Hormone-Sensitive Lipase Functions as an Oligomer†

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ABSTRACT: Hormone-sensitive lipase (HSL) is a cytosolic neutral lipase whose activity is regulated by reversible phosphorylation and which is thought to be the rate-limiting enzyme for the mobilization of FFA from adipose tissue. In the current studies the subunit structure of HSL has been explored using sucrose gradient centrifugation and in vivo and in vitro protein−protein interactions. Evidence is provided to demonstrate that HSL exists as a functional dimer composed of homologous subunits. Dimeric HSL displayed approximately 40-fold greater activity against cholesteryl ester substrate when compared with monomeric HSL without any differences in affinity for the substrate. Truncations of HSL identified the importance of the N-terminal 300 amino acids, as well as other regions, in participating in the oligomerization of HSL. These studies support the notion that the N-terminal region of HSL represents a docking domain for protein−protein interactions and provide an additional mechanism for the posttranslational control of HSL activity in the cell via oligomerization.

Hormone sensitive lipase (HSL)[1] is presumed to be the rate-limiting enzyme for the release of free fatty acids from adipose tissue and, as such, is felt to play a pivotal role in providing the major source of energy for most tissues (*1*). Evidence for the physiological importance of HSL is based on the facts that it is an intracellular neutral lipase that is highly expressed in adipose tissue and that it catalyzes the first two steps in the breakdown of stored triacylglycerols, e.g., the hydrolysis of triacylglycerol and diacylglycerol (*2*). HSL, however, has broader substrate specificity and tissue distribution, being able to catalyze the hydrolysis of 1(3)-monoacylglycerol (*3*) and mediate the hydrolysis of stored cholesteryl esters in steroidogenic tissues (*4*) and macrophages (*5*), and other lipid substrates, such as lipoidal esters of steroid hormones (*6*) and retinyl esters in adipose tissue (*7*), as well as water-soluble butyrate substrates (*2*). Another one of the unique features that differentiates HSL from other neutral lipases is that the activity of the enzyme is regulated acutely by reversible phosphorylation (*8*). HSL activity is stimulated by hormones, such as catecholamines, ACTH, and glucagon, through the activation of cyclic AMP-dependent protein kinase, which phosphorylates HSL, resulting in an increase in hydrolytic activity against triacylglycerol and cholesteryl ester substrates.

In addition to these functional characteristics that distinguish it from other lipases, HSL has a primary amino acid sequence that is unrelated to any of the other known mammalian lipases; however, it shares some sequence similarity with liver arylacetamide deacetylase within its catalytic domain (*9*). HSL also shares some sequence homology with several bacterial and fungal lipases and esterases (*10*−*15*). The C-terminal portion of HSL displays secondary structural homology with that of acetylcholinesterase and several fungal lipases (*12*) and bacterial brefeldin A esterase (*16*), consisting of parallel β-sheets flanked by α-helical connections, which has allowed these proteins to be classified as α/β hydrolases (*17*). The N-terminal portion of HSL has no similarity with any known proteins. Rat HSL is a 767 amino acid (aa) protein that has a molecular weight (MW) of 84 000 on SDS−PAGE (*3*). When it was initially purified from rat adipose tissue using detergent, its behavior on gel chromatography was consistent with the enzyme existing as a dimer or with an enzyme monomer within a detergent micelle (*3*). On the basis of this observation, it has been assumed that HSL functions as a dimer; however, no further evidence has been provided for the dimerization of HSL. In the current paper, we have reexamined the question of the subunit structure of HSL. We provide evidence demonstrating that HSL exists as a dimer and that its enzymatic activity is dependent on its forming an oligomer.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were obtained from the sources given in parentheses: bovine serum albumin (fraction V) (Intergen Co., Purchase, NY); sodium deoxycholate, Triton X-100, L−α phosphatidylcholine, cholesterol oleate, leupeptin, anti-rabbit IgG FITC conjugate, ruthenium(II) trisbipyridyl dication (Sigma Chemical Co., St. Louis, MO); cholesterol [1-^{14}C]oleate (E.I. Dupont de Nemours and Co., Boston, MA); fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA); Dulbecco's modified Eagle's medium,

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[1] Abbreviations: HSL, hormone-sensitive lipase; Ru(II)bpy$_3^{2+}$, ruthenium(II) trisbipyridyl dication; GST, glutathione *S*-transferase; GFP, green fluorescent protein; NCE, neutral cholesterol esterase; ALBP, adipocyte lipid-binding protein.

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lipofectin reagent (GIBCO BRL, Gaithersburg, MD); ECL Western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, [^{35}S]methionine (Amersham Life Sciences Products, Arlington Heights, IL); nitrocellulose paper (Schleicher and Schuell, Keene, NH); TNT transcription/translation system (Promega, Madison, WI); S*f*9 cells, TMN-FH insect medium, baculovirus transfer vector pAcGHLT-A, BaculoGold linearized baculovirus DNA (PharMingen, San Diego, CA); Ni−NTA agarose (Qiagen, Valencia, CA); anti-phosphoserine IgG (Zymed Labs Inc., South San Francisco, CA). All other chemicals were obtained from standard commercial sources.

Sucrose Gradient Centrifugation. Epididymal fat pads isolated from adult, male, Sprague−Dawley rats were homogenized in 20 mM Tris−HCl (pH 7.4), 1 mM EDTA, and 8% sucrose with 1 unit/mL leupeptin and then centrifuged at 100000*g* for 30 min. An aliquot of the supernatant (200 μL) was layered on top of a linear 5−45% (w/w) sucrose gradient (5 mL, prepared in 20 mM Tris−HCl (pH 7.4) and 1 mM EDTA). The tubes were centrifuged in a SW 50.1 rotor at 50000 rpm for 4 h. Samples were collected from the bottom of the tube in 20−22 fractions. Sucrose concentration in each aliquot was measured using a refractometer (Bausch & Lomb, Rochester, NY). Aliquots of each fraction were assayed for neutral cholesteryl ester hydrolase activity, as well as for catalase (11.6 S, MW $\approx$ 250 000), glyceraldehyde-3-phosphate dehydrogenase (7.7 S, MW $\approx$ 100 000), and alcohol dehydrogenase (4.5 S, MW $\approx$ 40 000), which served as internal markers (*18*).

Chemical Cross-Linking. Chemical cross-linking was performed using the photosensitive cross-linking system described by Fancy and Kodadek (*19*), where photolysis of ruthenium(II) trisbipyridyl dication (Ru(II)bpy$_3^{2+}$) in the presence of ammonium persulfate is used as a method to generate reactive intermediates that can cross-link associated proteins efficiently. An aliquot (200 μL) of the 100000*g* supernatant of extracts of rat adipose tissue was separated by sucrose gradient centrifugation. The fractions containing the presumed dimeric and monomeric forms of HSL were identified and incubated in 15 mM sodium phosphate (pH 7.5), 150 mM NaCl, and 0.125 mM Ru(II)bpy$_3$Cl$_2$. Ammonium persulfate (2.5 mM) was added to the incubation, and the tubes were exposed to light from an ordinary flashlight for varying lengths of time. The reaction was terminated by adding 2 × SDS−PAGE sample buffer (0.33 M Tris−HCl (pH 6.8), 2.7% SDS, 40% glycerol, and 2% β-mercaptoethanol). Samples were then electrophoresed on 4% SDS−PAGE under reducing conditions and immunoblotted with anti-rat HSL/fusion protein IgG.

Cell Culture and Transfection. COS cells were grown in 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 2 × 10^5 cells/well in six well plates the day prior to incubation with 1 μg of pEGFP-C1−HSL and pcDNA3-His-HSL in 10 μL of lipofectin reagent. Cells were harvested 40 h after transfection for measurement of both HSL and GFP activities.

Plasmid Construction. For pAcGHLT-A-HSL, full-length rat HSL cDNA was excised from pcDNA3-HSL (*20*) with HindIII/*Xba*I and blunt ended with Klenow and then cloned into the *Sma*I site of pAcGHLT-A containing a GST and 6 × His tag. For pcDNA3-His-HSL, full-length rat HSL cDNA was blunt end cloned into the *Eco*RI site of pcDNA3-HisA vector (Invitrogen, Carlsbad, CA). For pEGFP-C1−HSL, full-length rat HSL cDNA was excised from pcDNA3-HSL with HindIII/*Xba*I and cloned into the *EcoR*I/*Hind*III site of pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). Truncations of HSL were generated by cutting pcDNA3-HSL with *Eco*RI to produce HSL 1−625, with *Bam*HI to produce HSL 1−469, and with *Nhe*I to produce HSL 1−300; HSL 300−767 was produced by removing the HindIII−*Nhe*I fragment from pcDNA3-HSL, followed by religation using a HindIII/ATG/*Nhe*I adaptor, as described previously (*20*).

Insect Cell Culture and Production of Recombinant HSL. S*f*9 cells, derived from pupal ovarian tissue of *Spodopetera frugiperda*, were maintained as a monolayer culture in TMN-FH insect medium supplemented with gentamicin sulfate (50 μg/mL) and fungizone (2.5 μg/mL) at 27 °C. Purified recombinant baculovirus transfer plasmid containing HSL (pAcGHLT-A-HSL, 5 μg) was cotransfected into S*f*9 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 (as suggested by the manufacturer), and the virus was reamplified to a final titer of 1.5 × 10^7 pfu/mL. To produce recombinant proteins, S*f*9 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 3 days after infection. Production of GST−HSL fusion protein by the recombinant virus was assessed by immunoblotting cell extracts with anti-HSL antibodies and by assaying HSL activity.

In Vitro Translation and Protein−Protein Binding. Full-length rat HSL cloned into pcDNA3 vector (Invitrogen, Carlsbad, CA) was in vitro translated with [^{35}S]methionine using the TNT transcription/translation system as described previously (*20*). HSL was produced as a fusion protein with GST in the baculovirus system as described above. Extracts (100 μg) of S*f*9 cells infected with recombinant baculovirus that contains GST−HSL or GST alone were incubated with glutathione−agarose beads in buffer B (20 mM Tris (pH 8.0), 0.15 M NaCl, 1 mM EDTA, 0.5% NP40). After 1 h of incubation at room temperature, the beads were washed three times in buffer B, and then incubated with [^{35}S]methionine-labeled HSL. After 1 h of incubation at room temperature, the beads were washed five times in buffer B and proteins that were bound to the beads were then eluted in SDS−PAGE sample buffer, separated on 10% SDS−PAGE, and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Other Measurements. Measurement of HSL activity was performed using a cholesterol[^{14}C]oleate emulsion as described previously (*21*). GFP activity was determined by incubating lysates of COS cells transfected with pEGFP-C1-HSL and pcDNA3-His-HSL with Ni−NTA agarose in 1 mL of phosphate-buffered saline. After 1 h of incubation at 4 °C, the agarose beads were washed five times with phosphate-buffered saline, and then eluted with 0.1 M imidazole. An aliquot (100 μL) was assayed in a model 450 fluorometer (Barnsted/Thermolyne, Dubuque, IA) with an excitation of 460 nm and emission of 500 nm. A GFP standard curve was generated using COS cells that were transfected with pEGFP. Immunoreactive HSL was detected following electrophoresis of samples on 10% SDS−PAGE

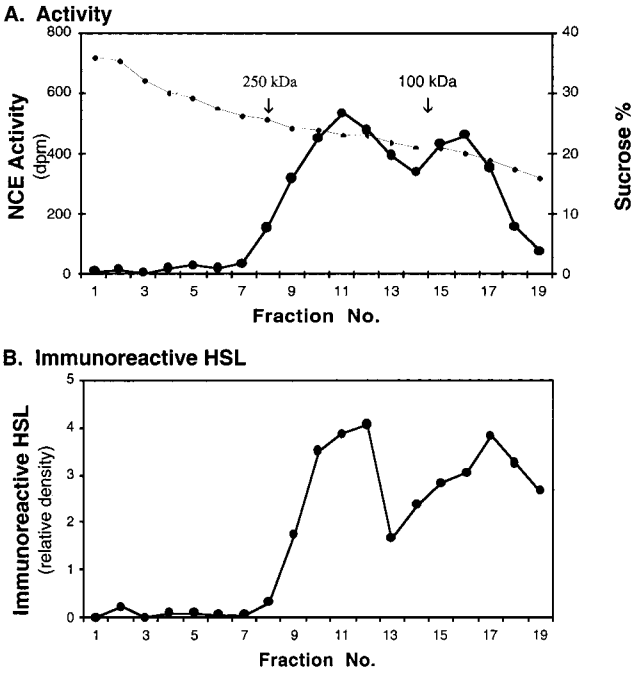

FIGURE 1: Fractionation of HSL activity from rat adipose tissue by sucrose gradient centrifugation. Extracts of rat adipose tissue were layered on top of a linear 5−45% (w/w) sucrose gradient and centrifuged at 230000*g* for 4 h as described in the Experimental Procedures. Sequential fractions were collected from the bottom of the tube. Panel A: Fractions were assayed for neutral cholesterol esterase (NCE) activity and sucrose concentration as described in the Experimental Procedures. Arrows denote the migration of molecular weight markers. Panel B: Fractions were immunoblotted with anti-HSL antibodies as described in the Experimental Procedures, and the relative intensity of the HSL band was plotted. The results are representative of four separate experiments.

under reducing conditions, transfer to nitrocellulose, incubation with anti-rat HSL/fusion protein IgG, and visualization by chemiluminescence, as described previously (*22*). Immunoprecipitation of HSL was performed using an immunomatrix consisting of rabbit polyclonal anti-HSL/fusion protein IgG and protein A, as described previously (*20*). Phosphorylation of HSL was detected by resuspending the immunoprecipitated HSL complex in 0.063 M Tris−HCl (pH 6.8) containing 1% β-mercaptoethanol, 1% SDS, and 13% glycerol, followed by electrophoresis on 8% polyacrylamide gels containing 0.1% SDS, transfer to nitrocellulose paper, and immunoblotting with rabbit anti-phosphoserine IgG.

Statistical Analysis. Data are expressed as the mean ± SEM. Statistical analyses were performed by analysis of variance and comparisons among groups by Bonferroni/Dunn using StatView software (ABACUS Concepts, Berkeley, CA) on a Power Macintosh computer.

RESULTS

Fractionation of HSL Species by Sucrose Gradient Centrifugation. As the initial step to evaluate whether HSL exists as a dimer, the cytosolic fraction from rat adipose tissue was layered on top of a linear sucrose gradient (5−45%) and subjected to centrifugation (Figure 1). Fractions were collected from the bottom of the tube and assayed for HSL activity by measuring neutral cholesterol esterase activity (panel A) to avoid any confounding interference from other lipases that possess neutral triacylglycerol hydrolase activity.

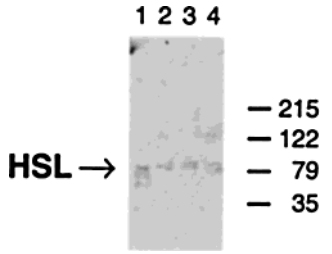

FIGURE 2: Phosphorylation of monomeric and dimeric HSL. Fractions containing the presumed monomeric (lanes 1 and 2) and dimeric (lanes 3 and 4) forms of HSL were identified and immunoprecipitated with anti-rat HSL/fusion protein IgG. The immunocomplex was then separated on 8% SDS−PAGE and immunoblotted with anti-phosphoserine antibodies, as described in the Experimental Procedures.

HSL activity appeared in two populations, one with a peak activity in fraction 10, which has a calculated molecular weight of ~160 000, which is consistent with the size of a dimer of HSL, and another population with a peak activity at fraction 16, which has a calculated molecular weight of ~85 000, equivalent to the size of a monomer of HSL. In addition, immunoreactive HSL was detected with a pattern similar to that seen for neutral cholesterol esterase activity (panel B). Thus, by sucrose gradient ultracentrifugation HSL appears to exist as both a monomer and a dimer in extracts of adipose tissue.

To explore whether the monomeric and dimeric species of HSL differed in their degree of phosphorylation, pooled fractions containing either monomer or dimer isolated from the sucrose gradient ultracentrifugation were first immunoprecipitated with anti-HSL IgG, and the immunoprecipitated complex was then immunoblotted with anti-phosphoserine IgG (Figure 2). Interestingly, the anti-phosphoserine antibodies detected both monomeric and dimeric HSL species, suggesting that a portion of both monomeric and dimeric HSL is phosphorylated. However, only a single phosphorylated band was observed with monomeric HSL, while dimeric HSL appeared as a doublet. While it is possible that the doublet appearing in the fraction from the dimeric-sized HSL represents HSL and another serine phosphorylated protein of similar size which coprecipitated with anti-HSL antibodies, this seems unlikely since a similarly sized dimer is seen when baculovirus-produced recombinant HSL is separated by sucrose density centrifugation (data not shown). Thus, these data suggest that phosphorylation of HSL appears to be important in enabling dimerization to occur.

To explore whether the dimerization of HSL is associated with any differences in the kinetics of the enzyme, complete substrate concentration curves with cholesteryl ester were analyzed with pooled fractions containing either monomer or dimer (Figure 3). Both HSL dimer (panel A) and monomer (panel B) displayed saturation kinetics and had identical affinities for cholesteryl ester substrate (K_m = 2.2 μM). In contrast, the maximum hydrolysis of cholesteryl ester by HSL dimer was more than 40-fold greater than seen with the monomer ($p < 0.001$). When aliquots of the fractions used in the enzyme kinetic studies were immunoblotted with anti-rat HSL antibodies (panel C), it was apparent that similar amounts of immunoreactive HSL were utilized within the assays. Therefore, these results suggest that the dimerization of HSL is associated with an increased capacity for the hydrolysis of lipid substrate, without any alterations in the

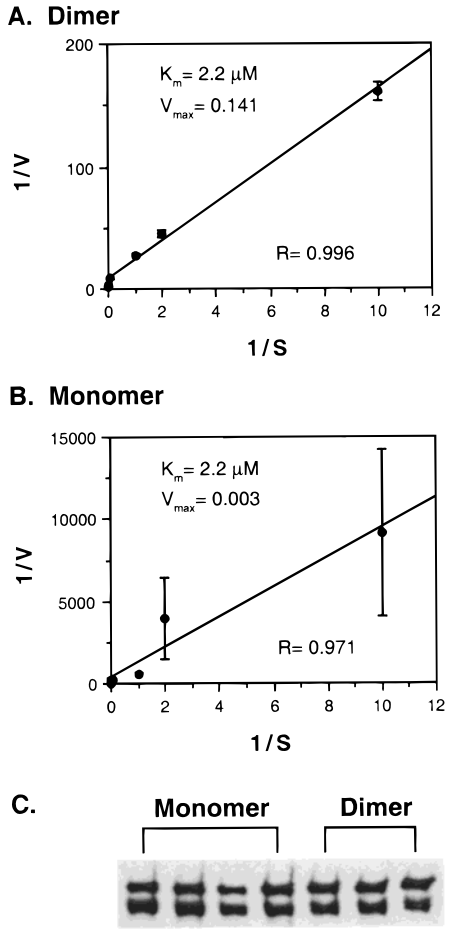

FIGURE 3: Kinetics of cholesteryl ester hydrolysis by monomeric and dimeric HSL. Panel A: Fractions corresponding to numbers 10 and 11 in Figure 1 were pooled and incubated with cholesteryl ester substrate in concentrations of 0.1, 0.5, 1, 10, 100, and 500 μM as described in the Experimental Procedures. The data were transformed by a double reciprocal plot to calculate V_{max} (nmol/h/mg of protein) and K_m (μM) values. R = correlation coefficient. The results are the mean ± SEM of four separate experiments. Panel B: Fractions corresponding to numbers 15 and 16 in Figure 1 were pooled and incubated with cholesteryl ester substrate in concentrations ranging from 0.1 to 500 μM as described in the Experimental Procedures. V_{max} (nmol/h/mg of protein) and K_m (μM) values were then calculated after linear transformation of the data. R = correlation coefficient. The results are the mean ± SEM of four separate experiments. Panel C: Immunoblot of HSL in samples used in panels A and B.

affinity of the enzyme for the substrate, and further suggest that active HSL preferentially exists as a dimer.

Chemical Cross-Linking of HSL Species. To confirm the dimerization of HSL, the cytosolic fraction from rat adipose tissue was separated by sucrose gradient centrifugation. The fractions containing the presumed dimeric and monomeric forms of HSL were identified and chemically cross-linked using a photosensitive cross-linking system with Ru(II)bpy_3^{2+} in the presence of ammonium persulfate (*19*). As shown in Figure 4, when the dimeric fraction was incubated with cross-linking reagent and exposed to light, two species of HSL were observed following separation under reducing conditions and immunoblotting with anti-rat HSL antibodies. One of the species had an MW of ~84 000, corresponding to a monomer of HSL, and the other had an MW of ~168 000, corresponding to the size of a homodimer of HSL. When cross-linking reagent or light (data not shown) was omitted

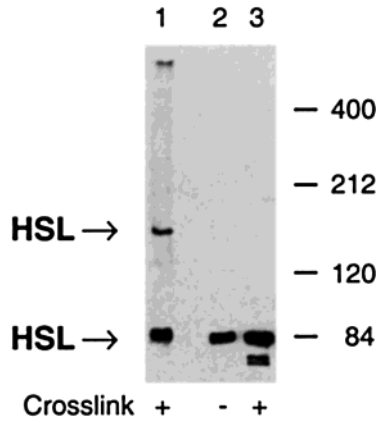

FIGURE 4: Chemical cross-linking of HSL. Extracts of rat adipose tissue were separated by sucrose gradient centrifugation. The fractions containing the presumed dimeric (lanes 1 and 2) and monomeric (lane 3) forms of HSL were identified and chemically cross-linked using Ru(II)bpy_3^{2+} in the presence of ammonium persulfate and exposure to light for 20 s, as described in the Experimental Procedures. Fractions were immunoblotted for immunoreactive HSL with anti-rat HSL/fusion protein antibodies, as described in the Experimental Procedures.

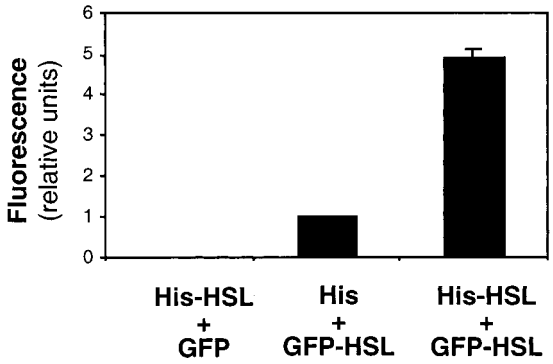

FIGURE 5: Oligomerization of HSL in vivo. COS cells were cotransfected with pcDNA3-His (vector alone) and pEGFP-C1 (vector alone) (column 1) or pcDNA3-His-HSL and pEGFP-C1 (vector alone) (column 2) or with pcDNA3-His-HSL and pEGFP-C1-HSL (column 3). Cell extracts were coupled with Ni−NTA agarose beads. Proteins that were bound to the beads were eluted and assayed for relative fluorescence.

from the reaction, only the MW 84 000 species was seen. Similarly, when the monomeric fraction was incubated with cross-linking reagent and exposed to light, only the MW ≈ 84 000 species was seen. Thus, the HSL subunits in the dimer are in sufficient proximity to allow cross-linking to occur, while monomeric HSL cannot be cross-linked under these conditions.

Oligomerization of HSL in Vivo. As another means to document the ability of HSL to self-associate to form oligomers, COS cells were transiently cotransfected with an HSL vector containing a 6 × His tag (pcDNA3-His-HSL) and with an HSL vector containing a GFP tag (pEGFP-C1-HSL). The His-tagged HSL protein in the cell extracts was bound to Ni−agarose beads and washed, and the proteins that were bound to the beads were eluted and assayed for fluorescence (Figure 5). In cells cotransfected with His-tagged HSL along with GFP vector alone, no fluorescence was detectable, while cells cotransfected with His vector alone along with GFP-tagged HSL displayed a low level of background fluorescence. In contrast, cells cotransfected with His-tagged HSL along with GFP-tagged HSL displayed a

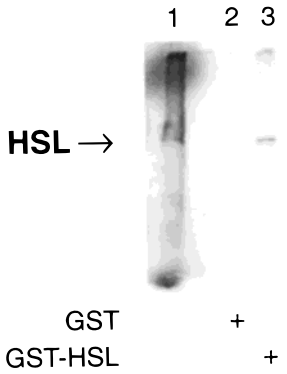

FIGURE 6: Interaction of GST−HSL with in vitro translated HSL. pcDNA3-HSL was in vitro translated with [^{35}S]methionine using the TNT transcription/translation system (lane 1). Extracts (250 μg) of S*f*9 insect cells transformed with pAcGHLT-A-HSL (GST−HSL, lane 3) or with pAcGHLT-A alone (GST, lane 2) were coupled with glutathione−agarose beads and then incubated with [^{35}S]methionine-labeled HSL as described in the Experimental Procedures. Proteins that were bound to the beads were eluted, separated on 10% SDS−PAGE, and visualized using a PhosphorImager.

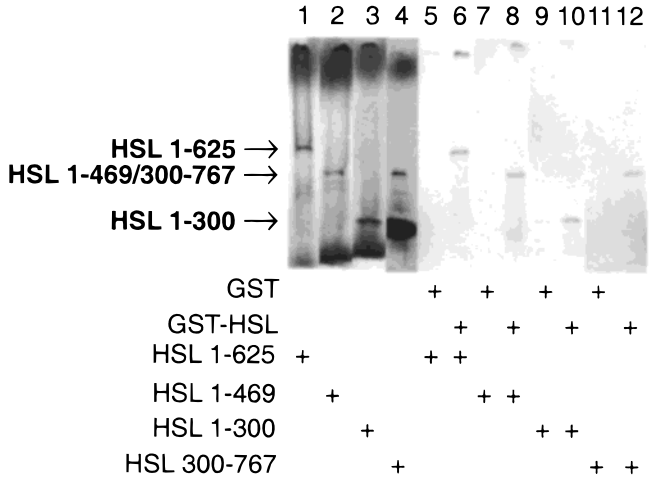

FIGURE 7: Identification of the region of HSL involved in oligomerization. Truncations of HSL were produced as described in the Experimental Procedures. pcDNA3-HSL 1−625 (lanes 1, 5, and 6), pcDNA3-HSL 1−469 (lanes 2, 7, and 8), pcDNA3-HSL 1−300 (lanes 3, 9, and 10), and pcDNA3-HSL 300−767 (lanes 4, 11, and 12) were in vitro translated with [^{35}S]methionine and incubated with GST (lanes 5, 7, 9, and 11) or GST−HSL (lanes 6, 8, 10, and 12) and with glutathione beads, as described in Figure 6. Proteins that were bound to the beads were eluted, separated on 10% SDS−PAGE, and visualized as described using a PhosphorImager.

5-fold increase in fluorescence above the background ($p < 0.001$). These results show that a GFP-tagged HSL fusion protein was able to interact specifically with a His-tagged HSL fusion protein, and that the interaction is dependent on the expression of HSL.

Oligomerization of HSL in Vitro. To verify the self-interaction of HSL, the ability of baculovirus-produced rat GST−HSL fusion protein to interact with HSL that was [^{35}S]methionine-labeled by in vitro translation was examined (Figure 6). Lane 1 shows the migration of the ~84 kDa in vitro translation product of pcDNA3-HSL alone. When [^{35}S]methionine-labeled HSL and GST−HSL were incubated with glutathione−agarose beads and the proteins that were bound to the beads were washed, eluted, and separated on SDS−PAGE, labeled HSL was seen (lane 3). However, when GST was substituted for GST−HSL, no labeled HSL was brought down with the glutathione−agarose beads (lane 2). The results from this additional, independent method confirm that HSL is capable of interacting with itself.

To determine the region within HSL that is important for its interaction with itself, we performed a series of deletions of HSL and tested the ability of the [^{35}S]methionine-labeled in vitro translation products of the truncated HSLs to interact with full-length GST−HSL. As shown in Figure 7, [^{35}S]methionine-labeled truncations of HSL and GST alone or GST−HSL were incubated with glutathione−agarose beads, and the proteins that were bound to the beads were washed, eluted, and separated on SDS−PAGE. As the C-terminal portion of HSL is removed from the 767 aa intact protein to HSL 1−625 (translation product MW $\approx$ 68 000), to HSL 1−469 (translation product MW $\approx$ 51 000), and to HSL 1−300 (translation product MW $\approx$ 33 000), the truncated portion of HSL continues to be able to interact specifically with GST−HSL, while no interaction is observed with GST alone. From these data it appears that the first 300 amino acids of HSL are important for the oligomerization of HSL. To determine whether the first 300 amino acids of HSL are exclusively responsible for oligomerization, an HSL construct lacking the initial 300 amino acids (HSL 300−767, translation product MW $\approx$ 51 000) was in vitro translated and its ability to interact with full-length GST−HSL was examined. HSL 300−767 was able to interact specifically with GST−HSL, while no interaction was observed with GST alone, suggesting that HSL oligomerization might occur via an interaction of its N- and C-terminal regions.

DISCUSSION

Initial studies with HSL detergent purified from rat adipose tissue suggested that, on the basis of its behavior on gel chromatography, the enzyme may exist as a dimer; however, the results were also compatible with an enzyme monomer contained within a detergent micelle (*3*). In the current study we provide several lines of evidence to demonstrate that HSL in adipose tissue exists as functional oligomers composed of homologous subunits. First, two populations of HSL were detected from nondetergent extracts from adipose tissue separated by sucrose gradient centrifugation, one with the size of a monomer and one with a size compatible with a homodimer. This finding is consistent with a recent report showing that the sedimentation equilibrium of purified, recombinant HSL conforms to that of a homodimer (*23*). Second, the dimer could be chemically cross-linked and isolated, while the monomer did not form larger species when incubated under cross-linking conditions. The appearance of both monomeric and dimeric HSL following cross-linking of the dimeric species suggests either that the cross-linking reaction was not completely efficient or that the monomer and dimer are in equilibrium, with only the dimer capable of being cross-linked. Third, the cotransfection of COS cells with two different HSL constructs, one containing a His tag and one tagged with GFP, directly demonstrated a noncovalent self-association of HSL in vivo. Fourth, in vitro translated HSL was shown to interact specifically with GST-tagged HSL in vitro. Fifth, truncations of HSL identified the importance of the N-terminal 300 amino acids, as well as amino acids 300−767, in participating in the oligomerization of HSL.

Using limited proteolysis, it has been suggested that HSL is composed of two major structural domains (*11*, *23*). On the basis of sequence alignment, structural homology with fungal lipases, and mutational analyses (*12*, *21*), the C-terminal domain has been shown to contain the catalytic triad and other residues important in hydrolytic activity, as well as a 150 aa insert that has been termed the regulatory module because several serines located within this region have been shown to be phosphorylated (*21*, *24*). The N-terminal domain in rat HSL constitutes the first 323 amino acids, which are encoded by exons 1−4 and which display no sequence or structural similarity with any other known proteins (*11*, *23*). The function of this N-terminal region of HSL has not been clearly defined. However, we have recently shown that HSL interacts specifically with adipocyte lipid-binding protein (ALBP) through this N-terminal domain, and on the basis of that observation, we proposed that the N-terminal 300 aa region represents a docking domain for the interaction of HSL with ALBP (*20*). Our current findings that HSL self-associates to form oligomers and that the N-terminal 300 amino acids are important in participating in this oligomerization permit this concept to be extended more generally to propose that the N-terminal region of HSL represents a docking domain for protein−protein interactions. Whether the same amino acids within this region are responsible for HSL oligomerization and for its interaction with ALBP is currently unknown and will require additional studies. However, it seems apparent that in contrast to its interaction with ALBP, where an HSL construct lacking the initial 300 amino acids (HSL 300−767) was unable to interact with ALBP (*20*), HSL 300−767 was able to interact with full length HSL, suggesting that regions in addition to the N-terminal 300 amino acids are important in HSL oligomerization. The identity of the sequences within the 300−767 region that are important in oligomerization remain to be determined, but the fact that dimeric HSL appeared as a phosphorylated doublet while monomeric HSL appeared as a single phosphorylated band raises the possibility that N-terminal amino acids might interact with portions of the regulatory module.

A functional significance for the oligomerization of HSL is suggested by the differences in enzyme activity observed between the species. Dimeric HSL displayed approximately 40-fold greater activity against cholesteryl ester and triacylglycerol (data not shown) substrates when compared with monomeric HSL without any differences in affinity for the substrate. Even though dimeric HSL appeared to be more highly phosphorylated than monomeric HSL, this difference in phosphorylation alone is unlikely to explain the marked differences in activity between the monomer and dimer since phosphorylation of HSL in vitro is generally associated with only a 2-fold increase in activity. This observation suggests the intriguing possibility that HSL activity is posttranslationally regulated via control of oligomerization of the protein. Furthermore, this is particularly interesting in light of the disparity between the 2-fold increase in HSL activity when phosphorylated in vitro compared to the 50−100-fold stimulation of lipolysis observed in intact fat cells. This discrepancy has been explained by the translocation of phosphorylated HSL from an aqueous cytosolic compartment to the lipid droplet (*25*, *26*). It has been suggested that translocation or exposure of the lipid droplet to HSL is facilitated by the interaction of HSL with specific cellular proteins, such as perilipin (*27*, *28*) or adipocyte differentiation-related protein (*29*, *30*). Indeed, a novel docking protein, lipotransin, which possesses intrinsic ATPase activity, has recently been identified to potentially direct HSL distribution (*31*). Our current observations suggest another potential mechanism; i.e., upon stimulation of cells by lipolytic agents, HSL might self-associate to form a larger species, which possesses dramatically increased hydrolytic efficiency. HSL phosphorylation or the phosphorylation of another protein(s) that interacts with HSL could potentially control oligomerization. Moreover, the extent of oligomerization might possibly influence translocation. Further experiments will be required to explore these possibilities.

In conclusion, our results show that HSL functions as a dimer, that the hydrolytic efficiency of the enzyme is greater as a dimer than as a monomer, and that the N-terminal region, as well as other regions, is important in mediating oligomerization. Thus, the data from these studies support the notion that the N-terminal region of HSL represents a docking domain for protein−protein interactions and provide additional information on the structure−function relationship of HSL. Furthermore, our findings provide an additional mechanism for the posttranslational control of HSL activity in the cell via oligomerization and, therefore, add a new layer of complexity toward understanding the mechanisms regulating lipolysis.

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