CHARACTERIZATION OF THE FUNCTIONAL INTERACTION OF ADIPOCYTE LIPID-BINDING PROTEIN WITH HORMONE SENSITIVE LIPASE

Wen-Jun Shen, Yu Liang, Richard Hong, Shailja Patel, Vanita Natu, Kunju Sridhar, Ann Jenkins, David A. Bernlohr, and Fredric B. Kraemer

1 Division of Endocrinology, Department of Medicine, Stanford University, Stanford, CA and VA Palo Alto Health Care System, Palo Alto, CA; 2 Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, MN

Address correspondence to:
Fredric B. Kraemer, M.D.
Division of Endocrinology, S-005
Stanford University
Stanford, CA 94305-5103
phone: 650-493-5000#63184
fax: 650-852-3263
email: fbk@stanford.edu

Running Title: ALBP:HSL Interaction
SUMMARY

Hormone sensitive lipase (HSL) is an intracellular lipase that plays an important role in the hydrolysis of triacylglycerol in adipose tissue. HSL has been shown to interact with adipose lipid-binding protein (ALBP), a member of the family of intracellular lipid-binding proteins that bind fatty acids and other hydrophobic ligands. The current studies have addressed the functional significance of the association and mapped the site of interaction between HSL and ALBP. Incubation of homogeneous ALBP with purified, recombinant HSL in vitro resulted in a 2-fold increase in substrate hydrolysis. Moreover, the ability of oleate to inhibit HSL hydrolytic activity was attenuated by co-incubation with ALBP. Co-transfection of CHO cells with HSL and ALBP resulted in greater hydrolytic activity than transfection of cells with HSL and vector alone. Deletional mutations of HSL localized the region of HSL that interacts with ALBP to amino acids 192-200, and site-directed mutagenesis of individual amino acids in this region identified H194 and E199 as critical for mediating the interaction of HSL with ALBP. Interestingly, HSL mutants H194L and E199A, each of which retained normal basal hydrolytic activity, failed to display an increase in hydrolytic activity when co-transfected with wild-type ALBP. Therefore, ALBP increases the hydrolytic activity of HSL through its ability to bind and sequester fatty acids and via specific protein-protein interaction. Thus, HSL and ALBP constitute a functionally important lipolytic complex.
INTRODUCTION

Hormone-sensitive lipase (HSL) is an intracellular neutral lipase that is highly expressed in adipose and steroidogenic tissues (1). The enzyme has broad substrate specificity, displaying hydrolytic activity against triacylglycerol, diacylglycerol and cholesteryl ester (2). Observations from HSL null mice have shown that HSL is responsible for ~50% of the neutral triglyceride lipase activity and all of the neutral cholesteryl ester hydrolase activity in white adipose tissue (3). Thus, HSL plays an important role in regulating lipolysis and the release of fatty acids from adipose tissue. The sequence of HSL is unrelated to other mammalian lipases, but it shares sequence and structural similarity with several bacterial and fungal lipases (4-11). This structural similarity is based on the ability to model a large portion of the C-terminal ~450 amino acids of HSL as an α/β hydrolase (7); however, the initial ~320 amino acids of the protein share no sequence or structural homology with any known proteins. Within the C-terminal region of the protein lies a 150 amino acid sequence that contains a number of sites phosphorylated in response to lipolytic stimulation (7,12,13). In this regard HSL is unique among lipases for the ability of its activity to be up-regulated by phosphorylation. In addition to phosphorylation, HSL activity appears to be regulated by oligomerization, with the dimeric enzyme exhibiting markedly increased activity (14).

Utilizing a yeast two-hybrid screen of a rat adipose tissue library, we previously demonstrated that HSL specifically interacts with adipose lipid-binding protein (ALBP or aP2) and identified the N-terminal 300 amino acids of HSL as the region responsible for this
interaction (15). ALBP is highly expressed in adipose tissue and is a member of the family of intracellular fatty acid-binding proteins (FABP) that bind fatty acids, retinoids and other hydrophobic ligands (16). It has been proposed that FABPs function to sequester fatty acids, thus serving as an intracellular buffer or participating in facilitating the movement of fatty acids within the cell. In view of our observation that HSL and ALBP interact, we proposed that ALBP might prevent feedback inhibition of HSL by high local concentrations of free fatty acids released at the site of hydrolysis. Consistent with this view, adipocytes from ALBP null mice exhibit markedly reduced basal and stimulated lipolysis both in situ and in vivo (17,18). In the present studies we have addressed the functional significance of the interaction of HSL with ALBP and provide evidence that the interaction of ALBP with HSL constitutes an additional mechanism whereby the hydrolytic activity of HSL is regulated. Furthermore, we have explored the identification of the sequences in HSL that mediate its interaction with ALBP.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents

All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Bovine serum albumin (fraction V) was from InterGen Co., Purchase, NY; fetal bovine serum from Gemini Bio-Products, Inc., Calabasas, CA; Coon’s F12/Dulbecco’s Modified Eagles media, lipofectin reagent from GIBCO BRL, Gaithersburg, MD; ECL western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, cholesteryl [1-14C] oleate, L-[2,3,4,5-3H] arginine monohydrochloride, L-[4,5-3H] leucine from Amersham Life Sciences Products, Arlington Heights, IL; nitrocellulose paper from Schleicher and Schuell, Keene, NH; TnT® Transcription/Translation System from Promega, Madison, WI; S/9 cells, S/21 cells, TMN-FH insect medium, baculovirus transfer vectors pAcGHLT-A, pAcHLT-A, BaculoGold™ linearized baculovirus DNA from PharMingen, San Diego, CA; Ni-NTA Agarose from Qiagen, Valencia, CA); Quick-Change mutagenesis kit from Stratagene, La Jolla, CA.

Organic solvents were from J.T. Baker (Phillipsburg, NJ).

Construction of deletional and mutational constructs of HSL

C-terminal deletion fragments of HSL were generated by PCR using a common 5’ primer (5’GAG AAC CCA CTG CTT ACT) and the following 3’ primers: HSL 1-270, 5’-CGG CAA GCT AAG CAG GCG GCT; HSL 1-240, 5’-GGC TTT CCA GAA GTG CAC GTC CAG; HSL 1-230, 5’-TGA TGC GCT CAA ATT CAG; HSL 1-220, 5’ GGT CTA TGG CGA ATC GGC; HSL 1-209, 5’-ACT TGC AGT CAC ACT GAG; HSL 1-197, 5’-GTA GTG TTC CCC GAA
GGA. Each of the DNA fragments generated from PCR was confirmed by DNA sequencing using an ABI prism DNA sequencer. Site-directed mutagenesis of HSL was carried out using Strategene “Quick-Change” mutagenesis kit. The primer pairs used and the resulting amino acids for different mutants are listed in Table 1. The identities of the mutant constructs were confirmed by DNA sequencing using an ABI prism DNA sequencer.

**Production and purification of recombinant HSL and ALBP**

Recombinant GST-HSL was produced in Baculovirus as described previously (14).

Recombinant His-HSL was generated by cloning full-length rat HSL cDNA into the Smal site of pAcHLT-A containing a 6xHis tag. pAcHLT-A-HSL (5 µg) was co-transfected into Sf21 cells with 1 µg of BaculoGold DNA using the transfection kit from the manufacturer. The titer of the recombinant virus was determined using an end-point dilution assay, and the virus was re-amplified to a final titer of 1.5×10^7 pfu/ml. To produce recombinant proteins, Sf21 cells were grown in 150 mm petri dishes and each 2×10^7 cells were infected with 100 µl of the high titer recombinant virus; cells were harvested three days after infection. In order to metabolically label HSL, Sf21 cells were grown in complete TMN-FH insect medium supplemented with L-[2,3,4,5-^3^H] arginine (100 µCi) and L-[4,5-^3^H] leucine (100 µCi) for 3 days following infection with baculovirus. After harvesting and cell extraction, His-HSL was purified on a Ni-agarose column. Recombinant ALBP and ALBP mutants were produced in *E. coli* and purified as described (19,20).

**In vitro protein-protein interaction**
After sequence confirmation of the identity of HSL mutants, the HSL mutants were *in vitro* translated with $[^{35}\text{S}]$ methionine by using the TNT transcription/translation system (15). GST-ALBP or GST alone were incubated with glutathione-agarose beads in buffer B (20mM Tris, pH 8.0/0.15M NaCl/1mM EDTA/0.5% Nonidet P-40). After 1h incubation at room temperature, the beads were washed three times in buffer B, and then incubated with $[^{35}\text{S}]$ methionine labeled HSL. After 1h incubation at room temperature, the beads were washed five times in buffer B, and proteins that bound to the beads were eluted in SDS/PAGE sample buffer, separated on SDS/10% PAGE, and visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Cell culture and transfection**

CHO cells were grown in Coon’s F12/Dulbecco's modified Eagle’s medium supplemented with 10% FCS at 37°C under 5% CO$_2$. For transient transfection experiments, cells were subcultured at a density of 2X10$^5$ cells/well in six well plates the day prior to incubation with 0.75 µg of pcDNA3-HSL or pcDNA3-HSL mutants, as well as 0.75 µg of pGFP-ALBP and 0.25 µg of pCMV β-GAL in 10 µl of lipofectin reagent. Cells were transfected following the procedure from GibCO-BRL and harvested 40h after transfection for measurement of HSL activity.

**HSL activity**

HSL activity was determined as neutral cholesterol ester hydrolase activity using either a cholesteryl$[^{14}\text{C}]$oleate emulsion for measurement of cellular activity, as described previously (13), or cholesteryl$[^{14}\text{C}]$oleate dispersed in ethanol for measurement of recombinant enzyme activity. Aliquots of 1-5µg of recombinant GST-HSL were added to 140 µl solutions of 50 mM
Tris-HCl (pH 7.0), 100 µl of TES containing different concentrations of BSA or ALBP, and 1.96
nmol of cholesteryl [14C]oleate dispersed in ethanol. After incubation at 37°C for 1h, the reaction
was terminated by adding 3.25 ml of methanol/chloroform/heptane (1.41:1.25/1.0, v/v/v),
followed by 1.05 ml of 50 mM sodium carbonate:50 mM sodium borate. The reaction mixtures
were then vortexed and centrifuged for 30 min at 1500x g. A 0.5 ml fraction of the upper phase
was then removed for liquid scintillation counting. The enzyme activities were determined as
pmol of hydrolyzed cholesteryl oleate per minute per µg of enzyme.

Sucrose gradient centrifugation

[3H]-labeled His-HSL was incubated in the presence of ALBP or GST and then layered on top of
a linear 10-40% (w/w) sucrose gradient (5ml, prepared in 20 mM Tris-HCl (pH 7.4) and 1 mM
EDTA), as described previously (14). The tubes were centrifuged in a SW 50.1 rotor at 50,000
rpm for 4 h. Samples were collected from the bottom of the tube in 20 to 22 fractions and
aliquots taken for liquid scintillation counting and determination of sucrose concentration by
refractometry.
RESULTS

Since we had previously demonstrated that HSL and ALBP physically interact (15), we sought to determine whether this physical interaction might influence the hydrolytic activity of HSL. As an initial approach, we assayed the effects of increasing concentrations of recombinant ALBP on the ability of baculovirus-produced rat HSL to hydrolyze cholesteryl ester substrate dispersed in ethanol. As shown in Figure 1A, addition of ALBP caused a dose dependent increase in cholesteryl ester hydrolytic activity of HSL, which resulted in up to a 2-fold increase in activity at saturating concentrations of ALBP. To explore whether this effect of ALBP on HSL hydrolytic activity was specific for ALBP and whether it depended on the ability of ALBP to bind fatty acids, we compared the effects on HSL activity of a nonspecific protein, GST, and a mutant form of ALBP (R126L, Y128F), which possesses only 1% of the fatty acid binding capacity of the wild type protein (20), but binds normally to HSL in GST pull down experiments (data not shown). Based upon the ALBP crystal structure (21), the side chains of R126L and Y128F extend into the fatty acid binding cavity. As such, they are not anticipated to participate in protein-protein interactions involving ALBP and HSL. In preliminary experiments we observed that the addition of proteins to the reaction resulted in some nonspecific or surface effects on the interaction of the enzyme with its substrate and caused an increase in activity above that seen in the absence of any other proteins. However, we found that inclusion of insulin, a completely nonspecific protein with regard to fatty acid binding or interaction with HSL (Figure 1B), in the incubation dampened these nonspecific or surface effects. Thus, insulin
(0.2 µM) was included in all subsequent assays. As shown in Figure 1C, the addition of GST caused only a small increase in HSL hydrolytic activity above control. Addition of equimolar amounts of the fatty acid-binding mutant of ALBP to the reaction increased HSL activity ~2-fold above that seen with GST alone to the level observed with wild type ALBP. These results suggest that under the current conditions the increase in HSL activity seen in the presence of ALBP might be due primarily to its ability to interact with HSL and that the ability of ALBP to bind fatty acid does not seem to be required for this effect. As an independent means of confirming that ALBP can modulate HSL activity, we transiently co-transfected CHO cells with HSL and either vector alone or ALBP, and then assayed the cellular extracts for HSL hydrolytic activity. As shown in Figure 1D, overexpression of ALBP together with HSL led to an approximate two-fold increase in HSL hydrolytic activity compared with cells transfected with HSL and vector alone. These data demonstrate that the presence of ALBP can lead to higher hydrolytic activity of HSL against its substrate both \textit{in vitro} and within cells.

We have shown that HSL exists as functional dimers composed of homologous subunits and that dimeric HSL displays dramatically greater activity against cholesteryl ester substrate when compared with monomeric HSL (14). Therefore, to test whether the increase in HSL activity observed in the presence of ALBP was due to a change in the dimerization of HSL, we examined the effects of ALBP on HSL subunit structure. To perform these experiments recombinant His-HSL was metabolically labeled by incubating S/21 cells, which had been infected with HSL-containing baculovirus, with \textsuperscript{[3]}H arginine and \textsuperscript{[3]}H leucine. Purified \textsuperscript{[3]}H-
labeled His-HSL was incubated in the presence of ALBP or GST and the HSL species were then fractionated by sucrose density gradient centrifugation. As shown in a representative experiment in Figure 2, two different sized populations of HSL were observed, consonant with a monomer and dimer; however, no consistent increase in HSL dimerization was seen in the presence of ALBP. Thus, the ALBP-induced increase in HSL activity does not appear to be due to any changes in the dimerization of HSL.

We had originally proposed that the interaction of ALBP with HSL might protect HSL hydrolytic activity from product inhibition by fatty acids released during lipolysis. To examine this directly we assayed residual neutral cholesteryl ester hydrolase activity of purified, recombinant rat HSL to which increasing concentrations of oleate were added in the presence or absence of ALBP. As reported previously (22), addition of oleate resulted in a dose-dependent inhibition of HSL activity (Figure 3A). The presence of ALBP in the incubation in the absence of any oleate increased HSL activity ~50% (Figure 3B). Furthermore, ALBP attenuated the oleate-induced inhibition of HSL hydrolytic activity, shifting the inhibition to the right. In order to control for potential nonspecific or surface effects of added proteins on the interaction of HSL with its substrate, we compared the ability of GST, the fatty-acid binding mutant of ALBP (mALBP), and native ALBP to prevent oleate-induced inhibition of HSL activity (Figure 3C). Although some nonspecific effects were observed, ALBP was significantly more effective in preventing inhibition of HSL activity at concentrations up to 25 µM oleate. Since the fatty acid binding mutant did not preserve HSL activity as effectively as native ALBP when exposed to...
higher concentrations of oleate (15-25 µM), these data suggest that ALBP protects HSL from
product inhibition, and that this protective effect is dependent, at least partially, on the ability of
ALBP to bind fatty acid.

Having documented that ALBP can modulate HSL hydrolytic activity, we sought to
identify the structural determinants within HSL that mediate its interaction with ALBP. Previous
studies had initially localized the interaction of ALBP with the 300 N-terminal amino acids of
HSL (15). To further define the region of HSL responsible for interacting with ALBP, a series
of C-terminal deletions from the N-terminal 300 amino acids of HSL were generated, and tested
for the ability of the [35S]methionine-labeled in vitro translated products to bind to ALBP. As
shown in Figure 4, 35S-methionine-labeled truncations of HSL and GST alone or ALBP-GST
were incubated with glutathione-agarose beads and the proteins that bound to the beads were
washed, eluted and separated on SDS-PAGE. The truncations to HSL 1-240, HSL 1-230, HSL
1-220, HSL 1-209 continued to be able to interact specifically with ALBP-GST, while no
interaction was observed with GST alone. However, the truncation to HSL 1-197 showed
similar binding to GST alone and ALBP-GST, while HSL 1-187 failed to bind either GST alone
or ALBP-GST. To confirm that the initial 187 amino acids are not involved in interacting with
ALBP, a form of HSL was generated which lacked the initial 187 amino acids, HSL 187-767.
This truncated HSL interacted specifically with ALBP-GST, while no interaction was observed
with GST alone. From these data it appears that the amino acids between 187 and 209 are
responsible for the interaction of HSL with ALBP.
Analyzing the predicted secondary structure of HSL using the PDH prediction program and the GCG prediction program suggested that amino acids 192-200 have the highest probability of being located at the surface of the protein and that they might form a loose or turn structure between two β-sheets. Internal deletion of amino acids 191-199 eliminated the binding of HSL to ALBP, but also resulted in a protein without hydrolytic activity (data not shown).

Likewise, deletion of amino acids 191-196 or 194-199 also resulted in HSL proteins that failed to bind to ALBP and lacked hydrolytic activity (data not shown). Therefore, we generated a series of single and double mutants of HSL between amino acids 193-199. As shown in Figure 5, $^{35}$S-methionine-labeled mutations of HSL were incubated with GST alone or ALBP-GST and then with glutathione-agarose beads, and the proteins that bound to the beads were washed, eluted and separated on SDS-PAGE. Mutants E193A, Y195A, Y195D, Y195F, K196V/R197L, and R197A all continued to be able to interact specifically with ALBP-GST, while no interaction was observed with GST alone. In contrast, mutants H194L and E199A failed to bind either GST alone or ALBP-GST, suggesting that these sites are either directly involved in the interaction of HSL with ALBP or that mutation at either site is sufficient to alter the secondary structure of HSL in order to eliminate its binding to ALBP.

In order to assess the functional significance of these mutations, we transiently co-transfected CHO cells with the HSL mutants and an empty vector, and then assayed the cellular extracts for HSL hydrolytic activity. As shown in Figure 6, when co-transfected with vector alone, HSL mutants E193A, H194L, Y195F, R197A, and E199A retained similar hydrolytic activity.
activity against cholesteryl ester as wild type HSL; however, HSL mutants Y195A, Y195D, and K196V/R197L resulted in reduced, though measurable, basal hydrolytic activity. Since these mutants retained hydrolytic activity, but mutations at 194 and 199 failed to bind to ALBP, we could now examine whether the specific interaction of ALBP with HSL is required for the ALBP-induced increase in HSL activity. CHO cells were transfected with the mutant HSLs and ALBP, and then assayed for neutral cholesterol esterase activity. Co-transfection of ALBP with wild type HSL increased hydrolytic activity \( \sim 50\% \). Likewise, co-expression of ALBP with E193A also increased hydrolytic activity \( \sim 50\% \). Co-expression of ALBP with Y195A, Y195D, or K196V/R197L appeared to increase hydrolytic activity \( \sim 50\% \); however, because the basal activities of these mutants were reduced, the results should be interpreted cautiously. Interestingly, HSL mutants H194L, Y195F or E199A, each of which retained normal basal activity, failed to display an increase in hydrolytic activity when co-expressed with ALBP.
DISCUSSION

Free fatty acids are an essential source of energy for many tissues. The flux of FFA is primarily dependent on the lipolysis of stored triacylglycerol in adipose tissue (23). Multiple mechanisms are involved in controlling lipolysis; however, HSL appears to play a critical role, mediating the initial hydrolysis of triacylglycerol to diacylglycerol, followed by the hydrolysis of diacylglycerol to monoacylglycerol (2). The final step, hydrolysis of monoacylglycerol to glycerol and fatty acid, is mediated by another enzyme, monoacylglycerol lipase (24). One of the mechanisms regulating lipolysis is control of the intracellular localization of HSL, with lipolytic stimulation leading to the translocation of HSL from the cytosol to the lipid droplet in some physiological settings (25-27). In addition, the hydrolytic activity of the enzyme is increased following phosphorylation by protein kinase A (12,13,28).

Our previous observation that HSL specifically interacts with ALBP led us to propose that ALBP might prevent feedback inhibition of HSL caused by high local concentrations of fatty acids released at the site of hydrolysis (15). An alternate concept would be that ALBP delivers fatty acids to HSL, thereby mediating the inhibition of catalytic activity by lipids. In the present studies we have provided evidence that the interaction of ALBP with HSL constitutes an additional mechanism whereby the hydrolytic activity of HSL is regulated. Thus, incubation of ALBP with purified, recombinant HSL in vitro resulted in an increase in substrate hydrolysis. This ALBP-induced increase in hydrolytic activity was due to at least two components: first, a small nonspecific effect of added protein, perhaps to alter the surface tension of the substrate...
and, thus, its interfacial interaction with the enzyme, and second, a specific effect. The specific effect of the ALBP-induced increase in HSL hydrolytic activity appeared to be due primarily to the ability of ALBP to interact with HSL. The ability of ALBP to bind fatty acid did not seem to be required for this effect, since a fatty acid binding mutant of ALBP, which binds normally to HSL, displayed a similar capacity to increase activity as wild type ALBP. There was no evidence that the ALBP-induced increase in HSL activity was due to any changes in the dimerization of HSL. Furthermore, the ability of ALBP to increase HSL hydrolytic activity was also demonstrated in situ by observing an increase in HSL activity in cells co-transfected with HSL and ALBP as compared to HSL and vector alone. Importantly, the ability of fatty acids to inhibit HSL hydrolytic activity was attenuated by co-incubation with ALBP. This protection of HSL hydrolytic activity was also due both to a nonspecific component and to a specific component. In this case, the specific effect of ALBP to protect HSL from fatty acid induced inhibition appeared to depend in part on the ability of ALBP both to interact with HSL and to bind fatty acids since the ALBP fatty acid binding mutant did not preserve HSL activity as effectively as native ALBP when exposed to higher concentrations of fatty acids. It should be noted that the level of ALBP in vivo is extremely high (estimated to be 250-400 µM), suggesting that, within the cellular context, the ability of ALBP to relieve product inhibition by sequestration of fatty acids is likely to be much greater than measured in vitro or in situ. Indeed, based upon the amount of fatty acids released from isolated adipocytes and the estimated water volume of an adipocyte, the calculated intracellular concentration of fatty acids is estimated to be
approximately 600 ± 75 µM (29). The near 1:1 stoichiometry of the total fatty acid pool with ALBP and the high affinity of fatty acids for the protein, measured by a combination of titration calorimetry and fluorescence displacement assays, suggest that the fatty acids within the cell are largely, if not exclusively, found protein bound. Thus, the concentration dependence measured for HSL appears to be well within the physiological range.

By analysis of a series of deletional mutants of HSL in GST pull-down experiments, we were able to localize the region of HSL that interacts with ALBP to amino acids 192-200. While it is still possible that regions >209 could affect binding through some secondary interactions, normal binding of HSL and ALBP was detected when >209 was eliminated, but not detected when >187 was eliminated, supporting the localization of the primary binding determinants within amino acids 187-209. Site-directed mutagenesis of individual amino acids in this region showed that mutation of either H194 or E199 eliminated the ability of HSL to interact with ALBP. Thus, these two residues, either directly or indirectly, are critical for mediating the interaction of HSL with ALBP. Interestingly, HSL mutants H194L and E199A, each of which retained normal basal hydrolytic activity, but were unable to bind to ALBP, failed to display an increase in hydrolytic activity when co-transfected with wild-type ALBP. The fact that these mutants retained normal basal hydrolytic activity argues against mutations at these sites affecting HSL dimerization, since monomeric HSL displays only ~1/40 the activity of the dimer (14). Moreover, this is consistent with our finding that no apparent increase in HSL dimerization occurred with incubation with ALBP. Consequently, it appears that the capacity of ALBP to
increase the hydrolytic activity of HSL is not due solely to the ability of ALBP to bind and sequester fatty acids, but appears to be dependent on the physical interaction of ALBP with HSL. This finding would suggest that the binding of ALBP to HSL might either alter the conformation of the enzyme allowing substrate to access the catalytic site more efficiently or prevent fatty acids from inhibiting the enzyme through a conformational change or steric inhibition. It is apparent, however, that the binding of ALBP to HSL may not be sufficient to allow an increase in hydrolytic activity since HSL mutants Y195F and R197A retained the ability to bind to ALBP and retained basal hydrolytic activity, yet failed to display an increase in hydrolytic activity when co-transfected with wild-type ALBP. This discrepancy might be due to conformational alterations associated with the mutations that either prevent ALBP-induced conformational changes or do not allow for ALBP-induced steric effects.

Therefore, it appears that ALBP can increase the hydrolytic activity of HSL through its ability to bind and sequester fatty acids and through its specific protein-protein interaction, perhaps leading to a conformational change or steric effects on HSL. Evidence that these functional effects of the interaction of ALBP with HSL occur in vivo is supported by experiments with ALBP null mice (17,18). In the absence of ALBP, intracellular free fatty acid concentrations are increased in adipose cells, and basal and isoproterenol-stimulated lipolysis are decreased ~40%, or to a similar degree suggested by our in vitro and cell experiments. Thus, the current observations are consistent with the proposal that ALBP and HSL constitute a lipolytic complex. This complex functionally results in an increase in the hydrolytic activity of HSL brought about
by the physical interaction of HSL with ALBP and by the ability of ALBP to bind and sequester fatty acids. Thereby, lipolysis and intracellular trafficking of fatty acids are controlled in an organized fashion.
REFERENCES

1. Kraemer, F. B., Patel, S., Saedi, M. S., and Szalryd, C. (1993) J Lipid Res 34, 663-671

2. Fredrikson, G., Stralfors, P., Nilsson, N. O., and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320

3. Osuga, J.-i., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., Shionoirai, F., Yahagi, N., Kraemer, F. B., Tsutsumi, O., and Yamada, N. (2000) Proc. Natl. Acad. Sci. USA 97, 787-792

4. Hemilä, H., Koivula, T. T., and Palva, I. (1994) Biochim Biophys Acta 1210, 249-253

5. Bertolini, M. C., Schrag, J. D., Cygler, M., Ziomek, E., Thomas, D. Y., and Vernet, T. (1995) Eur J Biochem 228(3), 863-869

6. Østerlund, T., Danielsson, B., Degerman, E., Contreras, J. A., Edgren, G., Davis, R. C., Schotz, M. C., and Holm, C. (1996) Biochem J 319, 411-420

7. Contreras, J. A., Karlsson, M., Østerlund, T., Laurell, H., Svensson, A., and Holm, C. (1996) J Biol Chem 271(49), 31426-31430

8. Choo, D.-W., Kurihara, T., Suzuki, T., Soda, K., and Esaki, N. (1998) Appl Environ Microbiol 64, 486-491

9. Kanaya, S., Koyanagi, T., and Kanaya, E. (1998) Biochem J 332, 75-80

10. Manco, G., Adinolfi, E., Pisani, F. M., Ottolina, G., Carrea, G., and Rossi, M. (1998) Biochem J 332, 203-212
11. Manco, G., Giosue, E., D’Auria, S., Herman, P., Carrea, G., and Rossi, M. (2000) *Arch Biochem Biophys* **373**(1), 182-192

12. Anthonsen, M. W., Rönnstrandt, L., Wernstedt, C., Degerman, E., and Holm, C. (1998) *J Biol Chem* **273**, 215-221

13. Shen, W.-J., Patel, S., Natu, V., and Kraemer, F. B. (1998) *Biochemistry* **37**, 8973-8979

14. Shen, W.-J., Patel, S., and Kraemer, F. B. (2000) *Biochemistry* **39**, 2392-2398

15. Shen, W.-J., Sridhar, K., Bernlohr, D. A., and Kraemer, F. B. (1999) *Proc. Natl. Acad. Sci. USA* **96**(10), 5528-5532

16. Bernlohr, D. A., Simpson, M. A., Vogel Hertzel, A., and Banaszak, L. J. (1997) *Annu Rev Nutr* **17**, 277-303

17. Coe, N. R., Simpson, M. A., and Bernlohr, D. A. (1999) *J Lipid Res* **40**, 967-972

18. Scheja, L., Makowski, L., Uysal, K. T., Wiesbrock, S. M., Shimshek, D. R., Meyers, D. S., Morgan, M., Parker, R. A., and Hotamisligil, G. S. (1999) *Diabetes* **48**(10), 1987-1994

19. Xu, Z. H., Buelt, M. K., Banaszak, L. J., and Bernlohr, D. A. (1991) *J Biol Chem* **266**(22), 14367-14370

20. Sha, R. S., Kane, C. D., Xu, Z., Banaszak, L. J., and Bernlohr, D. A. (1993) *J Biol Chem* **268**(11), 7885-92

21. Xu, Z., Bernlohr, D. A., and Banaszak, L. J. (1993) *J Biol Chem* **268**(11), 7874-84

22. Jepson, C. A., and Yeaman, S. J. (1992) *FEBS Lett* **310**(2), 197-200

23. Gordon, R. S., Jr., and Cherkes, A. (1956) *J. Clin. Invest.* **35**, 206-212
24. Fredrikson, G., Tornqvist, H., and Belfrage, P. (1986) *Biochim Biophys Acta* **876**, 288-293

25. Egan, J. J., Greenberg, A. S., Chang, M.-K., Wek, S. A., Moos Jr, M. C., and Londos, C. (1992) *Proc Natl Acad Sci USA* **89**, 8537-8541

26. Clifford, G. M., Londos, C., Kraemer, F. B., Vernon, R. G., and Yeaman, S. J. (2000) *J Biol Chem* **275**(7), 5011-5015

27. Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C., and Londos, C. (2000) *Biochim Biophys Acta* **1483**(2), 251-262

28. Huttunen, J. K., Steinberg, D., and Mayer, S. E. (1970) *Proc Natl Acad Sci USA* **67**, 290-295

29. Bernlohr, D. A., Jenkins, A., Hertzel, A. V., Frohnert, B. I., Coe, N. R., Witthuhn, B., Smith, A. J., Johnson, J., and Matarese, V. A. (2000) in *Adipocyte Biology and Hormone Signaling* (Ntambi, J., ed), pp. 31-39, IOS Press, Amsterdam
ACKNOWLEDGEMENTS

This work was supported in part by research grants from the Research Service of the Department of Veterans Affairs (FBK), by grant DK 46942 (FBK) from the National Institutes of Health, by grants MCB 9506088 and MCB 9816575 from the National Science Foundation (DAB), and by a Research Award from the American Diabetes Association (WJS). K.S. was supported by a postdoctoral training grant (DK 07217) from the National Institutes of Health.
FIGURE LEGENDS

Figure 1. Effects of ALBP on HSL hydrolytic activity. Panel A: The indicated amounts of recombinant ALBP were added to recombinant GST-HSL and HSL hydrolytic activity was measured as described in the Experimental Procedures using cholesteryl\(^{14}C\)oleate dispersed in ethanol as substrate. Panel B: HSL hydrolytic activity was measured after the indicated amounts of insulin were added. Panel C: HSL hydrolytic activity of recombinant His-HSL was measured after the addition of 0.2 μM insulin and the indicated amounts of GST protein, mutant ALBP (mALBP) or wild type ALBP. Panel D: CHO cells were co-transfected with pcDNA3-HSL, pGFP-ALBP or pGFP (vector alone), and 0.25 μg of pCMV β-GAL. Cells were harvested 40h after transfection for measurement of HSL activity. Results are the mean ± SEM of duplicate or triplicate samples and are representative of 3-5 independent experiments.

Figure 2. Effect of ALBP on HSL dimerization. \(^{3}H\)-labeled His-HSL was incubated with equimolar amounts of ALBP or GST and then layered on top of a linear 10-40% (w/w) sucrose gradient and centrifuged at 230,000g for 4h as described in the Experimental Procedures. Sequential fractions were collected from the bottom of the tube and taken for scintillation counting and determination of sucrose concentration. Bars denote the migration of molecular weight markers. The results are representative of three separate experiments.

Figure 3. Interaction of FFA and ALBP on HSL activity. Panel A: The indicated amounts of oleate were added to recombinant GST-HSL and HSL hydrolytic activity was measured as described in Figure 1. Panel B: The indicated amounts of oleate were added to 5μg of
recombinant GST-HSL in the presence or absence of 500 nM ALBP and HSL hydrolytic activity was measured as described above. Panel C: HSL hydrolytic activity of recombinant His-HSL was measured in the presence or absence of the indicated concentrations of oleate after the addition of insulin (0.2 µM) and either 20 nM GST protein, 20 nM mutant ALBP (mALBP) or 20 nM wild type ALBP. Results are the mean ± SEM of duplicate or triplicate samples and are representative of 3-5 independent experiments.

Figure 4. Identification of the region of HSL that interacts with ALBP. Truncations of HSL were generated as described in the Experimental Procedures. pcDNA3-HSL 1-240 (lanes 1, 8, 9), pcDNA3-HSL 1-230 (lanes 2, 10, 11), pcDNA3-HSL 1-220 (lanes 3, 12, 13), pcDNA3-HSL 1-209 (lanes 4, 14, 15), pcDNA3-HSL 1-197 (lanes 5, 16, 17), pcDNA3-HSL 1-187 (lanes 6, 18, 19), pcDNA3-HSL 187-767 (lanes 7, 20, 21), were in vitro translated with 35S-methionine using the TnT® Transcription/Translation System and incubated with GST (lanes 8,10,12, 14, 16, 18, 20) or ALBP-GST (lanes 9, 11, 13, 15, 17, 19, 21) and with glutathione-agarose beads. Proteins that bound to the beads were eluted, separated on 10% SDS-PAGE, and visualized using a PhosphorImager®.

Figure 5. Interaction of HSL mutants with ALBP. Mutations of HSL were generated as described in the Experimental Procedures. Constructs were in vitro translated with 35S-methionine and incubated with GST or GST-ALBP and with glutathione beads, as described in Figure 3. Proteins that bound to the beads were eluted, separated on 10% SDS-PAGE, and visualized as described using a PhosphorImager®.
Figure 6. Effect of ALBP on hydrolytic activity of HSL mutants. CHO cells were co-transfected with pcDNA3-HSL or pcDNA3-HSL mutants, pGFP-ALBP or pGFP (vector alone), and pCMV β-GAL. Cells were harvested 40h after transfection for measurement of HSL activity. Results are the mean ± SEM of triplicate samples and are representative of 5 independent experiments.
# Tables

Table 1. Primer pairs used for mutagenesis of HSL.

| Mutation     | Primers                                                                 |
|--------------|------------------------------------------------------------------------|
| E193A        | 5’ -GTG TCC TTC GGG GCC CAC TAC AAA CGC AAC GCG ACG GGC                |
|              | 3’ -CAC AGG AAG CCC CGG GTG ATG TTT GCG TTG CGC TGC CCG                |
| H194L        | 5’ -TCC TTC GGG GAG CTC TAC AAA CGC AAC GCG ACG GGC                  |
|              | 3’ -AGG AAG CCC CTC GAG ATG TTT GCG TTG CGC TGC TGC CCG               |
| E193A/H194L  | 5’ -TCC TTC GGG GCC CTT TAC AAA CGC AAC GCG ACG GGC                |
|              | 3’ -AGG AAG CCC CGG GAA ATG TTT GCG TTG CGC TGC TGC CCG               |
| K196V/R197L  | 5’ -GGG GAA CAC TAC GTG CTC AAC GCG AGC GGC CTC AGT                  |
|              | 3’ -CCC CTT GTG ATG CAC GAG TTG CGC TGC CCG GAG TCA                  |
| E199A        | 5’ -CAC TAC AAA CGC AAC GCG ACG GGC CTC AGT GTG                        |
|              | 3’ -GTG ATG TTT GCG TTG CGC TGC CCG GAG TCA CAC                      |
| Y195A        | 5’ -TCC TTC GGG GAA CAC GCC AAA CGT AAC GCG ACG GGC                |
|              | 3’ -AGG AAG CCC CTT GTG CGG TTT GCA TTG CGC TGC CCG                |
| Y195D        | 5’ -TTC GGG GAA CAC GAC AAA CGT AAC GCG ACG GGC                       |
|              | 3’ -AAG CCC CTT GTG CTG TTT GCT TTG CGC TGC CCG                       |
| Y195F        | 5’ -TCC TTC GGG GAA CAC TAC AAA CGT AAC GCG ACG GGC                |
|              | 3’ -AGG AAG CCC CTT GTG AAG TTT GCA TTG CGC TGC CCG                |
|       | 5′ -GGG GAA CAC TAC AAA GCC AAC GCG ACA GGC CTC |
|-------|----------------------------------------------|
|       | 3′ -CCC CTT GTG ATG TTT CGG TTG CGC TGT CCG GAG |
| Y195A/R197A | 5′ -TCC TTC GGG GAA CAC GCC AAA GCC AAC GCG ACA GGC CTC |
|        | 3′ -AGG AAG CCC CTT GTG CGG TTT CGG TTG CGC TGT CCG GAG |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Characterization of the functional interaction of adipocyte lipid-binding protein with hormone sensitive lipase
Wen-Jun Shen, Yu Liang, Richard Hong, Shailja Patel, Vanita Natu, Kunju Sridhar, Ann Jenkins, David A. Bernlohr and Fredric B. Kraemer

J. Biol. Chem. published online October 26, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104095200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts