Mutational Analysis of the "Regulatory Module" of Hormone-Sensitive Lipase†

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ABSTRACT: Hormone-sensitive lipase (HSL) is a rate-limiting enzyme in lipolysis that displays broad substrate specificity. HSL function is regulated by reversible phosphorylation that occurs within a 150 aa "regulatory module" of the protein. The current studies used mutational analysis to dissect the contribution of the "regulatory module" in HSL activity and substrate specificity. Deletion of the entire "regulatory module" or replacement of the "regulatory module" with the "lid" of lipoprotein lipase resulted in enzymatically inactive proteins. Deletion of sequentially longer stretches of the "regulatory module" resulted in a stepwise reduction in hydrolytic activity. Analysis of 7−19 amino acid deletional mutants that spanned the "regulatory module" showed that the N-terminal partial deletion mutants retained normal hydrolytic activity and activation by PKA. In contrast, the C-terminal partial deletion mutants displayed reduced hydrolytic activities, with preferential loss of activity against lipid-, as opposed to water-soluble, substrates. Single amino acid mutations of F650C, P651A, and F654D reduced activity against lipid-, but not water-soluble, substrates. The current results suggest that the length of the "regulatory module" and specific sequences within the C-terminal portion of the "regulatory module" of HSL (amino acids 644−683) are crucial for activity and appear to be responsible for determining lipase activity.

In mammals, free fatty acids derived from the breakdown of stored triacylglycerols in adipose tissue are a major energy source for most tissues. The critical enzyme that catalyzes this step is thought to be hormone-sensitive lipase (HSL).[1] HSL activity is tightly regulated by hormones and catecholamines to provide appropriate FFA on demand (*1*). Two distinctive features of HSL are its regulation by reversible phosphorylation, which is unique among known lipases, and its broad substrate specificity. HSL hydrolyzes triacylglycerols, diacylglycerol, 1(3)monoacylglycerol, cholesteryl esters, lipoidal esters of steroid hormones, and retinyl esters in adipose tissue as well as water-soluble butyrate substrates; however, HSL lacks phospholipase activity (*1*).

The primary sequence of HSL is unrelated to any other known mammalian lipases; however, it shares amino acid sequence homology with several fungal and bacterial lipases and esterases (*2*−*6*). Limited proteolysis and denaturation studies (*7*, *8*) suggested that HSL, like other lipases (*9*), contains two major domains. The N-terminal domain has been shown to interact with adipocyte lipid-binding protein (*10*) and has been proposed to function as a docking domain for protein−protein interactions. The C-terminal portion of HSL is composed of α/β hydrolase folds that accommodate the catalytic sites (*11*); Ser-423, Asp-703, and His-733 constitute the catalytic triad for rat HSL (*12*). Also located within this C-terminal portion is a ∼150 amino acid stretch that contains known phosphorylation sites and has been termed the "regulatory module". Early evidence suggested that S563 was phosphorylated by PKA (*13*, *14*); however, later studies have shown that PKA phosphorylates S559 and S660, which are required for the phosphorylation-induced increase in hydrolytic activity toward triacylglycerol substrate (*15*), as well as the translocation of HSL to the lipid droplet (*16*). The model of the topological organization of HSL places the "regulatory module" between the β6 and the β7 sheets (*11*). In other lipases, these surface loops often form "lids" that shield the catalytic site (*17*). The "lid" of many lipases confers functions, in addition to interfering with substrate access to the catalytic site, by contributing to the substrate specificity of the lipase (*18*−*20*). By exchanging the "lids" of lipoprotein lipase (LPL) and hepatic lipase, it has been demonstrated that the "lid" of LPL confers preferential triacylglycerol hydrolysis and the lid of hepatic lipase confers preferential phospholipase activity (*21*). Additionally, the surface loop may contribute to interfacial binding (*22*), although other elements appear to be more important in interfacial activation (*23*).

The current work used mutational analysis to investigate the function of the "regulatory module" in influencing HSL hydrolytic activity and substrate specificity. We found that the N-terminal portion of the "regulatory module" (amino acids 535−647) contributed minimally to HSL activity, whereas the distal C-terminal portion of the "regulatory module" (amino acids 644−683) was crucial for HSL activity and appears to be responsible for determining lipase, but not esterase, activity.

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[1] Abreviations: HSL, hormone-sensitive lipase; PKA, cyclic AMP-dependent protein kinase; CE, cholesterol ester; TG, triacylglycerol; *p*NPB, *p*-nitrophenyl butyrate; LPL, lipoprotein lipase.

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Table 1: Primer Pairs Used for Mutagenesis of HSL

mutation	primers
regulatory module deletion	5′-GCCCCTGACGTCATGCTGAAG
	3′-CCCACTTAACTCCAGGAAGGAG
LPL lid fragment	5′-TGCAACATTGGAGAAGCCATTCGT
	3′-GCACTTCACCAGCTGGTCCACATC
mutant I	5′-CGG CAAGCTAAGCAGGCGGCT
	3′-GAG AACCCACTGCTTACT
mutant II	5′-ACACTTGGCCCCTCCACACC
	3′-GAG AACCCACTGCTTACT
mutant III	5′-GCTGCCTTCCCTGATGGTTTC
	3′-GAG AACCCACTGCTTACT
mutant IV	5′-CACATGCCCCTCTACTCGTCA
	3′-GAG AACCCACTGCTTACT
mutant 1	5′-TTGCCTGCAACAGAGACACTG
	3′-CCCACTTAACTCCAGGAAGGAG
mutant 2	5′-AGGAGTGTGTCTGAGGCAGCC
	3′-CAATGGGGTCTTATGGGGCTT
mutant 3	5′-CTGGGCACAGATTCCTTGAAG
	3′-CCTGCGCATAGACTCCGTAAG
mutant 4	5′-GGCAACTCAGAGCCATCAGAC
	3′-ATCTGTGCCCAGCAAGCCCT
mutant 5	5′-ACACTTGGCCCCTCCACACC
	3′-TGAGTTGCCCTTAAAGCTCAAGTC
mutant 6	5′-AATTCCCAGGAAGAGGCTGAA
	3′-TGTCTCCATTGACTGTGACATCTC
mutant 7	5′-GCTGCCTTCCCTGATGGTTTC
	3′-CTCTTCCTGGGAATTCCCGGA
mutant 8	5′-TCAAGCCAAGGTGTCCTCCAC
	3′-GATTCCGTCCATGGGGCTTAT
mutant 9	5′-CACATGCCCCTCTACTCGTCA
	3′-TGGGTGGAAACCATCAGGGAA
mutant 10	5′-GCCCCTGACGTCATGCTGAAG
	3′-GTGGAGGACACCTTGGCTTGA
F650C	5′-GACGGAATCCCTAGGGTGCGCGCTGCCTGCCCTGATGGT
	3′-ACCATCAGGGCAGGCAGCGCGCACCCTAGGGATTCCGTC
P651A	5′-GCTGCCTTCGCTGATGGTTTCCACCCTAGGCGCTCA
	3′-TGAGCGCCTAGGGTGGAAACCATCAGCGAAGGCAGC
F654D	5′-TTCCCTGATGGTGATCACCCTAGGCGCTCAAGC
	3′-GCTTGAGCGCCTAGGGTGATCACCATCAGGGAA

EXPERIMENTAL PROCEDURES

Materials. All chemicals were from Sigma 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 Eagle's media, pfx DNA polymerase, Lipofectin reagent from Invitrogen, Carlsbad, CA; ECL Western blotting detection reagents, horseradish peroxidase-linked whole antibody anti-rabbit IgG, cholesteryl [1-^{14}C]oleate, glycerol tri[9,10(n)^{3}H]oleate, L-3-phosphatidylcholine, 1, 2-di[1-^{14}C] palmitoyl from Amersham Life Sciences Products, Arlington Heights, IL; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine from Avanti Polar Lipids, Inc. Alabaster, AL; nitrocellulose paper from Schleicher and Schuell, Keene, NH; QuikChange mutagenesis kit from Stratagene, La Jolla, CA. Organic solvents were from J. T. Baker (Phillipsburg, NJ); cyclic AMP-dependent protein kinase (Sigma Chemical Co., St. Louis, MO).

Chimeric, Deletional, and Mutational Constructs of HSL. The previously described pcDNA3-rat HSL (*14*) served as the template in the construction of all the HSL mutants. For the HSL−LPL chimeric protein, we created an EcoRV site and a KpnI site at amino acid positions 644 and 684, respectively, for cloning convenience. An oligonucleotide encoding the 22 amino acid lid of LPL (*18*) with a Kpn I site at the C-terminus was synthesized, and the HSL−LPL chimera was obtained by ligating the LPL lid oligonucleotide to pcDNA3-HSL644−683. All substitution and deletion mutations were prepared using specific oligonucleotide primers (Table 1) and the polymerase chain reaction with pfx DNA polymerase. Single amino acid mutations of HSL were carried out using the Stratagene "QuikChange" mutagenesis kit. The identity of all mutant constructs was verified by restriction endonuclease analysis and DNA sequencing.

Cell Culture and Transfection. COS7 cells were grown in Coon's F12/Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. For transient transfection experiments, cells were subcultured at a density of 1×10^6 cells/well in 100 mm plates the day prior to incubation with 6 μg of pcDNA3-HSL or pcDNA3-HSL mutants in 30 μL of Lipofectin reagent. Cells were transfected following Invitrogen instructions and harvested 48 h after transfection for measurement of HSL activity or immunoblotting.

Immunoblotting. Cells were scraped and briefly sonicated (3 s) in 0.3 mL of ice-cold lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA and 1 unit/mL leupeptin. Homogenates were centrifuged at 10000g 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, or with anti-full-length rat His-

HSL, and visualized by chemiluminescence as described previously (*24*).

Measurement of HSL and LPL Activity. Measurement of HSL lipase activity was performed using cholesteryl ester and triacylglycerol substrates as described previously (*14*). Esterase activity was measured using *p*-nitrophenyl butyrate (*p*NPB) as substrate, which is water soluble under the conditions employed. Briefly, aliquots (15−20 μL) of COS cell homogenates and 10 μL of substrate (200 mM *p*NPB in acetonitrile) were mixed in 0.9% NaCl, 0.1 M sodium phosphate, pH 7.25 in a total volume of 1 mL. After incubation for 10 min, the reactions were terminated by addition of 3.25 mL of methanol-chloroform-heptane (10: 9:7, v/v/v). After vortex mixing (10 s per tube) and centrifugation (20 min at 800*g*), the tubes were heated at 42 °C for 3 min and the absorbance (optical density) of the upper phase was measured at 400 nm. An extract from cells transfected with the expression vector alone served as a negative control. LPL lipase activity of the chimeric protein were measured using triacylglycerol as substrate with preincubation with or without serum and high or low NaCl concentration, as described previously (*25*).

Activation of HSL by PKA. COS7 cells that were transiently transfected with different HSL constructs were grown in COON's F12/Dulbecco's Modified Eagles media until confluent, and then harvested in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 U/mL leupeptin). After a brief sonication, the cells were centrifuged at 14000*g* for 10 min at 4 °C. In vitro phosphorylation was carried out as described previously (*14*). A total of 50 μL of cell lysate was mixed with reaction buffer (3.33 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 2 mM DTT, 0.1% C13E12, 0.2 mM ATP, and 180 unit/mL of the catalytic subunit of cyclic AMP-dependent protein kinase). 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, and HSL activity against cholesteryl ester substrate was assayed as described above.

Statistical Analysis. Data are expressed as mean ± SD. Statistical analyses were performed by unpaired two-tailed Student's *t* test using StatView (Abacus Concepts, Berkeley, CA) and InStat (GraphPad Software, San Diego, CA) software for Macintosh.

RESULTS

Deletion of the "Regulator Module" and Generation of an HSL−LPL Chimera. The model of the topological organization of HSL predicts that the "regulatory module" is located between the β6 and β7 sheets and comprises amino acids 535 to 683 of rat HSL (*11*). In other lipases these surface loop elements often form lids that shield the catalytic site or confer substrate specificity (*9*, *17*). To begin to explore the role of the "regulatory module" in influencing HSL activity and potential substrate specificity, we first generated a deletional mutant in which the entire "regulatory module" (amino acids 535−683) was removed. When this mutant protein (HSLΔ535−683) was expressed in COS7 cells, the deletion of the "regulatory module" resulted in a completely inactive protein that failed to hydrolyze lipid- (cholesteryl ester) or water-soluble (*p*NPB) substrates (Figure 1). Thus, it appears that the "regulatory module" is essential for enzymatic activity of HSL. To determine whether lid sequences from other lipases could substitute for the "regulatory module", we replaced the "regulatory module" of HSL with the "lid" of LPL, creating a chimeric protein. When expressed in COS7 cells, the HSL−LPL "lid" chimera also failed to hydrolyze lipid- (cholesteryl ester) or water-soluble (*p*NPB) substrates (Figure 1). In addition, the chimera failed to hydrolyze triacylglycerol in the absence or presence of serum (data not shown), conditions under which LPL is normally activated. These results suggest that "the regulatory module" is required for proper folding and activity of HSL and that analogous surface loop sequences from LPL are unable to substitute.

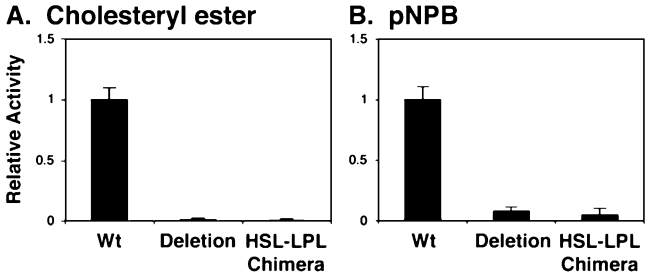

FIGURE 1: Hydrolytic activity of a deletion mutant of the full-length "regulatory module" of HSL and of an HSL−LPL "lid" chimera. Cell extracts from COS7 cells transfected with pcDNA3-HSL (control) or with pcDNA3-HSLΔ535−683 or with pcDNA3-HSL−LPL "lid" were incubated with 100 μM cholesteryl ester (CE) (A) or 2 mM *p*NPB (B) and enzymatic activity determined as described in the Experimental Procedures. The activity of full-length HSL was 85 nmol h^{-1} mg^{-1} for CE and 34 mmol h^{-1} mg^{-1} for *p*NPB and was set as 1. The results are the mean ± SD from three independent experiments.

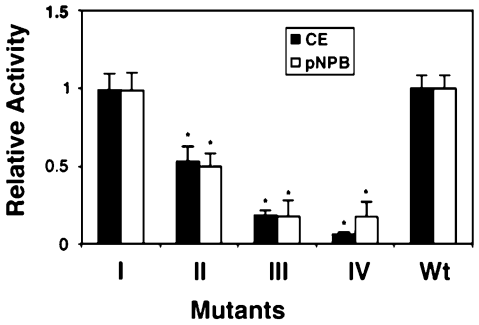

FIGURE 2: Hydrolytic activity of progressively longer deletions of the "regulatory module". Cell extracts from COS7 cells transfected with pcDNA3-HSL (control, Wt) or with pcDNA3-HSLΔ535−575 (I), pcDNA3-HSLΔ535−608 (II), pcDNA3-HSLΔ535−647 (III), or pcDNA3-HSLΔ535−664 (IV) were incubated with 100 μM cholesteryl ester (CE) or 2 mM *p*NBP substrates as described in the Experimental Procedures. Activity is expressed relative to control wild-type HSL, which was 79 nmol h^{-1} mg^{-1} for CE and 36 mmol h^{-1} mg^{-1} for *p*NPB and was set at 100. Results are the mean ± SD of duplicate wells and are representative of 3−4 independent experiments. *, $p < 0.001$ compared to control.

Influence of Length of the "Regulatory Module". In view of the discrepancy in length of the "regulatory module" (∼150 amino acids) of HSL and the surface loop of LPL (22 amino acids), it was possible that the "lid" of LPL was too short to provide proper folding of HSL. To address whether there is a critical length of the "regulatory module" that is required for activity, progressively longer deletions of the "regulatory module" were introduced. As shown in Figure 2, deletion of the first 41 amino acids (∼25%) of the "regulatory module" yielded a protein (HSLΔ535−575) with

Table 2: Partial Deletions of the "Regulatory Module" of HSL

	amino acid deletion		
construct	position	number deleted	sequence
mutant 1	535−541	7	RKPHKTP
mutant 2	543−561	19	PATETLRPTDSGRLTESMR
mutant 3	563−575	13	SVSEAALAQPEGL
mutant 4	580−592	13	SLKKLTIKDLSFK
mutant 5	596−608	13	EPSDSPEMSQSME
mutant 6	610−625	16	LGPSTPSDVNFFLRSG
mutant 7	631−647	17	AETRDDISPMDGIPRVR
mutant 8	644−658	15	PRVRAAFPDGFHPRR
mutant 9	657−664	8	RRSSQGVL
mutant 10	666−683	18	MPLYSSPIVKNPFMSPLL

activity against lipid- and water-soluble substrates that was not significantly different from wild-type HSL. Deletion of the first 74 amino acids (∼50%) of the "regulatory module" yielded a protein (HSLΔ535−608) with activity against lipid- and water-soluble substrates that was reduced ∼50% ($p < 0.001$), whereas deletion of the first 113 (∼75%, HSLΔ535−647) or 130 (∼85%, HSLΔ535−664) amino acids of the "regulatory module" yielded proteins with activity against lipid- and water-soluble substrates that were reduced ∼90% ($p < 0.001$). Thus, the length of the "regulatory module" appears to be important for normal HSL activity.

Sequence-Specific Influence of the "Regulatory Module". Since up to 40 amino acids could be deleted from the "regulatory module" without a significant impact on HSL function, a series of 10 mutants, in which 7−19 amino acids were deleted, was generated that spans the entire region of the "regulatory module" (Table 2). Each of these deletional mutants contains 90−95% of the full-length "regulatory module". All mutants were expressed in COS7 cells and were detected by immunoblotting using two different antibodies (Figure 3). The antibody used in panel A was generated from a fusion protein encoding amino acids 399−608, whereas the antibody used in panel B was generated using a full-length HSL protein. As detected equally by both antibodies, mutants 1, 4, 5, 6, 7, 8, 9 were expressed at levels comparable to wild-type HSL, whereas the apparent expression, i.e., detection, of mutants 2, 3, and 10 varied depending on the antibody used. Thus, the anti-fusion protein antibody weakly detected mutants 2 and 3, but their expression levels were comparable to wild-type HSL when anti-full-length HSL antibodies were used. In contrast, mutant 10 was detected by the anti-fusion protein antibody, but poorly detected by the anti-full-length HSL antibodies. These results indicate that amino acids 547−577 (deleted in mutants 2 and 3) and 666−683 (deleted in mutant 10) are either the direct epitopes or alter the conformation to disrupt the epitopes recognized by these different antibodies.

The relative activities of cell extracts of COS7 cells expressing the various HSL mutants were determined using cholesteryl ester, triacylglycerol, and *p*NPB substrates (Figure 4). Deleting 7−19 amino acid segments from amino acid 535 through 647 had no effect on hydrolytic activities against lipid- or water-soluble substrates (mutants 1−7). Moreover, none of these mutants showed a significant alteration in preference for cholesteryl ester or triacylglycerol as a substrate. In contrast, deletional mutations in the final 40 amino acids of the "regulatory module" resulted in marked alterations in hydrolytic activity. Thus, mutant 8 displayed

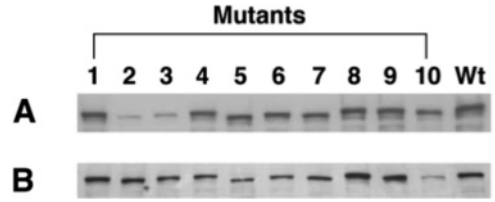

FIGURE 3: Immunoblot of HSL. COS7 cells were transfected with pcDNA3-HSL (control) or with pcDNA3-HSL deletional mutants (see Table 2). Cell extracts (10 μg (A) and 15 μg (B) of total protein) were electrophoresed on SDS−PAGE, transferred to nitrocellulose filters, immunoblotted with anti-HSL/fusion protein IgG (A) or with anti-full-length HSL IgG (B), and visualized by enhanced chemiluminescence. The films were developed after a 2−10 s exposure.

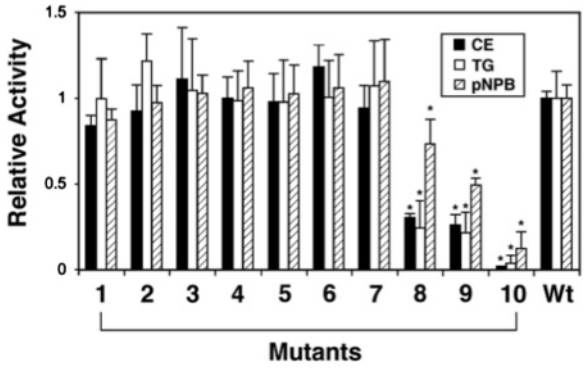

FIGURE 4: Hydrolytic activity of partial deletions of the "regulatory module" of HSL. Cell extracts from COS7 cells transfected with pcDNA3-HSL (control) or with deletional mutants (see Table 2) were incubated with 100 μM cholesteryl ester (CE), 1.67 mM triacylglycerol (TG), or 2 mM *p*NPB as described in the Experimental Procedures. Activity is expressed relative to control wild-type HSL, which was 95 nmol h^{-1} mg^{-1} for CE, 9 nmol h^{-1} mg^{-1} for TG, and 39 mmol h^{-1} mg^{-1} for *p*NPB and was set at 100. Results are the mean ± SD of triplicate wells and are representative of 3−5 independent experiments. *, $p < 0.001$ compared to control.

loss of its ability to hydrolyze cholesteryl esters ($p < 0.0001$) and triacylglycerol ($p < 0.001$), yet retained its ability to hydrolyze *p*NPB. Mutant 9 also displayed loss of its ability to hydrolyze cholesteryl esters and triacylglycerol ($p < 0.001$), as well as some loss of *p*NPB hydrolysis ($p < 0.001$). Mutant 10 appeared to be completely devoid of activity against any substrate. Since the hydrolytic activities of the mutants in these experiments were determined at only a single substrate concentration, substrate concentration curves were performed using cholesteryl ester, a lipid substrate, and *p*NPB, a water-soluble substrate, on the constructs displaying reduced activities, mutants 8, 9, and 10 (Figure 5). Under these conditions, mutant 8 and mutant 9 displayed ∼60−70% reductions in their ability to hydrolyze cholesteryl ester without any significant changes in the K_m of the enzyme (Table 3). However, both mutant 8 and mutant 9 displayed kinetics similar to wild-type control HSL against *p*NPB (Figure 5B and Table 3). In contrast, mutant 10 lost 90−95% of its activity against both cholesteryl ester and *p*NPB. The differential loss of activity against lipid- versus water-soluble substrates observed for mutants 8 and 9 suggests that a lipid binding site might exist in this region. Furthermore, the almost complete loss of activity in mutant 10, where 90% of the "regulatory module is intact, suggests that specific sequences within this portion are critical for proper function. As another means for assessing the structural integrity of

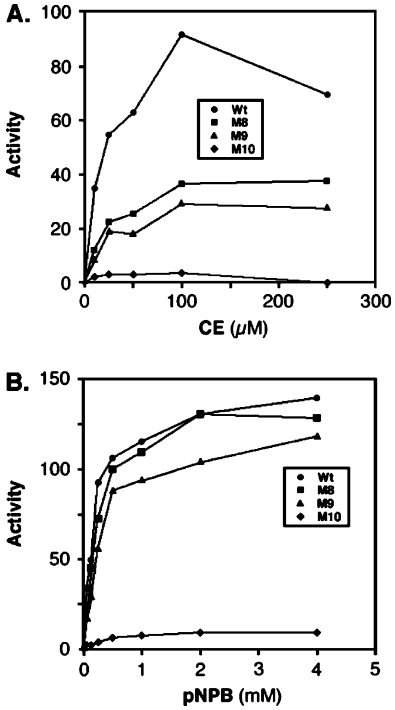

FIGURE 5: Substrate concentration curves of partial deletions of the "regulatory module" of HSL. Cell extracts from COS7 cells transfected with pcDNA3-HSL (Wt, control) or with deletional mutants M8, M9, or M10 were incubated with the indicated concentrations of cholesteryl ester (CE) (panel A), or *p*NPB (panel B) as described in the Experimental Procedures. Activity for CE substrate is expressed as nmol h^{-1} mg^{-1}; activity for *p*NPB substrate is expressed as mmol h^{-1} mg^{-1}. Results are representative of two independent experiments.

Table 3: K_m and V_{Max} Values of HSL Species[a]

	substrate			
	cholesteryl ester		*p*NPB	
HSL species	K_m (μM)	V_{max} (nmol h^{-1} mg^{-1})	K_m (μM)	V_{max} (mmol h^{-1} mg^{-1})
wild type	15 ± 1	82 ± 2	462 ± 21	162 ± 1
mutant 8	24 ±2	36 ± 11	389 ± 61	163 ± 36
mutant 9	25 ± 6	28 ± 9	427 ± 105	199 ± 60
mutant 10	21 ± 6	4 ± 3	268 ± 24	20 ± 13

[a] Cell extracts from COS7 cells transfected with pcDNA3-HSL (wild type, control) or with deletional mutants 8, 9, or 10 were incubated with the cholesteryl ester (0.1 to 250 μM) or *p*NPB (0.06 to 4 mM) as described in Experimental Procedures. K_m and V_{max} values were then calculated after linear transformation of the data. Results are the mean ± SE.

the mutant proteins, the ability of PKA induced phosphorylation to increase HSL activity against cholesteryl ester substrate was measured (Figure 6). Deletion mutants 1−7 could be activated to the same extent as native HSL, whereas mutants 8 and 9, albeit possessing lower basal hydrolytic activity, displayed no activation by PKA. This is consistent with the disruption or loss of the PKA sites S659 and S660 in mutants 8 and 9. Mutant 10 is not depicted in Figure 6 since its basal and stimulated activities were so low.

Lipid Binding Site. It has been suggested that HSL might contain a lipid binding groove within the "regulatory module" to accommodate the acyl chain (*26*). The observation that mutant 8 has markedly diminished activity against cholesteryl ester and triacylglycerol, while retaining activity against

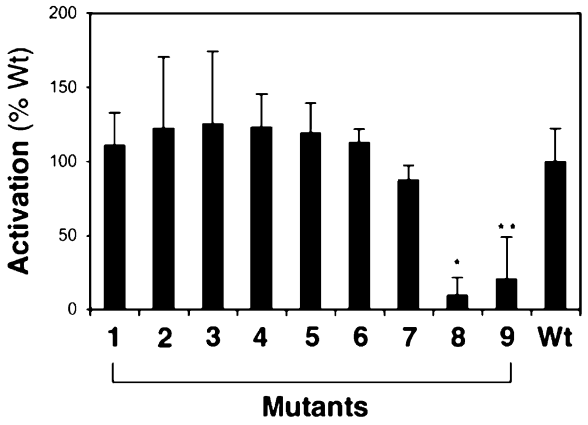

FIGURE 6: PKA-induced activation of hydrolytic activity of partial deletions of the "regulatory module" of HSL. COS7 cells were transfected with pcDNA3-HSL (wild type, control) or with deletional mutants (see Table 2). Homogenates were incubated with the catalytic subunit of PKA for 30 min at 37 °C prior to assessing activity against cholesteryl ester substrate. Activation (the ratio of activity of phosphorylated to nonphosphorylated HSL) is expressed relative to the activation of wild-type HSL (activation of wild-type HSL ranged from 50 to 200% among experiments). The results are the mean ± SD of duplicate or triplicate transfections and are representative of three independent experiments. *, $p < 0.01$ compared to control; **, $p < 0.05$ compared to control.

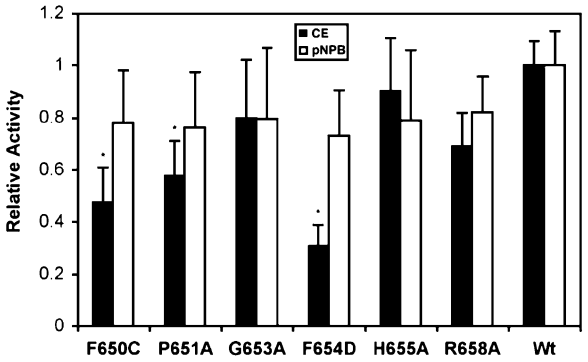

FIGURE 7: Hydrolytic activity of amino acid substitutions within the "regulatory module" of HSL. Cell extracts from COS7 cells transfected with pcDNA3-HSL (control) or with HSL-F650C, HSL-P651A, HSL-G653A, HSL-F654D, or HSL-H655A mutants were incubated with 100 μM cholesteryl ester (CE) or 2 mM *p*NBP as described in Experimental Procedures. Activity is expressed relative to control wild-type HSL, which was 73 nmol h^{-1} mg^{-1} for CE and 44 mmol h^{-1} mg^{-1} for *p*NPB and was set at 100. Results are the mean ± SD of duplicate wells and are representative of three independent experiments. *, $p < 0.001$ compared to control; **, $p < 0.05$ compared to control.

*p*NPB, suggested that a lipid binding site might exist within residues 644−658. Since large nonpolar amino acids are involved in the formation of lipid binding sites of *Candida rugosa* lipase, a fungal lipase related to HSL (*22*), site directed mutagenesis was used to change the hydrophobic residues within amino acids 644−658 (F650, P651, and F654). As shown in Figure 7, F650C, P651A, and F654D showed significantly reduced activities against cholesteryl ester ($p < 0.001$) as well as *p*NPB substrate ($p < 0.05$); however, there was a more pronounced loss of activity against cholesteryl ester. Experiments using a wide range of substrate concentrations confirmed that these mutations resulted in ∼50% reduction in activity against cholesteryl ester, but no significant alteration in activity against *p*NBP (data not shown). Therefore, F650, P651, and F654 appear

that they might be involved in a lipid binding pocket that allows HSL to recognize hydrophobic substrates.

Psychrotolerant Properties of HSL. A characteristic property of HSL is the retention of hydrolytic activity at low temperatures, a feature which most other lipases lack and which could be of importance in hibernating mammals (*27*). To explore whether any portions of the "regulatory module" contribute to the psychrotolerant properties of HSL, the activity of the deletion mutants which still possessed activity was compared at temperatures of 37, 22, 16, and 4 °C. At all temperatures tested, the "regulatory module" deletion mutants showed similar activity against cholesteryl ester and *p*NPB as wild-type HSL (data not shown). Thus, the regulatory module is not responsible for the psychrotolerant properties of HSL.

DISCUSSION

HSL is a member of the α/β hydrolase fold family of enzymes (*6*, *11*). Although the members of this family frequently do not share significant similarity in protein sequence, they do share structural similarities that provide them with the scaffold necessary to form the catalytic site of a variety of enzymes with varied substrate targets (*28*). The α/β hydrolase scaffold is able to accommodate a number of different insertions that vary from a few to many amino acids in length. These insertions form surface loops or lids that can shield the accessibility of substrate to the active site, contribute to substrate specificity, or allow interfacial activation, where lipase activity is enhanced by contact with a lipid−water interface (*22*, *28*). The regulatory module of HSL is found between the β6 and β7 sheets and is among the largest insertions found in the α/β hydrolase fold family; the lack of any sequence similarity has precluded analysis of the structure of the regulatory module. In the current work, we have investigated the function of the regulatory module in contributing to HSL activity and substrate specificity.

Deletion of the entire regulatory module resulted in complete loss of hydrolytic activity of HSL against both lipid- and water-soluble substrates. Among lipases within the α/β hydrolase fold family, some, such as pancreatic cholesterol esterase, contain no lid structure (*29*), whereas others contain lid structures, yet retain hydrolytic activity after deletion of the lid, such as human pancreatic lipase and acetylcholinesterase (*30*, *31*). Deletion of the lid from LPL has been reported to result in loss of hydrolytic activity against emulsified substrates, but enhanced activity against short chain fatty acid triglycerides (*18*). Interestingly, the lid region from LPL was unable to substitute for the regulatory module of HSL; the HSL−LPL lid chimera displaying no activity against either lipid- or water-soluble substrates. This contrasts with the exchange of lids between LPL and hepatic lipase where hydrolytic activity is retained, but substrate specificity altered (*21*).

It is noteworthy that the regulatory module of HSL is among the largest insertions found in the α/β hydrolase fold family. Although recent kinetic studies suggested that HSL might lack a functional "lid" (*32*), the current studies using mutational analysis support an important role for sequences within the regulatory module. Figure 8 is a representation of the structure of the C-terminal region of HSL, which contains the catalytic triad (Ser-423, Asp-703, His-733) and

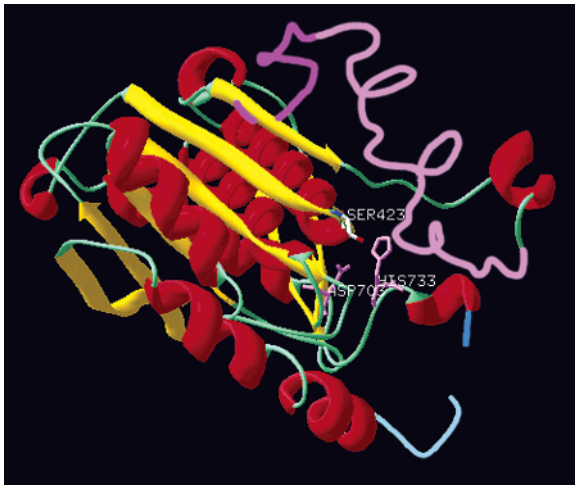

FIGURE 8: Structural model of rat HSL C-terminal region. HSL was modeled based on structure-aided sequence alignment with brefeldin A esterase (*6*). The thick red ribbons represent α-helices; the broad yellow arrows represent β-sheets. The α carbon trace is presented as a green ribbon with the N-terminus depicted in light blue and the C-terminus in dark blue. The "regulatory module" is shown in purple with the dark purple depicting the critical sequences. The structure of the "regulatory module" is hypothetical.

the "regulatory module", and was modeled based on structure-aided sequence alignment with brefeldin A esterase (*6*). However, since direct structural data for the "regulatory module" is lacking, the structure of the "regulatory module" is hypothetical and is based in part on the results from the deletional studies reported here. When progressively longer deletions were introduced from the N-terminal end of the regulatory module, there was a reduction in activity against both lipid- and water-soluble substrates that appeared to parallel the length of the segment of the regulatory module that was deleted. This finding suggests that the length of the regulatory module of HSL is important in maintaining the structural integrity of the catalytic triad. Although the length of the regulatory module seems to be important for basal activity, sequences within the C-terminal region of the regulatory module appear to be critical for activity against both lipid- and water-soluble substrates. Thus, small deletions (7−19 amino acid segments) within the first ~110 amino acids of the regulatory module had no effects on HSL activity. This topology of the regulatory module of HSL appears to differ from that of the closely related brefeldin A esterase, where the insertion between the β6 and β7 sheets contains two α helices in the N-terminal region that appear to form a cap over the active site (*6*). In contrast, small deletions within the C-terminal 40 amino acids of the regulatory module resulted in either preferential loss of activity against lipid substrates or loss of activity against both lipid- and water-soluble substrates. Site-directed mutagenesis of the hydrophobic residues within amino acids 644−658 confirmed that F650, P651, and F654 appear to be involved in a lipid binding pocket that allows HSL to recognize hydrophobic substrates, since there was a reduction in activity against lipid-soluble substrate, while normal activity against a water-soluble substrate was maintained. The production of a protein that is almost completely inactive following deletion of residues 666−683 suggests that this region is vital for the normal function of the enzyme. The fact that S659 and S660, the serines which are phosphorylated by PKA (*15*),

are located adjacent to these important regions within the regulatory module suggests possible mechanisms whereby phosphorylation results in increased activity against triacylglycerol and cholesteryl ester substrates by altering the conformation of these microdomains.

In conclusion, our results suggest that the N-terminal portion of the regulatory module (amino acids 535−647) contributes minimally to HSL activity, whereas the C-terminal portion of the regulatory module (amino acids 644−683) is crucial for HSL activity. The regulatory module does not contribute to the substrate specificity of HSL; however, amino acids within its C-terminal 40 amino acids appear to be involved in a lipid binding pocket and amino acids 666−683 are vital for the normal function of the enzyme.

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