize and traffic fatty acids throughout the aqueous cellular environment thereby increasing the diffusion of molecules between cellular compartments. The family members have 20-70% sequence identity yet maintain super-imposable crystal structures and similar fatty acid affinity and specificity. The divergent sequence among members confers subtle differences in ligand binding properties between the family members but may also define different protein-protein interaction partners depending upon the cellular context. Indeed, it has been shown that the adipocyte, epithelial and heart type fatty acid binding proteins interact with hormone sensitive lipase, while the intestinal and liver isoforms do not (10,13).

AFABP/aP2 is highly expressed in adipocytes and macrophages and has a role in lipid metabolism in both cell types. In adipocytes, AFABP/aP2 has no role in fatty acid influx but mediates lipolysis and lipid re-esterification that in turn affect glucose metabolism (3,5,6,8). In macrophages, AFABP/aP2's role in lipid metabolism is undefined but studies in null macrophages reveal a role in cholesterol metabolism and inflammation (9,14,15). The sum total of effects in adipose tissue results in changes in insulin sensitivity in the context of obesity and atherosclerosis in an ApoE null background (3,5-7,14-16). The complex nature of the biological effects revealed by the knockout models suggests a more direct role for AFABP/aP2 in cellular processes other than lipid transport. To that end, yeast two-hybrid analysis followed by a combination of studies including titration microcalorimetry, fluorescence resonance energy transfer and GST-pull downs have revealed that AFABP/aP2 interacts with a domain on HSL defined by amino acid residues 190-200 containing the core sequence motif ExYK (11,13,17-19). In addition, AFABP/aP2 has a similar sequence in the helix-turn-helix domain, DDYMK, that is necessary for the interaction with HSL (20).

We describe here a novel protein-protein interaction between AFABP/aP2 and Jak2. Jak2 is part of a family of non-receptor tyrosine kinases, which play a critical role in signal transduction from ligands that bind to members of the cytokine receptor superfamily. Upon ligand binding, one or more of the JAK kinases is activated and subsequently phosphorylates the receptor, other JAK kinases, and downstream signaling molecules such as the signal transducers and activators of transcription family or STAT

proteins (21,22). JAK2 has 49 potential tyrosyl phosphorylation sites, at least 10 of which have been identified as phosphorylated and two of these sites (Tyr1007 and Tyr1008) are critical for full activation of Jak2 (23-25). In light of this, we have characterized the interaction of JAK2 and AFABP/aP2. Data presented here indicate that the ExYK motif in AFABP/aP2 is critical for this interaction and may represent a common protein-protein interaction domain for FABPs.

EXPERIMENTAL PROCEDURES:

Materials:

Zeocin, geneticin, lipofectamine2000, and tissue culture reagents were obtained from Invitrogen (Carlsburg, CA). Pure oleic acid was from NuChek Prep (Elysian, MN). Antibodies for STAT3 (79D7), Phospho-STAT3 Tyr705 (3E2), and Jak2 (D2E12) were from Cell Signaling. Antibodies for total phospho-tyrosine (Py99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680 and Blocking Buffer were from LI-COR Biosciences (Lincoln, NE). IL-6 and IL-10 were from PeproTech (Rock Hill, NJ). DNA for pRK5-Jak2 and pRK5-Jak2-Y1007F/Y1008F were kind gifts from Dr. Bruce Witthuhn (University of Minnesota). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). The University of Minnesota Microchemical Facility carried out DNA sequencing and synthesis of oligonucleotides used for PCR.

Site-directed Mutagenesis:

Mutations in the proposed AFABP/aP2 Jak2 binding site were made in the template of pCDNA3.0-AFABP/aP2 or pRK5-Jak2 using the QuickChange site-directed mutagenesis technique of Stratagene Cloning Systems, La Jolla, CA. The mutational primers were as follows: E1006A Jak2: 5'- C TTG CCG CAG GAC AAA **GCA** TAC TAC AAA GTA AAG GAG C-3'. K1009A Jak2: 5'- G GAC AAA GAA TAC TAC **GCA** GTA AAG GAG CCA GGG-3'. K1009D Jak2: 5'- G GAC AAA GAA TAC TAC **GAC** GTA AAG GAG CCA GGG G-3'. K21A AFABP/aP2: 5'-C TTC GAT GAT TAC ATG **GCA** GAA GTG GGA GTG GGC TTT GCC-3'. D18K AFABP/aP2: 5'-CC AGT GAA AAC TTC GAT **AAG** TAC ATG AAA GAA GTG GGA GTG GGC-3'.

Yeast 2 Hybrid:

The yeast 2 hybrid screening with JAK2 was done as previously described (26). Briefly pEG202-JAK2 was used as the bait with a rat adipose cDNA library as the prey.

C8PA cross-linking:

Transient transfection of C8PA cells, HEK-293 cells stably expressing fatty acid transport protein 1 and perilipin A were used and described previously (19). Briefly, 4 ug of pCDNA3.0-AFABP/aP2, 4 ug of pRK5-Jak2 and 30 ul of Lipofectamine 2000 were used for each 10 cm plate. Transfected plates were fat-loaded 16 hours after initial transfection with DMEM plus 10% FBS containing 300 uM oleate: 100 uM BSA.

Twenty hours after fat-loading, cells were washed once in PBS and cross-linked. Cross-linking was done with 0.5% Formaldehyde in 10 ml PBS at room temperature for 10 minutes. The reaction was stopped with 1 ml of 1.25 mM Glycine for 5 minutes. The cells were washed twice in PBS and scraped in RIPA buffer (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 uM sodium floride, 1 mM sodium pyrophosphate, 1% Triton-X 100, 1% Sodium Deoxycholate, 0.1 % SDS) containing protease inhibitors and phosphatase inhibitors. The cell suspension was sonicated twice for 10 seconds at 4 C. The cell extract was centrifuged at 13,000 x g for 10 minutes to pellet cellular debris. The supernatant was used for subsequent western blotting and immunoprecipitation experiments.

Western blotting and co-immunoprecipitation:

Immunoprecipitation (IP) of Jak2 was done using rabbit anti-Jak2 antibody at 1:100 overnight at 4 C. Rabbit pre-immune serum was used as the control antibody at the same concentration as the Jak2 antibody, 600 ng/ml. IP's were incubated with protein A-agarose beads, 12.5 ul bead volume for 2 hours. Beads were washed four times in RIPA buffer and on the last wash transferred to a new tube. Ten ul of RIPA buffer and 10 ul of loading buffer were used to resuspend the beads and this mixture was boiled for 20 minutes. Total immunoprecipitations were loaded on SDS-PAGE gels. Gels were transferred to Immobilon-F PVDF membrane (Milipore) and blocked in Li-cor blocking buffer for one hour. Primary antibodies were incubated overnight at 4 C in 5% BSA in TBST. After washing in TBST, secondary IRDye antibodies were incubated for one hour

at room temperature in 5% BSA TBST. Blots were washed in TBST and then rinsed and stored in PBS. Li-cor Odyssey was used for detection and quantification. For Immunoprecipitation, Jak2, AFABP/aP2 and total phosphotyrosine (pY99) were blotted simultaneously on the same membrane. For signaling, peritoneal macrophages were stimulated with the appropriate cytokine for the indicated times and concentrations in the presence of 10% FBS. AFABP/aP2, STAT3 and tyrosine705 phosphoSTAT3 were blotted simultaneously on the same membrane.

Macrophages:

Immortalized AFABp/aP2-EFABP/mal1 double knockout and wild type murine macrophage cell lines were kind gift from Jill Suttles (University of Louisville) and generated as described previously (27).

Quantitative RT- PCR:

Expression of mRNAs were measured by quantitative RT-PCR. Total RNA was isolated from macrophages using Trizol reagent (Invitrogen Corp.) according to manufacturer's protocol. RNA was treated with DNase I to digest any genomic contamination and cDNA was synthesized using iScript cDNA synthesis kit (BioRad). iQ SYBRgreen Supermix and MyiQ detection system (BioRad) were used to monitor amplification. Primers used to amplify and detect mRNA are as follows and were designed using MacVector.
SOCS3: Forward 5'- GGGTTCTGCTTTGTCTCTCCTATG-3', Reverse 5'-

TGGCTGTGTTTGGCTCCTTGTG-3'. IL6R: Forward 5'-GCGACACTGGGGACTA TTTATGC-3', Reverse 5'- AAACAGCACAGCCTTCGTGGTTGG -3'.

RESULTS

Previous studies, using a yeast 2 hybrid approach identified AFABP/aP2 as an interaction partner with the Hormone Sensitive Lipase, HSL (18). Using a similar strategy in the course of investigating JAK2 interacting proteins, we identified AFABP/aP2 as an interaction partner (data not shown). To confirm this interaction in endogenous systems, we carried out co-immunoprecipitation experiments. When Jak2 was immunoprecipitated from adipose tissue of C57Bl/6J mice or from differentiated day 8 3T3-L1 adipocytes and then blotted for AFABP/aP2, specific association was detected (Figure 1). Immunoprecipitation with control antibodies did not reveal any non-specific complex formation.

AFABP/aP2 interacts with a domain on HSL containing the core sequence EHYKRNE (17). Analysis of JAK2 and AFABP/aP2 amino acid sequences revealed a similar sequence present in both proteins (Figure 2). In this context, JAK2 may interact with AFABP/aP2 in a similar manner as does HSL. Previous studies showed that the interaction of AFABP/aP2 with HSL is fatty acid dependent (11,13). Considering that most in vitro protein-protein interaction assays utilize one or more chaotropic agents to

eliminate non-specific interactions and that these agents strip fatty acids from the binding pocket of AFABP/aP2, we adopted a cross-linking protocol in C8PA cells to investigate the JAK2 interactions.

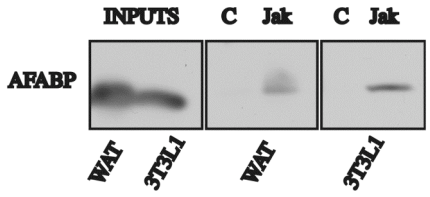

Figure 1. Endogenous interaction of AFABP/aP2 and Jak2. Immunoprecipitation with control IgG (C) or anti-JAK2 (Jak) from day 8 3T3-L1 adipocytes (3T3-L1) and C57B6/J epididymal adipose tissue (WAT) and western blot for AFABP/aP2. Input lanes represent samples prior to immunoprecipitation. The blot shown is representative of three independent experiments with similar results. The western blot shown is three portions from the same blot with intervening lanes omitted for clarity.

JAK2	$QDKEYYKVKEPG^{1114}$
HSL	$SFGEHYKRNETG^{201}$
AFABP	$NFDDYMKEVGVG^{26}$
	ExYK

Figure 2. Sequence alignment of JAK2, HSL and AFABP/aP2 ExYK motifs. Portions of the amino acid sequences of HSL, JAK2 and AFABP/aP2 that align with the ExYK motif. All alignment analysis was done using T-Coffee alignment software.

C8PA cells are HEK-293 cells stably expressing FATP1 and perilipin A and have the advantage of increased fatty acid uptake and storage and thus allow us to probe the JAK2-AFABP/aP2 interaction in an adipocyte-like cell system in the absence of endogenous AFABP/aP2 (19). Cells over expressing AFABP/aP2 and JAK2 were cross-linked in formaldehyde, lysed and cell extracts were used for subsequent immunoprecipitation experiments. As shown in Figure 3, wild type AFABP/aP2 co-

immunoprecipitated with JAK2 whereas control IgG does not bring down JAK2 or AFABP/aP2. In order to determine whether the interaction is ligand dependent, we tested the fatty acid binding mutant R126L/Y128F AFABP/aP2 for interaction with Jak2. Figure 3A shows that R126L/Y128F AFABP/aP2 does not associate with JAK2, suggesting that ligand binding to AFABP/aP2 is necessary for this interaction.

The sequence similarity between HSL and JAK2 suggests that this domain may be important for the Jak2 interaction. The JAK2 sequence, $EYYK^{1009}$ is located on the activation loop of the kinase, and phosphorylation of the two tyrosines is critical to full activation of JAK2 (24). To test the importance of phosphorylation and activity of JAK2 on the interaction, we mutated both tyrosines to phenylalanine. As expected Y1007F/Y1008F JAK2 has decreased tyrosine phosphorylation suggesting that JAK2 is less active. As shown on Figure 3B, AFABP/aP2 co-immunoprecipitates more avidly with Y1007F/Y1008F JAK2 than with wild type JAK2, suggesting that AFABP/aP2 interacts with JAK2 in the basal, unphosphorylated state. To extend the analysis of the $EYYK^{1009}$ region, we mutated E1006 and K1009 of JAK2, individually to alanine residues. Analysis of E1006A and K1009A JAK2 revealed decreased total phospho-tyrosine levels, suggesting that these mutations affect Jak2 activity in a similar manner as the Y1007F/Y1008F mutant. Co-immunoprecipitation experiments show that E1006A and K1009A JAK2 interact more avidly with AFABP/aP2 then does wild type Jak2. The sum of the mutations in the $EYYK^{1009}$ region suggests that AFABP/aP2 interacts with JAK2 in the basal state and that the $EYYK^{1009}$ is central to interaction.

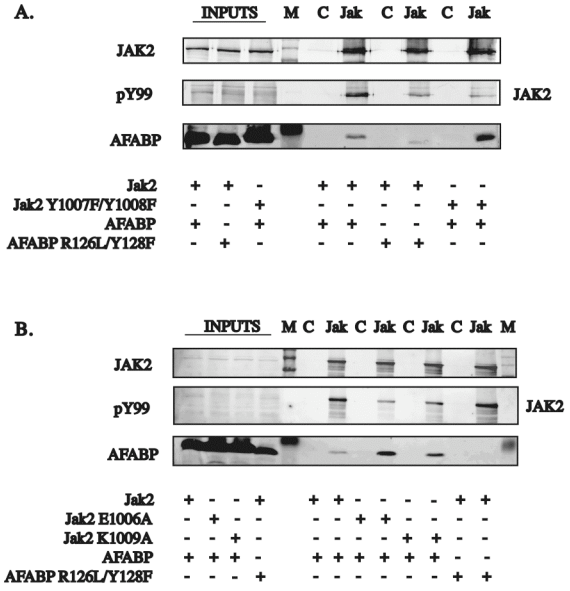

Figure 3. Mutational analysis of JAK2 and fatty acid dependence on interaction. C8PA cells were co-transfected with cDNA for AFABP/aP2 and JAK2, and 24 hours later loaded with oleate-BSA (3:1) for 24 hours. Cells were cross-linked in 0.5% formaldehyde for 10 minutes and then harvested. Immunoprecipitation with control IgG (C) and anti-JAK2 (Jak) was conducted and the resultant precipitate was boiled for 20 minutes to reverse cross-linking then run on SDS-PAGE and western blotted with anti-JAK2, anti-phosphotyrosine (pY99) and anti AFABP/aP2, protein marker (M). Input lanes represent the samples prior to immunoprecipitation. The blots shown are representative of at least three independent experiments with similar results. A. Y1007F/Y1008 JAK2 mutant and R126L/Y128F AFABP mutant. B. E1006A and K1009A JAK2 mutants.

Recent studies have revealed that D17, D18, K21 and R30 of AFABP/aP2 found on a single helix-turn-helix domain were necessary for the interaction with HSL and that mutation of these residues rendered AFABP/aP2 either unable to interact with HSL or have attenuated association (20). Importantly, mutations at these sites do not affect AFABP/aP2's ability to bind fatty acid ligand, functionally separating the ligand binding function of AFABP/aP2 from its protein-protein interaction domains (20). Moreover, the loss of HSL association in the D18K AFABP/aP2 mutant can be rescued by

corresponding mutation of HSL (K196E). The helix-turn-helix domain also has been shown to undergo main chain and side chain alterations upon ligand binding, suggesting a structural change that coincides with ligand association (28).

To further investigate the helix-turn-helix domain of AFABP/aP2 we performed a charge reversal experiment whereby we mutated D18 to K in AFABP and K1009 to D in Jak2. As shown in Figure 4A, expression of D18K AFABP/aP2 with wild type JAK2 into C8PA cells following by cross-linking and immunoprecipitation revealed decreased interaction between the two proteins suggesting that D18 and the helix-turn-helix domain of AFABP/aP2 are important for association. Interestingly, expression of K1009D JAK2 with wild type AFABP/aP2 resulted in increased interaction with AFABP similar to that observed with the K1009A mutant. However, K1109D of JAK2 (like K1009A) exhibited decreased total phosphotyrosine of JAK2 and, consistent with the results in Figure 3B, increased association. When D18K of AFABP/aP2 was co-expressed with K1009D of JAK2, association was returned to basal levels, suggesting that the interaction is ionically based and that the activation loop of JAK2 is the likely site of interaction. However, the site of interaction on AFABP/aP2 with JAK2 is not identical in all respects to the interaction of AFABP/aP2 with HSL. Figure 4B shows that mutation of lysine 21 to alanine (a mutation that blocks AFABP/aP2 interaction with HSL) had no effect on interaction with Jak2. These experiments demonstrate that the requirements for the interaction of AFABP/aP2 with JAK2 are not identical to those for HSL but are clearly overlapping.

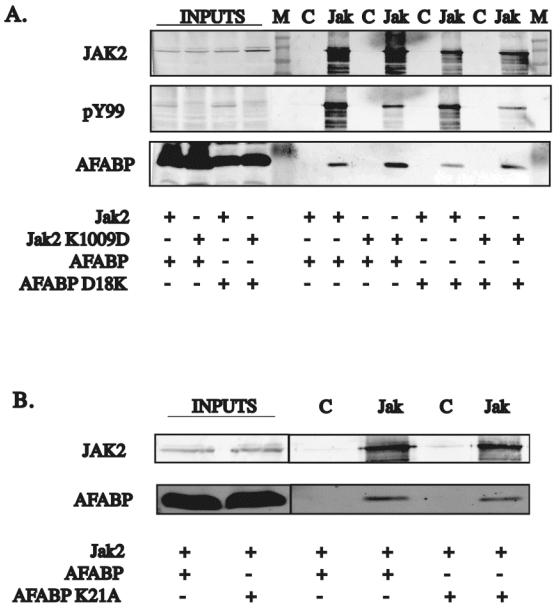

Figure 4. AFABP/aP2 DDYMK motif mutational analysis. C8PA cells were co-transfected with cDNA for AFABP/aP2 and JAK2, and 24 hours later loaded with oleate-BSA (3:1) for 24 hours. Cells were cross-linked in 0.5% formaldehyde for 10 minutes and then harvested. Immunoprecipitation with control IgG (C) and anti-JAK2 (Jak) was conducted and the resultant precipitate was boiled for 20 minutes to reverse cross-linking run on SDS-PAGE and western blotted with anti-JAK2, anti-phosphotyrosine and anti AFABP/aP2, protein marker (M). Input lanes represent the samples prior to immunoprecipitation. The blots shown are representative of at least three independent experiments with similar results. A. Charge reversal. B. K21A AFABP mutant.

Analysis of the phosphorylation deficient JAK2 mutants, Y1007F/Y1008F, E1006A, K1009A and K1009D (figure 3), indicates that AFABP/aP2 interacts with JAK2 in the basal state. In order to understand the physiological implications of this interaction, we made use of peritoneal macrophages from AFABP/aP2-EFABP/mal1 double knockout (DKO) mice. DKO mice are resistant to diet induce obesity, insulin resistance and atherosclerosis in an ApoE null background (15,16). Interleukin-6 and interleukin-10 signaling have been implicated in atherosclerosis development and a balance between these two cytokines is necessary for the prevention of atherosclerosis as Il-6 knockout

models in an ApoE null background have increased atherosclerosis (29-31). JAK2 plays an important role in the signaling mechanism for both of these cytokines in macrophages (32,33). Once activated, JAK2 recruits and phosphorylates STAT3 at Tyr705 (32). Therefore, we used STAT3 phosphorylation as an indicator of JAK2 activity in wild type and DKO macrophages. Initial assessment of these signaling pathways revealed that treatment of cells with IL-6 or IL-10 for 15 minutes resulted in Tyr705 phosphorylation of STAT3, Figure 5. IL-6 induced Tyr705 phosphorylation of STAT3 was potentiated 4-fold in the DKO cells compared to wild type, while IL-10 induced Tyr705 phosphorylation of STAT3 was unaffected, Figure 5. To more fully characterize the JAK2 signaling via the IL-6/IL-10 cytokines we evaluated the cytokine concentration dependence at a fixed time (15 minutes) and the time dependence of STAT3 phosphorylation at a fixed concentration of hormone (100 ng/ml IL-6). As shown in Figure 6, wild type cells induced Tyr705 STAT3 phosphorylation at 10 ng/ml of IL-6, while DKO cells were more sensitive, inducing Tyr705 STAT3 phosphorylation at 1 ng/ml of IL-6. The increased sensitivity to IL-6 in the DKO cells was observed at every concentration evaluated, suggesting that AFABP/aP2 attenuates a major determinate of this signaling pathway.

The kinetics of Tyr705 STAT3 phosphorylation exhibited by both wild type and DKO cells show maximally stimulated STAT3 phosphorylation at 15 minutes with a return to baseline at 90 minutes. DKO cells show an amplitude effect of the signal rather than duration, suggesting that AFABP/aP2 has an early effect on activation of the pathway but

did not play a role in desensitization. Importantly, the mRNA for IL-6 signaling receptors, gp130 and IL-6R, were unchanged between wild type and DKO macrophages (data not shown).

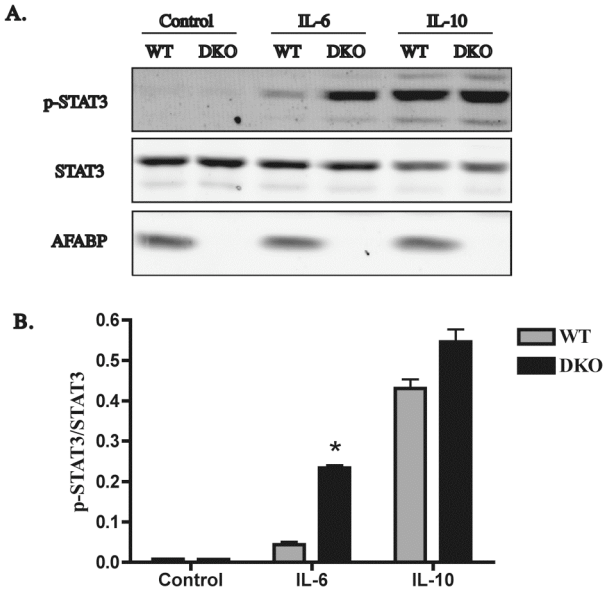

Figure 5. IL-6 and IL-10 signaling in AFABP/aP2-EFABP/mal1 double knockout macrophages. A. Wild type (WT) and AFABP/aP2-EFABP/mal1 knockout (DKO) peritoneal macrophages were either untreated (Control) or treated with 100 ng/ml IL-6 or IL-10 for 15 minutes. Cells were harvested and protein extracts were run on SDS-PAGE and western blotted for tyrosine 705 phosphorylation of STAT-3 (p-STAT3), total STAT3 protein (STAT3) and AFABP/aP2. P-STAT3 and STAT3 were blotted simultaneously on the same blot. A representative portion of one blot is shown. B. Quantification was done on the Licor Odyssey and is an n=3 for all conditions from the same blot. This experiment was repeated three times with similar results. Student t test was used for statistical analysis, * indicates $p < 0.01$.

STAT3 Tyr705 phosphorylation leads to dimerization and nuclear translocation where it can activate transcription of a number of genes. One of the STAT3 target genes is SOCS3, which then feedback inhibits the IL-6 signaling pathway (34-36). In order to

evaluate whether the increased phosphorylation of STAT3 in DKO cells has downstream effects we performed quantitative RT/PCR for SOCS3 in the presence or absence of 100 ng/ml IL-6. As shown in Figure 7, there was a more robust induction of SOCS3 expression in the DKO cells compared to the control wild type cells. The increase in SOCS3 expression in response to IL-6 correlated well with the increased STAT3 Tyr705 phosphorylation. These data are consistent with ligand-bound AFABP/aP2 interacting with Jak2 in the basal state and attenuating its activity, thereby reducing IL-6 effects in macrophages.

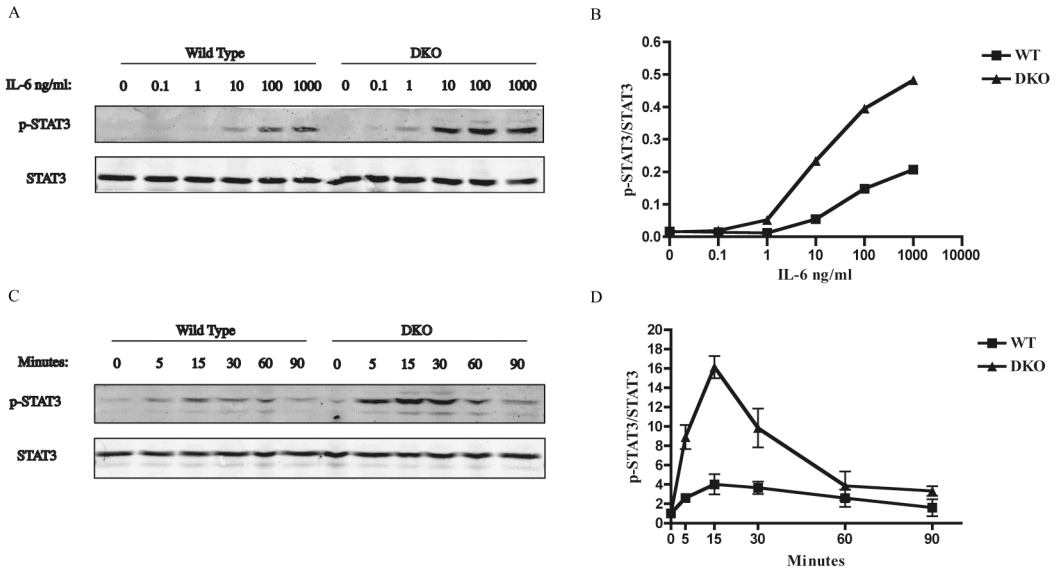

Figure 6. IL-6 concentration gradient and time course. Wild type (WT) and AFABP/aP2-EFABP/mal1 knockout (DKO) peritoneal macrophages were treated with IL-6. Cells were harvested and protein extracts were run on SDS-PAGE and western blotted for tyrosine 705 phosphorylation of STAT-3 (p-STAT3) and total STAT3 protein (STAT3). Quantification was done on the Licor Odyssey and is represented as phospho-STAT3 normalized to total STAT3 protein. P-STAT3 and STAT3 were blotted simultaneously on the same blot. These experiments were repeated three times with similar results. A. Concentration gradient of IL-6 treatment for 15 minutes. B. Quantification of the blot in A. C. Time course of 100 ng/ml IL-6 treatment for the indicated times. D. Quantification of three independent experiments, values are represented relative to control wild type cells.

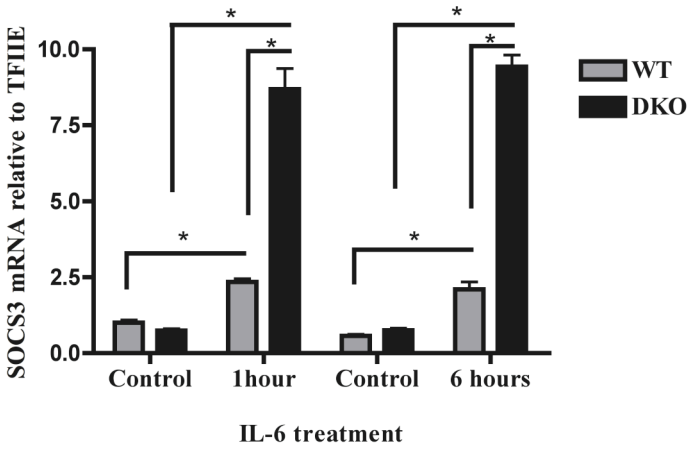

Figure 7. IL-6 induced SOCS3 mRNA in peritoneal macrophages. Wild type and DKO macrophages were treated with 100 ng/ml IL-6 or control treated for 1 or 6 hours. Quantitative RT/PCR for SOCS3 and TFIIE mRNA was done as described in materials and methods and is plotted normalized to TFIIE and relative to wt control 1 hour. N=3 for all groups, student T-test was used for statistical significance, * indicates $p<0.05$.

DISCUSSION

AFABP/aP2 is a fatty acid carrier solubilizing and enabling diffusion of fatty acids throughout the aqueous cellular environment. Knockout mice have revealed a role of AFABP/aP2 in whole body insulin resistance and atherosclerosis although the specific molecular mechanisms leading to these broad phenotypes are not well characterized.

The studies presented herein describe a novel interaction between AFABP/aP2 and JAK2. The studies also define four important considerations regarding this interaction; the requirement for fatty acids to be bound to AFABP/aP2, the tyrosyl phosphorylation

state of JAK2 to provide a suitable conformation for the interaction, and the mapping of the JAK2 binding site on AFABP/aP2 to a region surrounding the helix-turn-helix domain. Physiological implications for the interaction are revealed by the correlation that AFABP/aP2-EFABP/mal1 knockout macrophages show potentiated JAK2 signaling.

Traditional methods used to study protein-protein association have largely been unsuccessful in characterizing AFABP/aP2 interactions. Using a cross-linking system analogous to chromatin immunoprecipitation, performed in engineered lipocytes (C8PA cells) that mimic adipocytes, to some extent, we have examined the interaction between AFABP/aP2 and JAK2. The system was developed in response to the finding that association of AFABP with the hormone-sensitive lipase was fatty acid dependent (11). Therefore retention of either the bound lipid or preservation of the protein-protein interactions was key towards successful complex evaluation. Utilizing a cross linking strategy we were able to probe the interaction between AFAB/aP2 and JAK2 without the complication of losing the interaction due to chaotropic agents typically used for co-immunoprecipitation analysis. Moreover, C8PA cells are devoid of any FABP, implying we could introduce mutants of AFABP and/or JAK2 to define the requirements of specific amino acids. In this system we found that the fatty acid binding mutant R126L/Y128F AFABP/aP2 was unable to interact with JAK2. The ligand dependence of this interaction is likely to be the primary reason that standard protocols for protein-protein interaction analysis do not work for AFABP/aP2, as ligands would be stripped

from the protein with detergents used in such procedures. The fatty acid dependence allows AFABP/aP2 to act as a rheostat of intracellular fatty acid levels, regulating its interaction with JAK2. This could have major implications on JAK2 signaling pathways when fatty acid levels are elevated. Fatty acid levels would be increased in the obese condition in both adipocytes and macrophages resulting in more holo-AFABP/aP2. Under this condition we would expect more AFABP/aP2 interacting with JAK2, attenuating its signaling capacity. Considering, AFABP/aP2 has a role in obesity linked insulin resistance and atherosclerosis, two processes that result from increased lipid accumulation and metabolism, it may be a fatty acid sensor for JAK2 signaling pathways important for disease progression.

Previous work has revealed that AFABP/aP2 interacts with HSL within a sequence EHYKRNE^{199} (17). The ExYK motif is also found in AFABP/aP2 (DDYMK) and thus makes it an attractive motif to explore further. Studies have revealed that a charge-quartet comprised of D17, D18, K21 and R30 in AFABP/aP2 is necessary for the interaction with HSL and that a charge-charge interaction from D18 of AFABP/aP2 to K196 of HSL is central for this association (20). We show here that AFABP/aP2 interacts with a similar sequence in JAK2. Interaction was compromised with D18K AFABP/aP2, although the K21A AFABP/aP2 mutant had no effect on the interaction. Importantly, mutation at either of these sites results in similar ligand binding, excluding the possibility that decreased ligand binding is why D18K has decreased interaction. This suggests that the exact amino acids for interaction may be different than with HSL

but the helix-turn-helix domain is important for this association. Single mutations in this domain may not be sufficient to ablate this interaction due to other or more charged residues being important for this interaction with JAK2. Further investigation into this possibility is the subject of future studies.

JAK2 is constitutively active in the C8PA cell system as indicated by JAK2 phosphotyrosine levels. Y1007 and Y1008 are critical auto-phosphorylation sites necessary for activation loop flip by this kinase resulting in full activation of Jak2 (24). Mutation of all of the EYYK residues resulted in a significant reduction in the total phosphotyrosine levels, indicating that all of the mutations affect JAK2 activity in a similar manner. AFABP/aP2 interaction with these mutants was increased, suggesting that AFABP/aP2 interacts with JAK2 in the basal, unphosphorylated state. This would position AFABP/aP2 to attenuate signaling by not allowing JAK2 to auto-phosphorylate or to inhibit other JAK2 protein-protein interacting partners from binding.

In sum, the interaction data suggests that AFABP/aP2, when bound to a fatty acid ligand, interacts with unphosphorylated JAK2. In order to test the effect of AFABP/aP2 on JAK2 signaling, we made use of the AFABP/aP2-EFABP/mal1 double knockout peritoneal macrophages. DKO mice in an ApoE null background have significantly reduced atherosclerosis on a normal chow diet (15). Considering that AFABP/aP2 null bone marrow transplanted into an ApoE null animal results in a protection against atherosclerosis, suggests that expression of FABPs in the macrophage is important to

atherosclerosis development (7). Development of atherosclerosis is a complex process involving communication between many cell types through cytokine and inflammatory mediators. Il-6 and Il-10 both have anti-atherogenic potential (29-31). Considering the phenotype of the FABP null mice and the possible involvement of JAK2 signaling, we investigated IL-6 and IL-10 signaling in macrophages derived from AFABP/aP2-EFABP/mal1 deficient mice. The results showed a potentiation in IL-6 signaling while IL-10 signaling was unchanged in the DKO macrophages. This difference suggests that AFABP/aP2 has localized effects on JAK2 that may be due to the involvement of other signaling components. Indeed, IL-6 signaling and IL-10 signaling do have immediate upstream differences, such as the involvement of SHP-2 and SOCS3 as negative regulators in IL-6 signaling (32,36,37). Further analysis of other JAK2 signaling pathways is necessary to determine the specificity of the AFABP/aP2 effect. The inhibitory action of AFABP/aP2 on IL-6 induced STAT3 phosphorylation suggests that its role is at the level of JAK2, the immediate upstream event. These results are consistent with AFABP/aP2 directly inhibiting JAK2 kinase activity through protein-protein interaction.

In sum, we show here a novel interaction between AFABP/aP2 and JAK2. AFABP/aP2 interacts through the helix-turn-helix domain in a ligand dependent manner with unphosphorylated JAK2 and results in an attenuation of a JAK2 signaling pathway. Taken together this positions AFABP/aP2 as a fatty acid sensor for JAK2, resulting in the fine-tuning of JAK2 signaling as a consequence of the lipid state of the cell.

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CHAPTER 5:

PERSPECTIVES

This chapter was written by Brian Thompson.

The field of adipose biology and fatty acid metabolism has become increasingly popular with the rise in obesity. Understanding the complexity of lipid metabolism and its relation to disease states such as type II diabetes, atherosclerosis and cancer are critical to treatment of these diseases. It is clear that fatty acids are critical to the progression of obesity related disease states. Fatty acids can result in lipotoxicity in skeletal muscle and liver and inflammation in adipose tissue, both of which have been associated with insulin resistance and atherosclerosis. Understanding how fatty acids are trafficked within the cell becomes very important in light of these findings.

AFABP/aP2 has a major role in intracellular fatty acid metabolism, making it an attractive protein to study. Genetic and pharmacologic inhibition of AFABP/aP2 in mice results in insulin sensitivity and decreased atherosclerosis while still being obese (1,2). Therefore, obesity and insulin resistance are disconnected in these animals, or they are fat but don't know it. AFABP/aP2 could be sequestering or delivering fatty acids in a passive way, or regulating enzyme and signaling functions through protein-protein interaction, or a combination of the two. Either way, it is clear that AFABP/aP2 alters fatty acid and glucose metabolism in adipose tissue resulting in whole body metabolic changes. Therefore, the study of AFABP/aP2's molecular interactions and the consequences of these interactions is necessary.

This thesis presents two protein-protein interactions of AFABP/aP2, with HSL and JAK2. Three important considerations are presented for both interactions. First, AFABP/aP2 ligand binding is necessary for the interactions. Second, the helix-turn-helix domain and specifically, DDYMK of AFABP/aP2 is the site of interaction for HSL and JAK2. Third, an ExYK motif in HSL and JAK2 is important for their interaction with AFABP/aP2. In light of these observations we propose that AFABP/aP2 is a fatty acid sensor, sensing the levels of intracellular fatty acids, interacting through DDYMK with HSL and JAK2's ExYK motif, subsequently altering their functional outcomes.

The requirements for these interactions suggest that they are regulatory. AFABP/aP2 needs to be in the ligand bound state for the appropriate conformation for interaction, suggesting that cellular concentrations of ligand determine the extent of the interaction. On the hetero-partner side, both HSL's and JAK2's phosphorylation state regulate the interaction. For HSL, phosphorylation is a requirement for the interaction, while JAK2 needs to be in the basal, unphosphorylated state for the appropriate conformation for interaction. In regards to this, these interactions will be determined by the ligand concentration and the phosphorylation state of the hetero-partner of AFABP/aP2, suggesting that only a subset of AFABP/aP2 will interact with these partners at a given point in time. Further, this suggests that these interactions will result in the fine-tuning of these pathways and not an on/off switch. Fine-tuning of these pathways may result in maintaining a balance in cellular metabolism during a lipid challenge, allowing the cell to accommodate the increased lipid and respond appropriately in terms of signaling.

AFABP/aP2's role as a fatty acid sensor may extend beyond these two interactions. Analysis of the HSL sequence necessary for interaction with AFABP/aP2 resulted in finding an ExYK motif. BLAST searches and candidate protein alignments revealed more proteins with an ExYK motif. These include SH2-domain containing tyrosine phosphatase 2 (SHP-2), IKKβ, and PPARγ. Preliminary evidence suggests that these proteins all interact with AFABP/aP2 (data not shown). Further characterization of the site of interaction and fatty acid binding necessity is currently underway. PPARγ directly interacts with AFABP/aP2 and two independent domains of PPARγ are sufficient for the interaction, importantly one of them contains the ExYK motif (3). Interestingly, the ExYK motif is found on helix 12 in PPARγ. Helix 12 is the co-activator/co-repressor interaction domain and is critical to ligand induced transcriptional activation (4). It is intriguing to speculate that AFABP/aP2 may be necessary for activation or repression of PPARγ in a ligand specific manner, fine-tuning PPARγ activity depending on the specific ligand bound. AFABP/aP2 null macrophages show increased PPARγ activity, suggesting that AFABP/aP2's interaction may result in attenuation of PPARγ (5). Knockout animals and cell lines have revealed a role for AFABP/aP2 in the IKK-NFκB inflammatory pathway. Within this pathway it was shown that IKKβ kinase activity is diminished in the absence of AFABP/aP2 resulting in diminished NFκB activity and pro-inflammatory cytokine production (5). Defining these interactions and their physiological consequences may define AFABP/aP2 as a global fatty acid sensor, affecting inflammation, transcriptional reprogramming, and signal transduction through protein-

protein interactions. Searching the proteome for ExYK motifs has been extremely successful in determining protein-protein interacting partners of AFABP/aP2. This strategy has revealed a new function of AFABP/aP2 as a fatty acid sensor.

In the family of FABP's, heart and epithelial FABPs have similar sequence to AFABP/aP2's DDYMK while liver and intestinal FABPs do not. Considering this, the DDYMK motif may confer fatty acid sensing through interaction on a global scale. Indeed, EFABP and HFABP both interact with HSL, while LFABP and IFABP do not, suggesting that they may have similar interactions with JAK2 and the other ExYK proteins (6,7). LFABP and IFABP may also interact with proteins in a ligand dependent manner through an unidentified domain similar to A-, E- and H-FABP. Extending these interactions to other lipid metabolizing tissues, such as heart and liver may unveil critical functions for this family in other disease states. This would redefine this family as fatty acid sensors, important for many cellular processes when challenged with increased lipid.

Cellular based protein-protein interaction assays have been critical to study these two interactions. The complex nature of these interactions made it necessary to develop these cellular based assays, in order to study them in a "natural" setting. The cellular context is necessary for ligand binding, phosphorylation state and/or other requirements for this interaction that are not appreciated. Most in vitro protein-protein interaction assays utilize chaotropic agents to diminish non-specific interactions, and these agents strip ligand from AFABP/aP2, therefore disrupting the interaction. While in vitro protein-

protein interaction assays have proved difficult for understanding AFABP/aP2 interactions, cellular cross-linking and in situ FRET have revealed many of the requirements for the HSL and JAK2 interactions. As AFABP/aP2 is endogenously expressed in cell types with high lipid metabolism, it has been important to use a cell system, which mimics the levels of fatty acids seen endogenously. To this end, the C8PA cell system provides increased levels of fatty acid metabolism while having no endogenous AFABP/aP2. Through the combination of utilizing an adipocyte-like cell system in terms of lipid storage, without endogenous AFABP/aP2, and in situ interaction assays, we are able to probe these interactions. Future studies should utilize these cell based assays, in situ FRET and cellular cross-linking, to understand these interactions and to further characterize the other potential interacting partners of AFABP/aP2.

Defining the ligand specificity for these interactions is an exciting next step. AFABP/aP2 binds many hydrophobic molecules, and slight differences in binding have been shown for different ligands. Crystal structures for multiple ligands have been elucidated, and show slight conformational differences (8,9). This suggests that not every ligand will provide a suitable conformation for these interactions. Indeed, it has been shown that different ligands have different effects on AFABP/aP2's regulation of PPARγ and on nuclear import (10-14). Therefore, it is necessary to determine the specific ligands that confer interaction. This will be a challenge considering that cellular based interaction assays have been the most successful, and in this context it is very difficult to determine the specific ligand AFABP/aP2 is binding while interacting. Understanding the

requirements needed for these interactions may lead to better designed in vitro studies, which would allow for ligand screening. Adding to the complexity of these interactions is the possibility that these interactions may show different ligand specificity. While some ligands may confer a suitable confirmation for the HSL interaction this might not be true for the JAK2 interaction. As shown in Chapter 3, there is a difference in the importance of K21 with regard to the interaction with HSL and JAK2, suggesting that the acceptable conformations of AFABP/aP2 for these two interactions may be slightly different. Considering, pharmacologic agents disrupt fatty acid binding through competitive inhibition, it is important to know whether these agents act as pseudo-ligands in the context of interaction. Many of these agents may, in fact, increase these interactions resulting in phenotypes more similar to an overexpression system or lipid challenge. There also may be inhibitor specificity for each of these interactions, making it possible to separate the function of one interaction from the other. In this regard, re-defining inhibitors for AFABP/aP2 in terms of interactions may uncover more potent and efficacious treatments for insulin resistance. Furthermore, characterization of the impact of inhibitors on interaction may unveil a pharmacologic tool to study these interactions with exogenous ligand, allowing further analysis of the impact of these interactions in a physiologic context.

While the study of the requirements of these interactions is important to define AFABP/aP2 as a fatty acid sensor, the physiological consequences to these interactions is

the main goal. Adipose tissue is a very dynamic tissue, increasing and decreasing fatty acid metabolism with regard to whole body needs. This positions AFABP/aP2 to affect whole body energy status through its control of processes in adipocytes. JAK2 is activated by many cytokines and hormones in adipocytes and macrophages, such as IL-6, growth hormone, leptin, TNF-α, INFγ, and epinephrine, resulting in changes in lipid and glucose metabolism, inflammation, recruitment of macrophages, and survival (15-20). It is intriguing to speculate that some of the phenotypes seen in the AFABP/aP2 null mouse are a result from the disruption of its interaction with JAK2. In this regard it will be important to define the JAK2 signaling pathways that are affected by AFABP/aP2 and the impact on cellular metabolism when this interaction is disrupted. As discussed above, connecting all of these interactions with physiological consequences, such as, lipid transcriptional reprogramming and PPARγ, inflammation and IKKβ, lipolysis and HSL, and signal transduction and JAK2-SHP2, will be the important next steps to defining the role of AFABP/aP2 in the cell.

The complexity of metabolism provides an immense challenge to researchers. This being said, studying the molecular details of individual proteins can result in a better understanding of the roles they play in metabolism and result in a small step forward necessary for those giant leaps. AFABP/aP2 is a prime example, once thought of as a passive buffer for fatty acids, knockout animals revealed a more important regulatory role in whole body energy homeostasis. This thesis extends these findings, redefining

AFABP/aP2 as a fatty acid sensor, through ligand dependent protein-protein interactions, coordinating adipose tissue metabolism, signal transduction and transcription.

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