Identification of Novel Phosphorylation Sites in Hormone-sensitive Lipase That Are Phosphorylated in Response to Isoproterenol and Govern Activation Properties in Vitro

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Hormone-sensitive lipase (HSL) is the rate-limiting enzyme in lipolysis. Stimulation of rat adipocytes with isoproterenol results in phosphorylation of HSL and a 50-fold increase in the rate of lipolysis. In this study, we used site-directed mutagenesis and two-dimensional phosphopeptide mapping to show that phosphorylation sites other than the previously identified Ser-563 are phosphorylated in HSL in response to isoproterenol stimulation of 32P-labeled rat adipocytes. Phosphorylation of HSL in adipocytes in response to isoproterenol and in vitro phosphorylation of HSL containing Ser→Ala mutations in residues 563 and 565 (S563A, S565A) with protein kinase A (PKA), followed by tryptic phosphopeptide mapping resulted in two tryptic phosphopeptides. These tryptic phosphopeptides co-migrated with the phosphopeptides released by the same treatment of F654HPRSSQGVLMPLYSSPIVK675 phosphorylated with the phosphopeptides released by the same tryptic phosphopeptide mapping resulted in two tryptic phosphopeptides. These tryptic phosphopeptides co-migrated with the phosphopeptides released by the same treatment of F654HPRSSQGVLMPLYSSPIVK675 phosphorylated with PKA. Analysis of the phosphorylation site mutants, S659A, S660A, and S659A, S660A disclosed that mutagenesis of both Ser-659 and Ser-660 was necessary to abolish the activation of HSL toward a triolein substrate after phosphorylation with PKA. Mutation of Ser-563 to alanine did not cause significant change of activation compared with wild-type HSL. Hence, our results demonstrate that in addition to the previously identified Ser-563, two other PKA phosphorylation sites, Ser-569 and Ser-660, are present in HSL and, furthermore, that Ser-569 and Ser-660 are the major active regulatory sites of the activation of HSL.

Free fatty acids stored as triacylglycerols in the adipocytes comprise the quantitatively most important energy substrate in mammals. Hormone-sensitive lipase (HSL) catalyzes the first and rate-limiting step in the hydrolysis of stored triacylglycerols and is thereby a key enzyme in the mobilization of free fatty acids from adipose tissue (1). The hormonal and neural control of lipolysis is exerted by regulation of HSL activity, mediated by reversible phosphorylation (2, 3). In response to catecholamines and other fast-acting lipolytic hormones, HSL is activated through phosphorylation by protein kinase A (PKA). The major antilipolytic hormone insulin prevents cAMP-mediated phosphorylation and activation of HSL (3). The antilipolytic effect of insulin is brought about mainly by activation of phosphodiesterase 3B (4).

HSL has in intact rat adipocytes been reported to be phosphorylated at two sites (3). These sites were later identified as Ser-563 (5) and Ser-565 (6). In hormonally quiescent cells, only Ser-565 is phosphorylated (3). Stimulation with noradrenaline increases the phosphorylation extent of Ser-563 to that of Ser-565 (3). Ser-563 is also phosphorylated in vitro by PKA (5). Ser-565 has been shown to be phosphorylated in vitro by the 5’-AMP-activated kinase (6). This kinase, which phosphorylates and regulates the activity of other key enzymes in lipid metabolism (7), acetyl-CoA carboxylase (fatty acid synthesis), and 3-hydroxy-3-methylglutaryl-CoA (cholesterol synthesis), has also been suggested to be the kinase phosphorylating Ser-565 in vivo (8). Phosphorylation of Ser-565 does not directly affect the activity of HSL (6). Based on these observations, Ser-563 has been anticipated to be the activity-controlling site, although direct evidence for this has not been reported.

Phosphorylation of Ser-563 and phosphorylation of Ser-565 in HSL have been shown to be mutually exclusive (9), and phosphorylation of Ser-565 has been shown to inhibit subsequent phosphorylation and activation by PKA (6). Because of the negative interaction between phosphorylation of Ser-563 and Ser-565, phosphorylation of Ser-565 has been suggested to have an antilipolytic role (6). The mechanism behind activation of HSL upon phosphorylation by PKA is poorly understood, but it seems to involve translocation from the cytosol to the lipid droplet (10, 11) and possibly conformational changes and change in specific activity.

To establish the role of Ser-563 in phosphorylation and activation of HSL and to examine the interaction between the phosphorylation sites Ser-563 and Ser-565, we created a set of phosphorylation site mutants. Surprisingly, mutagenesis of Ser-563 did not abolish activation of HSL upon phosphorylation with PKA, indicating that a PKA phosphorylation site(s) in addition to Ser-563 exists in HSL. These results led us to perform two-dimensional tryptic phosphopeptide mapping of HSL phosphorylated in vitro with PKA or isolated from intact 32P-labeled adipocytes. Consequently, we report here the identification of two novel regulatory phosphorylation sites in HSL that are phosphorylated in response to isoproterenol. Mutation of both of these phosphorylation sites (to alanines) abolished the activation of HSL upon phosphorylation with PKA in vitro. Hence, we provide evidence that other PKA phosphorylation sites in addition to the previously identified Ser-563 exist in HSL and that these novel phosphorylation sites govern the activation properties of HSL in vitro.
**EXPERIMENTAL PROCEDURES**

**Construction of HSL Mutants and Expression in COS Cells**—The full-length rat HSL cDNA, subcloned into the expression vector pSVL (Pharmacia Biotech Inc.), was as described previously (12). The mutations were introduced using the overlap extension method (13) and high-fidelity Vent DNA polymerase (New England Biolabs), as described previously (12). The end primers used to generate fragments containing the original (wild) and the mutated (mut) nucleotide positions 2159 to 2160 and 2905–2926, respectively, in the rat HSL cDNA sequence. The mutagenic primers were located at nucleotide positions 2290–2317 (encoding replacement of Ser-563 and/or Ser-565) and 2576–2602 (encoding replacement of Ser-659 and/or Ser-660). Standard polymerase chain reaction thermal cycling parameters and 20 cycles in each of the two polymerase chain reactions were used. A cassette approach was employed to replace fragments of wild-type HSL cDNA by equivalent polymerase chain reaction-generated fragments containing the desired mutations (14). A 409-base pair cassette, obtained utilizing the unique sites for A/III and BesIII, was used for fragments encoding replacement of Ser-563 and Ser-656, and a 716-base pair cassette, obtained utilizing the unique sites for A/III and SstII, was used for fragments encoding replacement of Ser-659 and Ser-660. Constructs encoding replacements in Ser-563 and/or Ser-565 and Ser-659 and/or Ser-660 were generated by exchanging 308-base pair cassettes (from BesIII to SstII) between the corresponding pSVL constructs. The identity of each mutant and the absence of undesired mutations were verified by polymerase chain reaction–based automated sequencing, using Taq DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer) and a model 373A DNA sequencer (Applied Biosystems). Expression of HSL and HSL mutants in insect cells was performed as described using a baculovirus transfection kit (Pharmingen). Production of recombinant rat HSL in Transfections were carried out as described in the Baculovirus Expression Vector system manual from Pharmingen using the Baculogold transfection kit (Pharmingen). Production of recombinant rat HSL in the Baculovirus/insect cell system and subsequent purification by anion-exchange chromatography on Q-Sepharose FF (Pharmacia) was performed as described previously (16).

**Enzyme Assays and Phosphorylation by PKA—**HSL was routinely assayed using emulsified 1(3)-mono-[3H]-oleyl-2-acylglycerol (a diacylglycerol ether analogue) as substrate (16, 17). Phosphorylation and activation of HSL with PKA was carried out by incubation with the catalytic subunit of protein kinase A (Sigma or Promega; 0.2 units/μmol of 110 nM), 0.4 mM ATP, 5 mM MgCl₂, 1 mM dithioerythritol, and 1 mM okadaic acid for 30 min at 37 °C. The concentration of HSL ranged from 0.1 to 40 nM in different experiments. Within this range, no difference in phosphorylation state or degree of activation of the enzyme due to altered PKA:HSL ratios were observed. In controls, PKA was omitted or subjected to SDS-PAGE analysis to identify the band corresponding to 32P-HSL after digital imaging of 32P (Fuji BAS 2000, Fuji Inc.). 32P-HSL was excised and digested with trypsin (modified sequencing grade; Promega) as described (22, 23). One μg of trypsin in 200 μl of 50 mM ammonium bicarbonate was applied per piece of membrane and incubated for 12 h at 37 °C. About 90% of the 32P radioactivity was released from the membranes. Eluates were vacuum centrifuged and oxidized with formic acid as described (23). The oxidized phosphopeptides were desalted for 12 h at 37 °C in 140 μl of pH 1.9 buffer (formic acid:glacial acetic acid:H₂O, 4:145:1800 (v/v/v)) oxidized with formic acid:glacial acetic acid:H₂O, 4:145:1800 (v/v/v)) was added, and the mixture was centrifuged (10,000 × g for 10 min) and vacuum centrifuged. Finally, the phosphopeptides were dissolved in 10 μl of pH 1.9 buffer and subjected to two-dimensional phosphopeptide analysis using the Hunter thin layer electrophoresis apparatus (HTLE-7000, CMS Scientific). The first dimension was performed in pH 1.9 buffer for 23 min at 2000 V, and the second dimension of ascending TLC was performed in isobutyric acid buffer (isobutyric acid:n-butanol:pyridine:glacial acetic acid:H₂O, 1250:38:96:58:558 (v/v/v/v/v)). In some experiments, after labeling of 32P by digital imaging, 32P-phosphopeptides were scraped from the TLC plates, eluted in pH 1.9 buffer, and lyophilized. The phosphopeptides were either rerun in the two-dimensional system to assess co-migration with other peptides or subjected to additional digestion with chymotrypsin. One μg of chymotrypsin (sequencing grade; Boehringer Mannheim) was added to the phosphopeptides in 100 mM Tris-HCl, 10 mM CaCl₂ (50 μl) and incubated for 12 h at 25 °C. Trifluoroacetic acid (0.1%; 400 μl) was added, and the digests were applied to C18 disposable micromicelles (Chromobond, Macherey-Nagel) (24). The eluate was then subjected to two-dimensional phosphopeptide mapping.

**Phosphorylation and Trypsinization of a Synthetic Peptide Corresponding to Rat HSL Amino Acid Sequence—**The peptide FSGHPRRSSQGVLHMPLYSSPVK(25) was synthesized (Biomolecular Core Facilities, Lund University) on an Applied Biosystems model 430A using the Fmoc-(N-9-fluorenylmethoxycarbonyl) program and purified by preparative reversed phase chromatography (Kromasil C8 column). The purity of the peptide was checked by high performance liquid chromatography. The peptide was phosphorylated with PKA, subjected to chromatography on C18 disposable micromicelles, and vacuum centrifuged. The phosphopeptides were suspended in 50 μl of 50 mM ammonium bicarbonate buffer, subjected to the trypsinization, and further treated as described above.

**Western Blot Analysis—**Aliquots of COS cell homogenates, corresponding to 3 milligrams of HSL activity, were subjected to SDS-PAGE analysis, followed by Western blot analysis using the ECL system (Amersham Life Science, Inc.) with polyclonal chicken anti-rat HSL and horseradish peroxidase-conjugated anti-chicken IgG (Sigma).

**RESULTS**

**Analysis of HSL Phosphorylation Site Mutants S563A, S565A, and S563A,S565A—**To establish the role of and study the interaction between the different phosphorylation sites in HSL, the mutants S563A, S565A, and S563A,S565A were constructed and expressed in COS cells. The expressed mutants all had basal activities toward di- and triglycerides substrates that were comparable to those of wtHSL (data not shown). For the purpose of determining the activation of these mutants upon *in vitro* phosphorylation with PKA, emulsified triolein was used as substrate instead of the diacylglycerol analogue that is routinely used for measuring HSL activity because triolein and cholesterol olate are superior to diglyceride and monoglyceride substrates for discrimination between the activity of phosphorylated and nonphosphorylated HSL (25–28).

Ser-563 has been reported to be the only site phosphorylated by PKA *in vivo* and *in vitro*. Therefore, we expected a complete abolishment of the activation of S563A. However, as shown in Fig. 1, the activation of S563A and S565A, S565A was comparable to that obtained for wtHSL, suggesting that PKA phosphorylates site(s) other than Ser-563 exist in HSL. The activation of S565A was increased relative to that of wtHSL (Fig. 1), as would be expected based on the observation that phosphorylation of Ser-565 inhibits subsequent phosphorylation and activation by PKA (6) and assuming a substantial phosphorylation of Ser-565 in COS cells.

To obtain higher protein purity and to further characterize the phosphorylation site mutants, S563A, S565A, S563A,S565A, and wtHSL were overexpressed in Sf9 cells and purified to
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FIG. 1. Activation of S563A, S565A, S563A,S565A, and wtHSL (wild-type) Homogenates of COS cells transfected with expression vectors encoding wtHSL or different HSL mutants were phosphorylated with the catalytic subunit of PKA for 30 min at 37 °C. HSL activity was assayed against a 0.5 mM triolein substrate. Activation (activity of phosphorylated/ activity of nonphosphorylated HSL) is expressed as percentage activity relative to wtHSL. The results are representative of 15 independent experiments.

FIG. 2. Two-dimensional tryptic phosphopeptide maps of HSL purified from rat adipose tissue, recombinant rat HSL, S563A,S565A, and HSL from intact rat adipocytes stimulated with isoproterenol. Rat adipose HSL (A), recombinant rat HSL (B), and the HSL mutant S563A,S565A (C) were phosphorylated with PKA in the presence of [γ-32P]ATP. [32P]-HSL from the fat cake of isoproterenol-stimulated (D) and nonstimulated (E) adipocytes was isolated by SDS-PAGE and electroblotting to nitrocellulose membranes. Tryptic phosphopeptides were analyzed by two-dimensional TLE/TLC. tPPs are numbered 1–4 as follows: 1, tPP containing Ser-565; 2, tPP containing Ser-563; and 3 and 4, unidentified phosphopeptides tPP3 and tPP4.

Identification of Novel Phosphorylation Sites—HSL mutants were phosphorylated with PKA, and [γ-32P]ATP, radioactivity was incorporated into all of the mutants. Furthermore, the activation pattern for these partially purified mutants was the same as for the crude COS cell-expressed mutants shown in Fig. 1 (data not shown). The stoichiometry of phosphorylation of wtHSL was about 1 mole of phosphate per mole of enzyme, in accordance with previous results (2, 16). The incorporation of [32P] phosphate in S563A,S565A was about 40% compared with wtHSL (data not shown), consistent with the existence of another phosphorylation site(s) in HSL.

Phosphopeptide Mapping of HSL Phosphorylated in Vitro with PKA—Because of our results indicating the presence of sites in HSL other than Ser-563 that are phosphorylated by PKA, we decided to investigate the phosphorylation pattern of HSL. Two-dimensional tryptic [32P] phosphopeptide maps of recombinant wtHSL, HSL purified from rat adipose tissue, and S563A,S565A, phosphorylated in vitro with PKA are shown in Fig. 2, A–C. By comparison of the phosphopeptide maps in Fig. 2, the tryptic phosphopeptide (tPP) corresponding to Ser-563 (identified by Garton et al. (5)), could be tentatively assigned. This assignment was supported by subjecting the mutants S563A and S565A to phosphopeptide mapping analysis and also by radiosequencing, which showed that both tPP1 and tPP2 were monophosphorylated, with radioactivity released in cycle 3 for tPP1 and in cycle 1 for tPP2 (data not shown). This suggests that tPP1 and tPP2 are derived from different HSL molecules phosphorylated on either Ser-563 or Ser-565. Identical phosphopeptide maps were obtained from recombinant wtHSL and HSL purified from adipose tissue (Fig. 2), thus confirming our previous study showing that recombinant rat HSL exhibits enzymological properties identical to HSL purified from rat adipose tissue (16). In the tryptic phosphopeptide map of S563A,S565A, two tPPs, designated tPP3 and tPP4, were obtained. The extent of phosphorylation of tPP3 and tPP4 compared with the tPP containing the phosphorylated Ser-563 of S563A,S565A was about 25% for each.

Phosphopeptide Mapping of HSL Isolated from Isoproterenol-Stimulated Rat Adipocytes—To ascertain that phosphorylation of the additional phosphorylation site(s) observed in vitro was found in vivo, we compared tryptic [32P] phosphopeptide maps of S563A,S565A phosphorylated by PKA, with those of HSL isolated from intact [32P]-labeled, isoproterenol-stimulated adipocytes. Fig. 2, D and E, displays tryptic phosphopeptide maps of translocated HSL from the fat cake, isolated from quiescent and isoproterenol-stimulated [32P]-labeled adipocytes, respectively. The tPP3 and tPP4 from the tryptic phosphopeptide map of S563A,S565A (Fig. 2C) were eluted from the TLC plates, mixed with apparent equivalent HSL peptides isolated from the fat cake of isoproterenol-stimulated cells (Fig. 2D), and rerun using two-dimensional phosphopeptide mapping. Both tPP3 and tPP4 co-migrated with the respective phosphopeptides obtained from HSL isolated from isoproterenol-stimulated cells (Fig. 3). To further verify that the two apparently equivalent tPPs from isoproterenol-stimulated cells migrated identically to tPP3 and tPP4 from S563A,S565A, the tryptic phosphopeptides in question were reanalyzed with chymotrypsin. The chymotrypsin treatment clearly generated new phosphopeptides (Fig. 4). The chymotrypsin-treated tPP3 and tPP4 from isoproterenol-stimulated cells (Fig. 2D) were shown to co-migrate with tPP3 and tPP4 from S563A,S565A (Fig. 2C), which were also reanalyzed with chymotrypsin (Fig. 4, A–F). Thus, tPP3 and tPP4 from PHA-K-phosphorylated S563A,S565A were identical to tPP3 and tPP4 from isoproterenol-stimulated cells. Hence, the additional phosphorylation site(s) in HSL (which give rise to tPP3 and tPP4) were assumed to be of physiological importance.

Identification of the Novel Phosphorylation Sites—The amino acid sequences of HSL from different species (16) are compared, there are, in addition to Ser-563, three potential PKA sites based on the consensus sequence R(R/K)SS. These sites are coded for by exon 1 (Ser-99 in rat HSL) and exon 8 (Ser-659 and Ser-660 in rat HSL). From sequence alignments with lipase 2 from Moraxella TA144 (16) and from a three-dimensional model of HSL based on similarity in organization of secondary structure elements of HSL and a superfamily of esterases and lipases (29), it has been suggested that parts of...
Fig. 3. Comparison of tryptic phosphopeptides from rat adipocyte HSL phosphorylated in cells with S563A, S565A. Tryptic phosphopeptides tPP3 and tPP4, from rat adipocyte HSL (fat cake, isoproterenol-stimulated cells) and from the HSL mutant S563A,S565A phosphorylated in vitro with PKA were eluted from the TLC plates and rerun in two-dimensional TLE/TLC alone (A, B, D, and E) or mixed (C and F) as indicated. X marks the origin. ISO, isoproterenol; FC, fat cake.

Fig. 4. Comparison of chymotryptic-treated phosphopeptides from rat adipocyte HSL phosphorylated in cells with phosphopeptides from chymotryptic-treated S563A,S565A, tPP3 and tPP4, from rat adipocyte HSL phosphorylated in cells and from HSL mutant S563A,S565A phosphorylated in vitro with PKA were eluted from the TLC plates, digested with chymotrypsin and rerun in two-dimensional TLE/TLC alone (A, B, D, and E) or mixed (C and F) as indicated. ctPP3 and ctPP4 denote chymotryptic-digested tPP3 and tPP4, respectively. X marks the origin. ISO, isoproterenol; FC, fat cake.

exon 7 and exon 8 (which encode the previously identified phosphorylation sites) constitute a regulatory module that has been inserted in HSL during evolution. We therefore hypothesized that the identities of the novel phosphorylation sites were Ser-659 and Ser-660. The peptide F654HPRRSSQVGLHMP-LYSSPIVK675 was synthesized. After phosphorylation of this synthetic peptide with PKA and subsequent two-dimensional tryptic phosphopeptide mapping, two tPPs that appeared to migrate similarly to tPP3 and tPP4 in the two-dimensional separation system were obtained. In mixing experiments, the tryptic phosphopeptides obtained from F654HPRRSSQVGLHMP-LYSSPIVK675 were shown to co-migrate with tPP3 and tPP4 from HSL isolated from the fat cake from isoproterenol-stimulated cells (Fig. 5).

Radiosequencing of tPP3 and tPP4 revealed that the peptides were di- and monophosphorylated, respectively (Fig. 6). The radioactivity was released in cycle 3 in tPP4 and in cycles 2 and 3 in tPP3. Thus, Ser-659 and Ser-660 were found to be phosphorylated simultaneously in the same peptide. However, the rate of phosphorylation of the two sites probably differs. Based on kinetic measurements performed on peptides containing the motifs RRS and RRX (data not shown), Ser-660 can be assumed to exhibit the higher rate constant.

Analysis of Phosphorylation Site Mutants S659A, S660A, and S659A,S660A—To examine the function of the phosphorylation sites in the activation of HSL, the mutants S659A, S660A, S659A,S660A and mutants involving various combinations of these sites and Ser-563 and Ser-565 were generated and expressed in COS cells. A Western blot analysis of the expressed HSL and HSL mutants is shown in Fig. 7A. The increase in HSL activity after incubation with PKA assayed against a triolein substrate is shown in Fig. 7B. Mutating either Ser-659 or Ser-660 did not cause marked reduction of activation relative to wtHSL. Mutants involving Ser-659 generally displayed lower activation than mutants involving Ser-660. However, mutating both Ser-659 and Ser-660 resulted in
complete abolishment of activation (Fig. 7B). Tryptic phosphopeptide mapping analysis of the mutant S659A,S660A phosphorylated with PKA resulted in a disappearance of tPP3 and tPP4 (data not shown).

All of the mutants were expressed (Fig. 7A) in an apparently stable and enzymatically active form. Interestingly, because equal amounts of HSL activity were loaded on the gel and the intensity of the bands representing S565A, S565E, and S563A,S565A is significantly lower than for the other mutants, it can be concluded that these mutant proteins exhibit higher...
specific activity than wtHSL and the other mutants. The significance of this is not known.

**DISCUSSION**

Our results show that HSL in isoproterenol-stimulated adipocytes is phosphorylated at the novel sites Ser-659 and Ser-660, as well as at the previously identified Ser-563. Thus, HSL, like other metabolically important enzymes, such as glycogen synthase and acetyl-CoA carboxylase, is subject to complex activity regulation involving multisite phosphorylation. Using site-directed mutagenesis, the importance of the different phosphorylation sites in the activation of HSL upon phosphorylation with PKA was elucidated (Fig. 7B). The results clearly show that the novel phosphorylation sites Ser-659 and Ser-660 are the critical activity controlling sites, whereas Ser-563 plays a minor role in direct activation of HSL. The fact that the mutants S659A and S660A were both activated to about the same extent as wtHSL indicates that PKA can phosphorylate either Ser-659 or Ser-660 and give conformational changes resulting in comparable activation of HSL. The corresponding sequence in human cardiac troponin I, RRSS, has been shown to involve ordered phosphorylation of the adjacent residues Ser-23 and Ser-24 by PKA (30, 31). Quirk et al. (31) used nmr spectroscopy to show that a diphosphorylation of the adjacent serines induced a more extensive conformational transition than monophosphorylation. This provided an explanation on a molecular basis for the results of Zhang et al. (32) showing that phosphorylation of both of the adjacent serine residues in cardiac troponin I was required for its proper function. Furthermore, Quirk et al. (31) found that the rate of phosphorylation was more rapid for Ser-24 than for Ser-23. Radiosequencing of tPP$_3$ and tPP$_4$ (Fig. 6) from HSL from the fat cake of isoproterenol-stimulated cells disclosed that radioactivity was released in cycles 2 and 3 (tPP$_3$) and in cycle 3 (tPP$_4$). Thus, at maximal lipolysis, besides the diphosphorylated peptide, only the monophosphorylated form involving Ser-660 was found. Hence, the phosphorylation state of Ser-660 is presumably functionally more important in adipocytes at the conditions investigated, i.e. at maximal lipolysis. Furthermore, the activation experiments suggest that phosphorylation of Ser-659 and Ser-660 is not dependent on prior phosphorylation of Ser-563.

The exact activation mechanism of HSL is poorly understood, but it presumably involves both translocation of HSL (10, 11) and conformational changes in the HSL molecule. It is possible that different phosphorylation sites in HSL play different roles in the process of translocation and increase of specific activity of HSL. The in vitro system used in this study to measure HSL activity does not reflect the translocation of HSL that presumably occurs in vivo. This, together with the difference in nature of the substrates utilized in vivo and in vitro, could be the explanation for the discrepancy in the fold activation obtained in the in vitro assay (2–3-fold) and the in vivo assay (50-fold), as has previously been discussed extensively in the literature (33, 34). Thus, the activity of HSL in vivo is subject to more complex activity regulation involving several assets than given by the in vitro system. Hence, a detailed discussion of the relative values of activation for the different mutants (Fig. 7B) does not seem relevant. The role of different phosphorylation sites in the translocation of HSL will have to be investigated in studies using approaches different from those provided in this report.

Phosphorylation of Ser-565 has been reported to prevent...
phosphorylation of Ser-563 and to have an antilipolytic role (6, 9). Although our results confirm that phosphorylation of Ser-563 and phosphorylation of Ser-565 are mutually exclusive, the significance of this is difficult to appreciate until the role of phosphorylation of Ser-563 has been clarified. The effect of prior phosphorylation of Ser-565 on the subsequent phosphorylation of Ser-659 and Ser-660 has not been investigated in this study. However, it is interesting to note that the mutant S565A showed considerably higher activation than wtHSL, whereas the mutant S565E (in which a negative charge mimics the effect of phosphorylation) displayed lower activation than S565A, corroborating previous results that reported a negative effect of phosphorylation of Ser-565 on activation of HSL.

In conclusion, we describe the identification of two novel phosphorylation sites, Ser-659 and Ser-660, in rat HSL that are phosphorylated in response to isoproterenol stimulation of rat adipocytes and are also phosphorylated by PKA (effect of phosphorylation) displayed lower activation than S565A, corroborating previous results that reported a negative effect of phosphorylation of Ser-565 on activation of HSL.

Future studies will have to address the activity controlling properties of the novel sites in vitro, as well as the role of the previously identified Ser-563 and the interaction between the different phosphorylation sites.

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