Mutational Analysis of Structural Features of Rat Hormone-Sensitive Lipase†

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ABSTRACT: Hormone-sensitive lipase (HSL) is a cytosolic neutral lipase that hydrolyzes intracellular stores of triacylglycerols and cholesteryl esters. HSL activity is regulated via phosphorylation−dephosphorylation, with cyclic AMP-dependent protein kinase increasing activity following phosphorylation of a single serine and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylating another serine at a basal site. The current studies used site-directed mutagenesis to show that Ser-563 of rat HSL is phosphorylated by cyclic AMP-dependent protein kinase and that Ser-565 is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II. Mutation of Ser-563→Ala eliminated HSL hydrolytic activity against cholesteryl ester, triacylglycerol, and diacylglycerol substrates to the same extent as mutation of Ser-423→Ala, the presumed catalytic site. Mutation of Ser-565→Ala modestly decreased HSL activity. In contrast, mutation of Ser-563→Asp preserved HSL hydrolytic activity and even increased activity 20% above the control wild-type enzyme. Molecular modeling of the catalytic pocket of HSL suggested the involvement of Val-710. Mutation of Val-710→Ala resulted in an 85% loss of HSL hydrolytic activity. The results of these studies illustrate the importance of the presence of a hydroxyl group or negative charge at residue 563, either for proper conformation of rat HSL or for proper stabilization of substrate to allow maintenance of hydrolytic activity, as well as the importance of the involvement of additional amino acids in the catalytic pocket of the enzyme.

Hormone-sensitive lipase (HSL)[1] is a cytosolic neutral lipase that hydrolyzes intracellular stores of triacylglycerols in adipose tissue (*31*). As its name implies, hormones, such as catecholamines, ACTH, and glucagon, stimulate the activity of HSL (*27*). While HSL appears to be the rate-limiting enzyme for the mobilization of free fatty acids in adipose tissue through its ability to catalyze the hydrolysis of triacylglycerol to diacylglycerol, it has broader substrate specificity, also catalyzing the hydrolysis of diacylglycerol and 1,(3)-monoacylglycerol (*6*). In addition, HSL is capable of mediating the hydrolysis of cholesteryl esters in steroidogenic tissues, such as adrenal and ovary (*3, 4*), and in macrophages (*12*), as well as the hydrolysis of other substrates, such as lipoidal esters of steroid hormones (*15*) and retinyl esters in adipose tissue (*29*).

HSL activity against triacylglycerol and cholesteryl ester substrates appears to be rapidly modulated via phosphorylation−dephosphorylation. Cyclic AMP-dependent protein kinase activates HSL following the phosphorylation of a single site (*25*) that was identified as serine 563 in rat HSL (*7*). In addition to cyclic AMP-dependent protein kinase, other kinases such as glycogen synthase kinase-4 (*20*), Ca^{2+}/calmodulin-dependent protein kinase II, and AMP-activated protein kinase (*8*) phosphorylate HSL at a secondary basal site, serine 565 in rat HSL (*9*), which impairs the phosphorylation of serine 563 by cyclic AMP-dependent protein kinase (*9*). HSL activity can also be reversibly regulated by protein phosphatases. The most active phosphatases against serine 563 have been shown to be phosphatase 2A and 2C, while serine 565 is predominately dephosphorylated by phosphatase 2A (*19, 30*).

The primary sequence of HSL is unrelated to any of the other known mammalian lipases; however, it shares some sequence homology with lipase 2 of an antarctic bacterium, *Moraxella* TA144 (*14*). Sequence analysis and comparison with lipase 2 from *Moraxella* TA144 located a G-X-S-X-G motif which was proposed (*10*), and later confirmed by mutagenesis (*11*), to contain the catalytically active serine at position 423 in rat HSL. Alignment of HSL and lipase 2 from *Moraxella* TA144 also suggested that the regulatory and basal phosphorylation sites of HSL appear to be contained within a 150 amino acid insertion and that HSL might be distantly related to other lipases and esterases, such as acetylcholinesterase (*10*). Together with limited proteolysis and denaturation studies with recombinant HSL (*22*), it was suggested that the C-terminal portion of HSL, similar to several lipases with known structure, is composed of α/β-hydrolase folds that accommodate the catalytic site. In an attempt to develop a structural model for HSL, Contreras et al. (*2*) observed that, even in the absence of primary sequence homology, the organization of the secondary structure predicted for human HSL was similar to the secondary structure of acetylcholinesterase and of two fungal lipases from *G. candidum* and *C. rugosa*. This proposed model predicted that Ser-423, Asp-703, and His-733 constitute the

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[1] Abbreviations: HSL, hormone-sensitive lipase; CHO, chinese hamster ovary; PKA, cyclic AMP-dependent protein kinase; CaMK, Ca^{2+}/calmodulin-dependent protein kinase II; CE, cholesteryl ester; TG, triacylglycerol; DG, diacylglycerol.

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catalytic triad for HSL, and site-directed mutagenesis of Asp-703 and His-733 appears to support this conclusion (*21*). To facilitate the understanding of the regulation of HSL activity through phosphorylation, we have made point mutations at Ser-563 and Ser-565, as well as double mutations at both of these two serine residues, in rat HSL. We have also mutated an additional site that might be involved in catalysis.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following sources: bovine serum albumin (fraction V) (Intergen Co., Purchase, NY); sodium deoxycholate, Triton X-100, 10 tridecyl ether, L-α phosphatidylcholine, cholesteryl oleate, trioleolylglycerol, dioleolylglycerol, leupeptin, aprotinin, anti-rabbit IgG FITC conjugate, G418, cyclic AMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO); cholesteryl[1-^{14}C]oleate, glycerol tri[9,10(*n*)-^{3}H]oleate, phosphatidylcholine, L-α dioleolyl [dioleolyl-1-^{14}C] (E. I. Dupont de Nemours and Co., Boston, MA); fetal bovine serum (Gemini Bio-Products, Inc., Calabasas, CA); Coon's F12/Dulbecco's Modified Eagles media, lipofectin reagent, Ca^{2+}/calmodulin-dependent protein kinase II (GIBCO BRL, Grand Island, NY); ECL western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, [γ-^{32}P]-ATP (Amersham Life Sciences Products, Arlington Heights, IL); nitrocellulose paper (Schleicher and Schuell, Keene, NH). All other chemicals were obtained from standard commercial sources.

Plasmid Construction and Site-Directed Mutagenesis. Rat HSL cDNA was excised from pCEP4−HSL vector (*26*) with *Hin*dIII and *Xba*I and cloned into pcDNA3 (Stratagene). The resulting plasmid, pcDNA3−HSL, was used in the site-directed mutagenesis of all the HSL mutants. Mutagenesis of serine residues at 423, 563, and 565, as well as the double mutation of Ser-563/Ser-565, to alanine was carried out using the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). To facilitate the selection of positive mutants, an additional, neutral base-pair alteration, which either introduced or eliminated a restriction enzyme site in the cDNA while not affecting the normally encoded amino acid, was incorporated into the primers in the mutagenesis design. For mutagenesis of Ser-423 to Ala, the oligonucleotide (CCTTGCGGGGGACGCCGCAGGTGGGAACCTC) also introduced a novel Sac II site. For Ser-563 to Ala (CTATGCGCAGGGCTGTGTCTGAGGCAGCCC), Ser-565 to Ala (CTATGCGCAGGAGTGTGGCTGAGGCAGCCC), and Ser-563/Ser-565 to Ala/Ala (CTATGCGCAGGGCTGTGGATGAGGCAGCCC), the oligonucleotides also eliminated a Fsp I site. Mutagenesis of Ser-563 to Asp and Val-710 to Ala was carried out using the Quick Change Mutagenesis Kit (Stratagene). The oligonucleotides for mutagenesis of Ser-563 to Asp (GAGTCTATGCGGAGGGATGTGTCTGAGGCAGCCCTG) also eliminated a Fsp I site, while mutation of Val-710 to Ala (CCCATGCTGGATGATTCGGCCATGTTCGAGCGGCGA) also eliminated a Tth 111I site. All constructs were sequenced to confirm the desired mutations and to ensure that no additional mutations had been introduced.

Cell Culture and Transfection. Chinese Hamster Ovary (CHO) cells were grown in Coon's F12/Dulbecco's Modified Eagles media containing 1 gL glucose (50/50) and supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. To generate stably transfected cell lines, CHO cells were grown in 25 mL tissue culture flasks to 80% confluent and incubated with 10 μg of plasmid DNA in 10 μL of lipofectin reagent. After 48 h of incubation, a selection medium composed of Coon's F12/Dulbecco's Modified Eagles media supplemented with 10% FCS and G418 (500 μg/mL) was applied, and cells were maintained in this media thereafter. Before reaching confluence, antibiotic-resistant cells were trypsinized and plated at a cell density of 5 cells per well in 96 multiwell dishes. Screening for HSL expression was then performed by immunofluorescence staining using anti-rat HSL/fusion protein antibodies (*26*). Cells expressing HSL were subsequently subcloned at a cell density of 1 cell per 5 wells of a 96 multiwell dish to isolate clonal cell lines. For transiently transfected cells, CHO cells were seeded at a density of 2×10^5 cells/well in 35 mm dishes. The next day, cells were incubated with 1.8 μg of plasmid DNA along with 0.2 μg of control plasmid (pRSVB−gal) in lipofectin reagent. Cells were harvested 40 h after transfection for determination of both HSL and β-galactosidase activity (*18*).

Immunoblotting. Cells were scraped and briefly sonicated (3 s) in 1 mL of ice-cold lysis buffer containing 0.15 M NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, and 1 unit/mL leupeptin. Homogenates were centrifuged at 10000*g* for 15 min, and the supernatants were taken for electrophoresis. Samples were electrophoresed on 10% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, incubated with anti-rat HSL/fusion protein IgG, and visualized by chemiluminescence as described previously (*13*).

Measurement of HSL Activity. Measurement of HSL activity was performed using cholesteryl ester, triacylglycerol, and diacylglycerol substrates. Cells were scraped into 1 mL of 50 mM Tris-HCl and 1 mM EDTA containing 1 unit/mL leupeptin and homogenized. After centrifuging the homogenates at 14000*g* for 15 min, the supernatants were removed, and aliquots (50−100 μL) were assayed in duplicate (*13*). Substrate for the cholesteryl ester assay was prepared by adding 1.25 μCi of cholesteryl [1-^{14}C]oleate (purified by thin-layer chromatography), 0.043 mmol of phosphatidylcholine, and 0.011 mmol of cholesteryl oleate into 3 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM sodium taurocholate. The substrate solution was vortexed and then sonicated for 3 h with a Branson Sonifier/Cell Disruptor model W-350 on an output setting of 5.0 (50%). The substrate was centrifuged at 3000 rpm for 15 min to remove metallic fragments released by the probe and stored under nitrogen at 4 °C for up to one week. Substrate for the triacylglycerol assay was prepared identically except for the substitution of glycerol tri[9,10(*n*)-^{3}H]-oleate and trioleolylglycerol for cholesteryl [1-^{14}C]oleate and cholesteryl oleate, respectively, in the same molar amounts. Substrate for the diacylglycerol assay was prepared by generating glycerol di[1-^{14}C]oleate from phosphatidylcholine, L-α dioleolyl [dioleolyl-1-^{14}C] by treatment with phospholipase C and purification on thin-layer chromatography (*16*). The glycerol di[1-^{14}C]oleate was mixed with dioleolylglycerol, phosphatidylcholine, and sodium taurocholate in phosphate buffer as described above and sonicated for 4×30 s bursts. Aliquots of supernatant protein adjusted to 100 μL

with buffer to contain equivalent amounts of HSL protein were mixed with 140 μL of 0.05% bovine serum albumin in 100 mM potassium phosphate, pH 7.0. After the addition of 10 μL of substrate (0.1−500 μM), the assay was carried out at 37 °C for 60 min. The reaction was stopped by addition of chloroform:methanol:heptane (250:230:180). After the addition of borate/carbonate buffer (0.1 M, pH 10.5), the tubes were vortexed and centrifuged, and aliquots of the upper phase were taken for liquid scintillation counting in a Beckman scintillation counter. The results are expressed in nmol of cholesteryl oleate or trioleolylglycerol or dioleolylglycerol hydrolyzed/mg of protein.

In Vitro Phosphorylation. CHO cells that were stably transfected with different HSL mutants were grown in COON's F12/Dulbecco's Modified Eagles media until confluent, and then harvested in lysis buffer (0.15 M NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, 1 mM PMSF, 1 U/mL leupeptin, 0.2 mg/mL aprotinin, and 5 mM NaF). After a brief sonication, the cells were centrifuged at 14000*g* for 10 min at 4 °C. For in vitro phosphorylation, 50 μg of cell lysate were mixed with reaction buffer [3.33 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 2 mM DTT, 0.1% 10 tridecyl ether, 0.2 mM [γ-^{32}P]-ATP], and 180 unit/mL of either cyclic AMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase II was added to start the reaction. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding an excess amount of ice-cold 10 mM EDTA and 2 mM DTT. The volume was adjusted to 200 μL with lysis buffer and precleared with 20% Pansorbin. The supernatants were then incubated with rabbit anti-rat HSL/fusion protein IgG (0.5 μg/mL) overnight at 4 °C. The immune complexes were precipitated with 25% Pansorbin, and the pellets were collected and washed twice in 0.5 M LiCl, 0.1 M Tris-HCl (pH 7.5) and twice in phosphate-buffered saline (pH 7.4) with 0.1% (w/v) *N*-lauryl sarcosine at 4 °C. The final pellets were redissolved in 2% SDS, 0.1M Tris-HCl (pH 7.5), and the mixture was boiled for 5 min to facilitate resuspension. The resultant solutions were adjusted to 15 mM Tris-HCl (pH 7.5), 1 M NaCl, 1% (w/v) *N*-lauryl sarcosine, and 3% (v/v) Triton X-100 and incubated a second time overnight with 10 μg/mL rabbit anti-rat HSL/fusion protein IgG at 4 °C (*13*). The immune complexes were precipitated with 40% Pansorbin and the pellets were washed twice in 0.5 M LiCl, 0.1 M Tris-HCl (pH 7.5), followed by two more washes with phosphate-buffered saline (pH 7.5) and 0.1% (w/v) *N*-lauryl sarcosine. The final pellets were resuspended in 0.63 M Tris-HCl (pH 6.8) containing 8 M urea, 1% β-mercaptoethanol, 1% SDS, and 13% glycerol, boiled for 5 min, and electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. Autoradiographs were visualized in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Computer Modeling of HSL Structure. The Predictprotein PHD program from the European Molecular Biology Laboratories was used to predict the secondary structure of the proteins, and sequences were aligned with the esterase from *C. rugosa* according to the similarity of their secondary structures. The atomic coordinates of *C. rugosa* lipase were obtained from the Brookhaven Protein Data Bank and visualized on a Silicon Graphics workstation with the molecular modeling program X-LOOK (*17*). HSL protein structure was predicted using the homologous modeling function in the X-LOOK program, which performs comparative modeling according to homologous alignment with automatic refinement for satisfaction of spatial restraints.

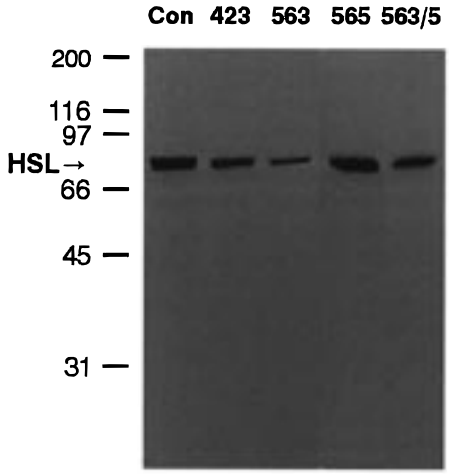

FIGURE 1: Immunoblot of HSL expression in stably transfected CHO cells. CHO cells were transfected with pcDNA3−HSL (control) and pcDNA3−HSL in which the HSL contains Ser-423→Ala (423), Ser-563→Ala (563), Ser-565→Ala (565), or Ser-563→Ala/Ser565→Ala (563/5) mutations, selected by G418 resistance, and stably transfected cell lines were cloned as described in Experimental Procedures. Cell extracts (25 μg) were electrophoresed on SDS−PAGE, transferred to nitrocellulose filters, immunoblotted with anti-HSL/fusion protein IgG, and visualized by enhanced chemiluminescence. The film was developed after a 10 s exposure.

Statistical Analysis. Data are expressed as 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

Establishment of Cell Lines That Stably Express Different HSL Mutants. Mutation of serines at 423, 563, and 565 to Ala in rat HSL was carried out and, after confirmation by sequencing, CHO cells were stably transfected with the different HSL mutant constructs, as well as native HSL. Clones expressing Ser-423→Ala, Ser-563→Ala, Ser-565→Ala, Ser-563→Ala/Ser-565→Ala, or normal HSL were identified by immunostaining using anti-rat HSL/fusion protein antibody, and the expression of HSL was confirmed by immunoblotting. As shown in Figure 1, anti-rat HSL/fusion protein antibody recognized a prominent protein of 84 kDa, the size of intact rat HSL (*13*), in cell extracts of each of the clones. To characterize the effects of the mutations on the phosphorylation properties of HSL, the ability of either cyclic AMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase II to phosphorylate HSL in vitro was examined (Figure 2). As displayed in panel A of Figure 2, cyclic AMP-dependent protein kinase stimulated the incorporation of [^{32}P]-PO_4 into normal HSL, which was not observed when the kinase was omitted from the incubation. Mutation of Ser-563→Ala as a single mutation (563), or as a double mutation with Ser-565→Ala (563/5), abolished the ability of cyclic AMP-dependent protein kinase to phosphorylate HSL; however, the mutation of Ser-565→Ala (565) did not affect the ability of HSL to be phosphorylated normally by cyclic AMP-dependent protein kinase. These results are consistent with Ser-563 being the site phospho-

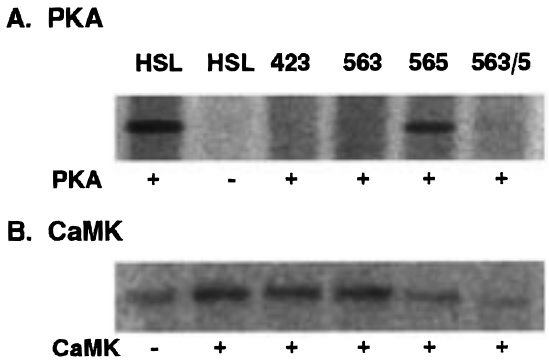

FIGURE 2: Phosphorylation of HSL by cyclic AMP-dependent protein kinase (panel A) or Ca^{2+}/calmodulin-dependent protein kinase II (panel B). Panel A: Cell lysates from CHO cells stably transfected with pcDNA3−HSL (control), Ser-423→Ala (423), Ser-563→Ala (563), Ser-565→Ala (565), or Ser-563→Ala/Ser565→Ala (563/5) mutations were incubated in 3.33 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 2 mM DTT, 0.1% 10 tridecyl ether, 0. 2 mM [γ-^{32}P]-ATP and in the presence or absence of 180 unit/mL of cyclic AMP-dependent protein kinase (PKA). Phosphorylated HSL was double immunoprecipitated, separated on SDS−PAGE, and autoradiographs visualized in a PhosphorImager. Panel B: Cell lysates were incubated as above in the presence or absence of 180 unit/ml of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK). Phosphorylated HSL was double immunoprecipitated and separated on SDS−PAGE, and autoradiographs were visualized.

rylated by cyclic AMP-dependent protein kinase (*7*). Interestingly, mutation of Ser-423→Ala (423) also failed to be phosphorylated by cyclic AMP-dependent protein kinase under these conditions, suggesting that this mutation might cause significant changes in the tertiary structure of HSL. As displayed in panel B of Figure 2, Ca^{2+}/calmodulin-dependent protein kinase II stimulated the incorporation of [^{32}P]-PO_4 into normal HSL; however, a small amount of basal phosphorylation was still observed when the kinase was omitted from the incubation. Ca^{2+}/calmodulin-dependent protein kinase II also stimulated the incorporation of [^{32}P]-PO_4 into Ser-423→Ala and Ser-563→Ala mutants of HSL. However, there was no increase in the phosphorylation of either the Ser-565→Ala or the Ser-563→Ala/Ser-565→Ala double mutant by Ca^{2+}/calmodulin-dependent protein kinase II, only the same low level of incorporation of [^{32}P]-PO_4 observed when the kinase was omitted from the incubation. These results are consistent with Ser-565 being a secondary phosphorylation site in HSL, but raise the possibility of an additional basal site for phosphorylation.

Activity of Mutant HSL. To explore the effects of the mutations on HSL activity, the relative activities of cell extracts of CHO cells expressing the various HSL mutants were determined using 100 μM cholesteryl ester, triacylglycerol, and diacylglycerol substrates (Figure 3). Consistent with its proposed function as the catalytic serine (*11*), mutation of Ser-423→Ala resulted in >99% loss of cholesteryl ester hydrolysis ($p < 0.0001$), >80% loss of triacylglycerol hydrolysis ($p < 0.0001$), and >90% loss of diacylglycerol hydrolysis ($p < 0.0001$). Surprisingly, mutation of Ser-563→Ala not only eliminated phosphorylation of HSL by cyclic AMP-dependent protein kinase, but also resulted in a similar loss of hydrolytic activity as seen with Ser-423→Ala, with >98, >80, and >90% loss of activity against cholesteryl ester, triacylglycerol, and diacylglycerol substrates, respectively ($p < 0.0001$). Mutation of Ser-565→Ala also decreased activity compared to wild-type

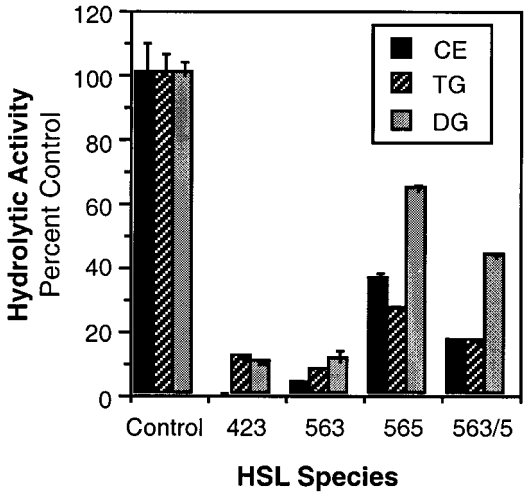

FIGURE 3: HSL activity in stably transfected CHO cells. Cell extracts from CHO cells stably transfected with pcDNA3-HSL (control), Ser-423→Ala (423), Ser-563→Ala (563), Ser-565→Ala (565), or Ser-563→Ala/Ser565→Ala (563/5) mutations were incubated with 100 μM cholesteryl ester (CE), triacylglycerol (TG), or diacylglycerol (DG) substrates as described in Experimental Procedures. Activity is expressed relative to control wild-type HSL which was set at 100. Results are the mean ± SEM of 9 wells for each HSL species for cholesteryl ester and diacylglycerol and 6 wells for triacylglycerol and are representative of 4 separate experiments.

Table 1: V_{max} and K_m Values of HSL Species[a]

	substrate					
	cholesteryl ester		triacylglycerol		diacylglycerol	
HSL species	V_{max} (nmol h^{-1} mg^{-1})	K_m (μM)	V_{max} (nmol h^{-1} mg^{-1})	K_m (μM)	V_{max} (nmol h^{-1} mg^{-1})	K_m (μM)
control	61.46	4.69	18.18	3.4	111.11	30.33
Ser-423	2.04	1.16	0	0	13.3	7.39
Ser-563	1.67	0.96	0	0	9.42	6.64
Ser-565	7.17	1.99	23.76	5.36	134.59	49.68
Ser-563/5	8.13	1.84	16.84	3.43	16.61	5.3

[a] Cell extracts from CHO cells stably transfected with pcDNA3-HSL (control), Ser-423→Ala, Ser-563→Ala, Ser-565→Ala, or Ser-563→Ala/Ser565→Ala mutations were incubated with cholesteryl ester, triacylglycerol, or diacylglycerol substrates in concentrations ranging from 0.1 to 500 μM as described in Experimental Procedures. V_{max} and K_m values were then calculated after linear transformation of the data.

HSL, with a 70, 35 and 30% loss of activity against cholesteryl ester, triacylglycerol, and diacylglycerol substrates, respectively ($p < 0.0001$); however, there was still substantially greater activity against all three substrates than observed with either Ser-423→Ala or Ser-563→Ala mutants ($p < 0.0001$). The double Ser-563→Ala/Ser565→Ala mutant displayed a loss of activity that was intermediate between the Ser-563→Ala and Ser-565→Ala single mutants.

Since the above studies were performed at a single substrate concentration, complete substrate concentration curves were analyzed with each of the mutants. V_{max} and K_m values for each substrate for the HSL mutants were calculated and are summarized in Table 1. Wild-type control HSL hydrolyzed ~3-fold more cholesteryl esters than triacylglycerol, and 11-fold more diacylglycerol, similar to previous results with purified rat HSL (*6*). The calculated V_{max} values for the different mutants paralleled the relative

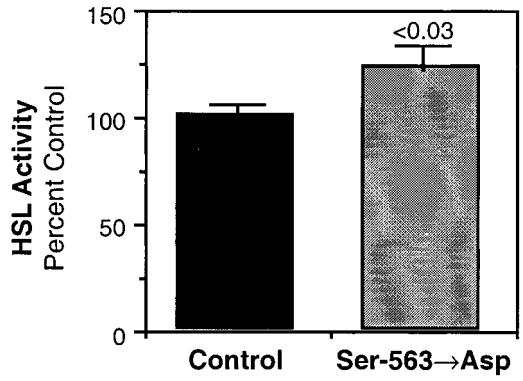

FIGURE 4: HSL activity in Ser-563→Asp mutant. Cell extracts from CHO cells transiently transfected with pcDNA3-HSL (control) or Ser-563→Asp HSL mutant and pRSVB-gal were assayed for neutral cholesteryl ester hydrolase and β-galactosidase activity as described in Experimental Procedures. Activity is expressed relative to control wild-type HSL, which was set at 100, and corrected for β-galactosidase activity. The results are the mean ± SEM of duplicate wells from 4 separate experiments. $p < 0.03$ compared to control wild-type HSL.

differences observed in Figure 3, with the Ser-423→Ala and Ser-563→Ala mutants having markedly reduced or absent activity against all three substrates. When analyzed with a full range of substrate concentrations, the Ser-565→Ala mutant again showed a reduced activity against cholesteryl ester, but preservation of hydrolytic activity against triacylglycerol and diacylglycerol. The double Ser-563→Ala/Ser565→Ala mutant again displayed a loss of activity that was intermediate between the Ser-563→Ala and Ser-565→Ala single mutants. None of the mutations was associated with substantial alteration in K_m for any of the substrates against which activity could be detected.

In view of the evidence showing that Ser-563 of rat HSL is the target for phosphorylation of HSL by cyclic AMP-dependent protein kinase, it was surprising to observe the almost complete loss of hydrolytic activity with mutation of this site to alanine. Although it is possible that phosphorylation of Ser-563 mediates the translocation of HSL between compartments within the cell (*5*), there is also evidence that phosphorylation of Ser-563 alters the intrinsic activity of the enzyme (*6*). Thus, it is possible that the Ser-563→Ala mutation altered the conformation of the protein with resultant loss of activity. The addition of a phosphate group to Ser-563 would be expected to change the pH of the microenvironment and, hence, induce a change in the conformation of the protein. To test this hypothesis, we mutated Ser-563 to a small acidic amino acid, Asp, and studied HSL activity in transiently transfected cells. In contrast to the Ser-563→Ala mutation, where >90% of hydrolytic activity was lost, the Ser-563→Asp mutant displayed an ~20% increase ($p < 0.03$) in cholesteryl ester hydrolysis compared to wild-type, control HSL (Figure 4), illustrating the importance of the presence of a hydroxyl group or negative charge at residue 563.

Computer Modeling of HSL Structure. Based on the observations of Contreras et al. (*2*), the Predictprotein PHD program was used to predict the secondary structure of HSL and confirmed the similarity of HSL to the lipase from the fungus *C. rugosa*. When the X-LOOK molecular modeling program was utilized to construct a model of the structure of HSL using the atomic coordinates of *C. rugosa* lipase as a template, the model revealed that, even though Asp-703 and His-733 are in the vicinity of Ser-423 and could comprise a catalytic triad as proposed (*2, 21*), Val-710 is actually in closer proximity to Ser-423 than Asp-703 (Figure 5). In this model, Ser-423, Val-710, and His-733 are all located within 4 Å of each other, while Asp-703 is located within 4 Å of Val-710 and His-733, but not Ser-423. To test whether Val-710 is important in the hydrolytic activity of HSL, Val-710 was mutated to Ala and HSL activity studied in transiently transfected cells. The Val-710→Ala mutation resulted in >85% reduction ($p < 0.001$) in cholesteryl ester hydrolysis compared to wild-type control HSL (Figure 6), supporting an important role for Val-710 in the catalytic activity of HSL.

DISCUSSION

Early studies with HSL noted that fast-acting lipolytic hormones induce an increase in the cellular concentration of cyclic AMP (*28*), activating cyclic AMP-dependent protein kinase, which then phosphorylates HSL (*28*) and results in an increase in hydrolytic activity (*23, 24*). A single mole of phosphate was found to be incorporated per mole of HSL by cyclic AMP-dependent protein kinase, suggesting that only a single site was phosphorylated (*25*). This site was identified in peptide fragments of bovine HSL to correspond to Ser-563 in rat HSL (*7*). In addition to phosphorylation of HSL by cyclic AMP-dependent protein kinase, glycogen synthase kinase-4 (*20*), Ca^{2+}/calmodulin-dependent protein kinase II, and AMP-activated protein kinase (*8*) were observed to phosphorylate HSL at a secondary site, which was identified in peptide fragments of bovine HSL to correspond to Ser-565 in rat HSL (*9*).

The current studies have explored the importance of these previously identified phosphorylation sites in maintaining the hydrolytic activity of HSL. Mutation of Ser-563→Ala eliminated the ability of cyclic AMP-dependent protein kinase to phosphorylate HSL; however, cyclic AMP-dependent protein kinase phosphorylated HSL normally when the mutation Ser-565→Ala was introduced. These results are consistent with Ser-563 being the site phosphorylated by cyclic AMP-dependent protein kinase (*7*). Likewise, Ser-565 appears to be the site phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II since mutation of Ser-565→Ala eliminated phosphorylation by this kinase, while the Ser-563→Ala mutant was phosphorylated normally. However, since some low-level incorporation of [^{32}P]-PO_4 was observed either when the kinase was omitted from the incubation or when mutations of Ser-563 and Ser-565 were combined, there could possibly be a phosphorylation site in addition to Ser-563 and Ser-565. Surprisingly, mutation of Ser-563→Ala and Ser-565→Ala decreased HSL hydrolytic activity significantly, with Ser-563→Ala having as little activity against cholesteryl ester, triacylglycerol, and diacylglycerol substrates as seen with mutation of Ser-423→Ala, the presumed catalytic site (*11*), while Ser-565→Ala retained most activity against triacylglycerol and diacylglycerol substrates. The fact that the combined Ser-563→Ala/Ser565→Ala mutation displayed activity that was intermediate between Ser-563→Ala and Ser-565→Ala further supports the importance of these serines for maintaining the proper conformational integrity of the protein or for proper stabilization of substrate in order to carry out its catalytic function. In contrast to the Ser-563→Ala mutation, where >90% of

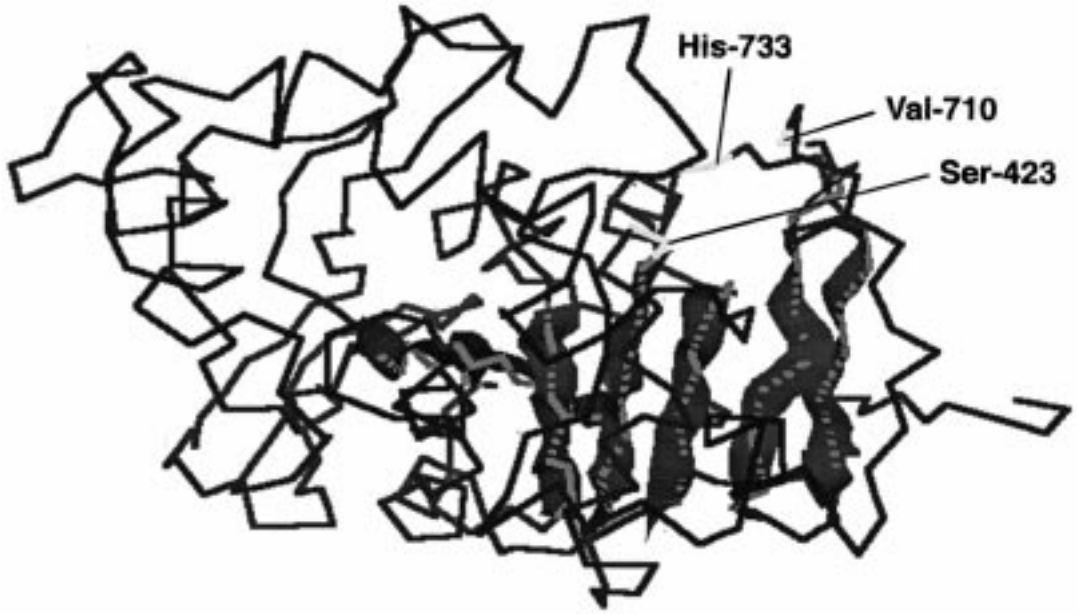

FIGURE 5: Structural model of rat HSL. Rat HSL was modeled using the X-LOOK modeling program by comparison with the structure of the lipase from *C. rugosa*. The broad dark bands represent β-sheets; Ser-423, His-733, and Val-710 are labeled and highlighted in grey, and comprise the catalytic pocket. The solid lines represent portions of HSL that could not be modeled.

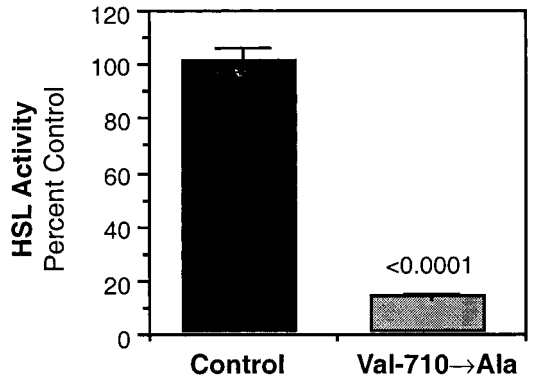

FIGURE 6: HSL activity in Val-710→Ala mutant. Cell extracts from CHO cells transiently transfected with pcDNA3-HSL (control) or Val-710→Ala HSL mutant and pRSVB-gal were assayed for neutral cholesteryl ester hydrolase and β-galactosidase activity as described in Experimental Procedures. Activity is expressed relative to control wild-type HSL, which was set at 100, and corrected for β-galactosidase activity. The results are the mean ± SEM of duplicate wells from 8 separate experiments. $p < 0.0001$ compared to control wild-type HSL.

hydrolytic activity was lost, mutation of Ser-563→Asp preserved HSL hydrolytic activity and even increased activity 20% above the control wild-type enzyme. Thus, the substitution of a small acidic amino acid appears to have mimicked the effects of adding a phosphate group to Ser-563, perhaps by changing the pH of the microenvironment and, hence, inducing a change in the conformation of the protein or in the stabilization of substrate to increase its catalytic efficiency. During the preparation of the manuscript, it was reported that mutation of Ser-563 had no effect on HSL activity, but that Ser-659 and Ser-660 were phosphorylated by cyclic AMP-dependent protein kinase in vitro and required for the phosphorylation-induced increase in hydrolytic activity against triacylglycerol substrate (*1*). It is very difficult to reconcile the differences between those studies and ours; however, there are some methodological differences. First, Anthonsen et al. (*1*) studied HSL mutants from transiently transfected COS cells from which the relative ratios of triacylglycerol hydrolysis were reported following the in vitro incubation of isolated HSL in the absence and presence of excess amounts of PKA; no absolute rates of hydrolysis of any of the HSL species were reported. Thus, it is possible that there were differences in basal enzyme activity among the mutants that precluded their observing any differences in the stimulation of activity when comparing phosphorylated to nonphosphorylated forms (Ser-563 for instance). Second, incubation with markedly excessive amounts of PKA in vitro might have resulted in the phosphorylation of additional sites (Ser-659, Ser-660) not normally phosphorylated. In contrast, we examined HSL mutants from stably transfected CHO cells and expressed our results as both relative and absolute rates of hydrolysis of triacylglycerol, cholesteryl ester, and diacylglycerol. Thus, although there are some methodological differences, the bases for the differences in the results are unclear; however, our current results are consistent with previous publications by other investigators which support a single site being phosphorylated by PKA. In addition to affecting the intrinsic catalytic activity of the enzyme, it has been suggested that phosphorylation by cyclic AMP-dependent protein kinase induces the translocation of HSL between compartments within the cell (*5*). We were unable to address the importance of phosphorylation of Ser-563 in this process in the current studies since CHO cells do not accumulate sufficient quantities of either cholesteryl esters or triglyceride to examine this issue.

While the primary sequence of HSL is unrelated to any of the other known mammalian lipases, it shares some sequence homology with lipase 2 of *Moraxella* TA144 (*14*). This homology aided in locating a G-X-S-X-G motif which was proposed (*10*), and later confirmed by mutagenesis (*11*), to contain the catalytically active serine at position 423 in rat HSL. Further expanding on this observation, it was noted that the C-terminal portion of HSL was similar to acetylcholinesterase, bile salt-stimulated lipase, and several fungal

lipases, and is composed of α/β-hydrolase folds that accommodate the catalytic site (*22*). A significant advance in understanding the structure of HSL was made by Contreras et al. (*2*), who observed that, even in the absence of primary sequence homology, the organization of the secondary structure predicted for human HSL was similar to the secondary structure of acetylcholinesterase and of two fungal lipases from *G. candidum* and *C. rugosa*. Using the molecular modeling program Modeler, these investigators proposed that Ser-423, Asp-703, and His-733 (numbered for rat HSL) constitute the catalytic triad for HSL, and later provided data from site-directed mutagenesis of Asp-703 and His-733 to support this conclusion (*21*). Utilizing the Predictprotein PHD program to predict the secondary structure of rat HSL and aligning the predicted secondary structure of rat HSL with the secondary structure of *C. rugosa* lipase, we used a different molecular modeling program, X-LOOK, to generate a structural model for rat HSL that differs in some aspects from that proposed by Contreras et al. (*2*). The current model revealed an interaction of the residues (Ser-423, Asp-703, and His-733) previously identified potentially as the catalytic triad, but also the close proximity of Val-710 to the catalytic residue, Ser-423. The importance of Val-710 in the activity of HSL was supported by the demonstration of the loss of hydrolytic activity following mutagenesis of this site. The involvement of this hydrophobic residue in the hydrolysis of lipophilic substrates is further supported by recent experiments in which a series of synthetic substrates display an increasing affinity for binding by HSL as their hydrophobicity is raised (D. Takeda, F. B. Kraemer, and C. Selassie, manuscript in preparation). It should be noted that Val-710 is located within a concensus α-helical region (707−717), and it is possible that alteration of the α-helix might have disrupted the structure of HSL sufficiently to result in loss of hydrolytic activity. Although this finding does not rule out the importance of the catalytic function of the Ser-423, Asp-703, and His-733 triad, it suggests a possible explanation for the differences in substrate characteristics between HSL and other lipases. Unfortunately, the absence of homology of the primary sequence of HSL and the lack of similarity of the predicted secondary structure of the phosphorylation domain of HSL with other known proteins precluded modeling the interaction of the phosphorylation domain with the catalytic portion of the enzyme.

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