Spatiotemporal Regulation of Early Lipolytic Signaling in Adipocytes

Hormone-sensitive lipase (HSL) is a key enzyme regulating the acute activation of lipolysis. HSL functionality is controlled by multiple phosphorylation events, which regulate its association with the surface of lipid droplets (LDs). We determined the progression and stability of HSL phosphorylation on individual serine residues both spatially and temporally in adipocytes using phospho-specific antibodies. Within seconds of β-adrenergic receptor activation, HSL was phosphorylated on Ser-660, the phosphorylated form appearing in the peripheral cytosol prior to rapid translocation to, and stable association with, LDs. In contrast, phosphorylation of HSL on Ser-563 was delayed, the phosphorylated protein was predominantly detected on LDs, and mutation of the Ser-659/Ser-660 site to Ala significantly reduced subsequent phosphorylation on Ser-563. Phosphorylation of HSL on Ser-565 was observed in control cells; the phosphorylated protein was translocated to LDs with similar kinetics to total HSL, and the degree of phosphorylation was inversely related to phospho-HSLSer-563. These results describe the remarkably rapid, sequential phosphorylation of specific serine residues in HSL at spatially distinct intracellular locales, providing new insight into the complex regulation of lipolysis.

The regulation of lipid storage and lipolysis in adipocytes has important implications for the maintenance of whole body lipid homeostasis (1, 2). Dysregulation is associated with obesity and the onset of metabolic disease, insulin resistance, and type II diabetes. An important goal for the future is to design strategies that allow us to manipulate lipid storage and so control weight gain. Fundamental to a targeted approach to controlling the storage and mobilization of lipid in adipose tissue is a detailed understanding of the cellular mechanisms and machinery that regulate lipolysis. Although the key players in this process have been identified, the precise regulation of the lipolytic machinery is not yet fully understood. In this study we have begun to address this by analyzing the earliest events in the activation of lipolysis at the cellular level.

Adipocytes are specialized lipid droplet (LD)laden cells that store large amounts of neutral lipid, predominantly as triglycerides (TG) (3, 4). In response to extracellular stimulation by catecholamines, adipocytes hydrolyze stored TGs to generate free fatty acids and glycerol. In rodent adipocytes, the hydrolysis of neutral lipids is tightly regulated by a series of signal transduction pathways from the G-protein-coupled β,adrenergic receptor that culminate at the surface of the LD (2, 5). A well characterized pathway from the β,adrenergic receptor results in the elevation of cAMP levels, activating cAMP-dependent protein kinase/protein kinase A (PKA), which in turn phosphorylates downstream targets, including the lipid droplet scaffold/ adaptor protein perilipin (6) and the primary diacylglycerol lipase hormone-sensitive lipase (HSL) (7). The array of phosphorylation sites present on HSL (7–11) suggests a complex regulation with important implications for the control of lipolysis. In this study, we have investigated the spatial and temporal characteristics of HSL phosphorylation in 3T3-L1 adipocytes.

In contrast to perilipin, which is constitutively associated with the LD surface, HSL is a cytosolic protein that translocates to LDs in response to catecholamine stimulation (12). Translocation of HSL is dependent upon phosphorylation (13) and exquisitely regulates the functional activity of HSL in vivo. Phosphorylation of HSL has little effect on the intrinsic activity of the enzyme (14). HSL is phosphorylated on up to three sites by PKA (Ser-563, Ser-659, and Ser-660 (7)) and at a single site by extracellular signal-regulated kinase (ERK) (Ser-600 (10)) in response to the activation of lipolysis. The phosphorylation of both Ser-659 and Ser-660 by PKA is necessary for activated lipolysis and the translocation of HSL to the LD surface (13). However, although Ser-563 is phosphorylated in response to catecholamines, the functional significance of this is not known, as it triggers neither HSL translocation to LDs (13) nor HSL activity in vitro (7). HSL is also phosphorylated on Ser-565 by 5′-AMP-activated protein kinase in unstimulated adipocytes, although the precise role of HSLSer-565 phosphorylation by 5′-AMP-activated protein kinase remains elusive. Mutation of Ser-565 abolishes translocation of HSL to LDs in stimulated cells indicating a role in the activation of lipolysis (13). Phosphorylation of peptides incorporating the Ser-565 site by 5′-AMP-activated protein kinase in vitro inhibits subsequent phosphorylation on the Ser-563 site by PKA (8), indicating a functional relationship between these two sites.

To examine the relationship between the specific phosphorylation events in HSL during activated lipolysis, we analyzed...
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the distribution of phosphorylated HSL biochemically and by immunofluorescence microscopy using phospho-specific antibodies. We found both spatial and temporal differences in the progression of phosphorylation events upon acute activation of lipolysis in adipocytes. Furthermore, we found evidence that not only the phosphorylation but also the dephosphorylation of specific serines in HSL was tightly controlled. In light of these findings, we propose that distinct signaling complexes are involved in the activation of lipolysis, and we hypothesize that the LD-associated phosphorylation complexes are important in regulating the stability and/or duration of lipolysis.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—3T3-L1 fibroblasts (American Type Culture Collection, Manassas, VA) were maintained and differentiated, as described previously (15), and used between days 8 and 15 post-differentiation. Differentiated cells were maintained in growth medium for at least 24 h prior to experimentation to establish control conditions for both indirect immunofluorescence and biochemical analyses or transferred into KRPH containing 2% fatty acid-free bovine serum albumin for the measurement of free glycerol and nonesterified fatty acid release.

Antibodies and Reagents—Rabbit anti-phospho-PKA substrate (RRX(S/P)/T) (9624), P-HSL anti-563 (4139), P-HSL anti-565 (4137), P-HSL anti-660 (4126), and HSL (4107) were obtained from Cell Signaling Technology, and rabbit anti-perilipin A (P1998) and mouse anti-α-tubulin clone DM 1A (T9026) were from Sigma. The rabbit antibody to HSL used for immunofluorescence and immunoelectron microscopy was a generous gift from Dr. Andrew Bicknell, University of Reading, Reading, UK (16). Alexa555-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR). Forskolin was obtained from Merck. The release of nonesterified fatty acids into the media was measured using the NEFA kit from Wako Pure Chemicals Industries Ltd. (Osaka, Japan), and free glycerol release was measured using free glycerol reagent (Sigma). Remaining reagents were obtained from Sigma unless stated otherwise.

Generation of His-HSL(S650A,S651A)—His-tagged murine HSL in the pcDNA-HisMax vector (Invitrogen) was a generous gift of Dr. Robert Zimmerman (University of Graz, Austria). The S650A,S651A mutation (equivalent to S659A,S660A in rat) was generated using the QuikChange Lightning site-directed mutagenesis kit (Stratagene) and the mutational primer 5’TTCAC-CCCCGGGCAGCAGCCCAAGGTGTCCTC-3’. All constructs were sequenced using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) in the Australian Genome Research Facility, University of Queensland.

Subcellular Fractionation and Western Blotting—3T3-L1 adipocytes were fractionated into cytosol, LD, and pellet fractions by centrifugation. Whole cell lysates were centrifuged at 60,000 rpm (TLA100.3) for 30 min. The infranatant (cytosol) was carefully removed from beneath the floating LD fraction, re-centrifuged, and transferred to clean tubes twice more to minimize LD contamination. The LDs were collected from the tube in 500 µl of buffer and washed in TNE twice by centrifugation to deplete cytosol. The membrane pellet was washed once with 500 µl of buffer. All samples were solubilized directly into Laemmli SDS-PAGE sample buffer and equivalent volume fractions analyzed by immunoblotting.

SDS-PAGE and Western blot analysis was carried out as described previously (15). Relative band intensity was measured using the ImageJ software and corrected for background using an equivalent area of blank film.

Cell Lysate Preparation and Immunoprecipitation—Cell lysates were prepared by homogenization in TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA), containing protease (Merck) and phosphatase (Roche Diagnostics) inhibitors and then solubilized in Laemmli sample buffer containing 25 mM dithiothreitol. For time course analysis, cells were activated at 37 °C as described under “Results,” swiftly washed in a large volume of ice-cold TNE, drained, and immediately plunged in liquid N₂, with a processing time of under 3 s. Cells were subsequently thawed on ice into SDS-extraction buffer (25 mM Tris-HCl, pH 7.4, 0.4 % NaCl, 0.5% SDS) containing protease and phosphatase inhibitors. For immunoprecipitation, cells were solubilized in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors at 4 °C for 30 min, and the solubilized proteins were immunoprecipitated using protein A-Sepharose as described previously (15).

His-tagged proteins were expressed in 3T3-L1 adipocytes by electroporation at 950 microfarads, 0.16 kV (Bio-Rad Gene Pulser II and Capacitance Extender Plus) for ~20 ms with 100 µg of DNA. Cells were solubilized in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 8 µl urea, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors. Following clarification by microcentrifugation at 4 °C, the Histagged proteins were isolated by immobilized metal affinity chromatography using His SpinTrap columns according to the manufacturer’s instructions (GE Healthcare).

Mouse Adipose Tissue Explant Culture—Adipose tissue was obtained from the epididymal fat pads of male C57Bl6 mice between the ages of 12 and 21 weeks, sacrificed by CO₂ asphyxiation. Tissue was chopped into small pieces, washed, and maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.1% fatty acid-free bovine serum albumin (PAA Laboratories GmbH, Austria) at 37 °C for 2 h prior to use. Following activation, explants were homogenized in TNE containing protease and phosphatase inhibitors using an Ultra-Turrax homogenizer (IKA, Kuala Lumpur, Malaysia).

Immunofluorescence Microscopy—Indirect immunofluorescence microscopy was performed as described previously (15). Permeabilization prior to fixation was performed using 0.05% saponin in phosphate-buffered saline for 5 s at room temperature, and the cells were immediately fixed by addition of an equal volume of 8% paraformaldehyde in phosphate-buffered saline. Labeling was analyzed using an Axiovert 200 M SP LSM 510 META confocal laser scanning microscope (Carl Zeiss Pty. Ltd., North Ryde, Australia) under oil, using either 100 or 63X oil immersion objectives. The data were processed using the
Profiles—Lipolysis in 3T3-L1 adipocytes was activated using either a specific β₂-adrenergic receptor agonist (CL316,243), a nonselective β-adrenergic receptor agonist (isoproterenol), or by the direct elevation of intracellular cAMP levels using a combination of forskolin to activate adenylate cyclase and IBMX to inhibit phosphodiesterase (Fsk/IBMX). In each case, a robust increase in lipolysis was observed, measured by the release of both NEFA and glycerol (Fig. 1A). For each treatment, the net release over a 2-h period was identical, indicating that activation of PKA by signal transduction from the cell surface and direct elevation in cAMP are capable of stimulating the maximal net release of lipolytic products.

The regulatory module of HSL contains five known phosphorylation sites targeted by at least three distinct protein kinases (Fig. 1B). The effect of activating lipolysis on the phosphorylation of HSL by both PKA (HSLSer-563 and HSLSer-660) and by 5'-AMP-activated protein kinase (HSLSer-565) was analyzed using phospho-specific antibodies. In unstimulated cells, only P-HSLSer-565 was detected, with no constitutive phosphorylation of either HSLSer-563 or HSSLer-660 with a decrease in the phosphorylation of HSSLer-565. However, although HSSLer-660 was phosphorylated to equal levels in response to different activation protocols, HSSLer-563 was more highly phosphorylated in the Fsk/IBMX-treated cells than following cell surface signaling (Fig. 1D). These data showed that the net degree of phosphorylation of HSL on the different target serines was differentially modulated by receptor-mediated activation and direct elevation of cAMP levels.

To examine phosphorylation of multiple serine residues on individual HSL proteins, cell lysates were solubilized, and both total and phospho-HSL were immunoprecipitated (Fig. 1E). Immunoprecipitation of total HSL from unstimulated adipocytes co-precipitated P-HSLSer-563, only, consistent with constitutive phosphorylation of this site. Following activation of lipolysis for 30 min, we were able to co-precipitate P-HSLSer-563 with P-HSLSer-660 and P-HSLSer-660 with P-HSLSer-660, but no interaction between P-HSSLer-563 and P-HSSLer-660 was detected. This is con...

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FIGURE 1. Isoproterenol, CL316,243, and Fsk/IBMX activate lipolysis to the same degree while generating different phosphoprotein profiles. A, net release of glycerol and NEFA over a 2-h period was measured following activation of lipolysis using either 10 μM isoproterenol (Isop), 10 μM CL316,243 (CL), or a combination of 10 μM Fsk/500 μM IBMX (n = 3, ± S.E.). B, schematic model of phosphorylation sites on rat HSL. C, whole cell lysates were prepared from 3T3-L1 adipocytes treated with either vehicle (DMSO), 10 μM isoproterenol, 10 μM CL316,243, or 10 μM Fsk/500 μM IBMX for 30 min. Samples were immunoblotted for total HSL, P-HSLSer-563, P-HSLSer-565, or α-tubulin. D, band intensities shown in C were quantified relative to tubulin and normalized to Fsk/IBMX levels (n = 5, ± S.E.). E, whole cell lysates were prepared from control 3T3-L1 adipocytes treated with either 10 μM isoproterenol (lane 2) or 10 μM Fsk/500 μM IBMX (lane 3) for 30 min, solubilized, and phosphorylated forms of HSL immunoprecipitated (IP). Immunoprecipitates were immunoblotted for total HSL, P-HSLSer-563, P-HSLSer-660, and P-HSLSer-565. A control immunoprecipitation was performed using rabbit IgG (C). F, immunodepletion of P-HSLSer-563, P-HSLSer-565, and P-HSLSer-660 from solubilized cell lysates by antibodies raised against P-HSLSer-563 and P-HSLSer-660 was determined by immunoblotting.
sistent with in vitro peptide data (8) and also suggests that oligo-
meric forms of HSL (18) are exclusively phosphorylated on either
Ser-563 or Ser-565.

To determine the relative level of phosphorylation of HSL on
Ser-563 and Ser-660 in lipolytically active cells, we performed
quantitative immunodepletion of solubilized whole cell lysates (Fig.
1F). We found that the antibody raised against the phosphorylated
Ser-660 site of HSL was able to quantitatively immunodeplete
total HSL, P-HSL Ser-563, and P-HSL Ser-565 from cells indicating
that the total cellular pool of HSL is either phosphorylated on Ser-
660 or forms oligomers in which one or all HSL molecules are phos-
phorylated on Ser-660. In contrast, although the antibody raised against
the phosphorylated Ser-563 site of HSL was capable of quantitative
immunodepletion of the Ser-563 phosphorylated protein, it resulted
in only ~10% depletion of total HSL and P-HSL Ser-660 and no
detectable depletion of P-HSL Ser-565 (Fig. 1F).

Phosphorylated HSL Localizes to Both Large LDs and Micro-LDs
upon Activation of Lipolysis—Previous studies have shown that LDs
undergo structural alterations during lipolysis, with the formation of
a multitude of micro-LDs in the cell periphery, the function and
origin of which are unknown (19–21). The association of phosphory-
lated forms of HSL with micro-LDs was determined by immuno-
fluorescence microscopy in response to either isoproterenol stimulation or
Fsk/IBMX (Fig. 2A). The distribu-
tion of P-HSL Ser-565 mirrored that of total HSL, translocating from the
cytosol of unstimulated cells to LDs upon activation of lipolysis (22). In
contrast, ~<1% of unstimulated cells was labeled for P-HSL Ser-563
or P-HSL Ser-660, but both forms of the protein were detected on large LDs
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ther characterized by quantitative immunoelectron microscopy (Fig. 2, C–I). In unstimulated cells, HSL was present in the cytosol, and a low level was associated with the surface of large LDs (Fig. 2C; Table 1). Following a 30-min activation using either isoproterenol (results not shown) or Fsk/IBMX (Fig. 2, E–I), HSL was largely absent from the cytosol and associated with the surface of large LDs and numerous micro-LD (operationally, micro-LDs were defined as <1 μm in diameter). The morphological identification of peripheral micro-LDs was confirmed by perilipin A labeling (Fig. 2D). The proportion of LDs classified as micro-LDs increased from 18% to 62% and 52% in isoproterenol- and Fsk/IBMX-activated cells, respectively (6–12 cells/treatment, n = 2 separate experiments).

No significant difference was observed between the labeling density of HSL on large LDs and micro-LDs in either isoproterenol- or Fsk/IBMX-activated cells (Table 1). Recruited HSL showed no preference for any morphologically identifiable surface domain of the LD and was neither enriched nor excluded from regions of the LD surface in close apposition to the endoplasmic reticulum (Fig. 2G).

### TABLE 1

| Gold labeling/μm² LD surface area | Control | 10 μM isoproterenol | 10 μM Fsk/500 μM IBMX |
|----------------------------------|---------|---------------------|------------------------|
| >1 μm diameter                   | 3.8 ± 4.2 | 6.3 ± 2.6 | 7.8 ± 2.4               |
| <1 μm diameter                   | 2.2 ± 2.2 | 7.4 ± 1.4 | 8.2 ± 1.3               |

*Data are classified as micro-LDs.

### FIGURE 3

**A.** Whole cell lysates were prepared from control 3T3-L1 adipocytes or cells treated with either 10 μM isoproterenol or 10 μM Fsk/500 μM IBMX for times between 5 s and 30 min. Samples were immunoblotted for total HSL, P-HSLSer-563, P-HSLSer-660, perilipin A, PKA substrates (RRX(S*/T*)), or α-tubulin. **B.** Intensity of protein bands corresponding to P-HSLSer-563, P-HSLSer-660, and P-perilipin A (PKA substrates) was measured and normalized to the intensity of α-tubulin (n = 3 ± S.E.). **C.** Intensity of protein bands corresponding to P-HSLSer-566 was measured and normalized to the intensity of α-tubulin in response to isoproterenol or Fsk/IBMX. (n = 3 ± S.E., p < 0.05).

### FIGURE 3

**Temporal analysis of HSL and perilipin A phosphorylation.** A, whole cell lysates were prepared from control 3T3-L1 adipocytes or cells treated with either 10 μM isoproterenol or 10 μM Fsk/500 μM IBMX for times between 5 s and 30 min. Samples were immunoblotted for total HSL, P-HSLSer-563, P-HSLSer-660, perilipin A, PKA substrates (RRX(S*/T*)), or α-tubulin. B, intensity of protein bands corresponding to P-HSLSer-563, P-HSLSer-660, and P-perilipin A (PKA substrates) was measured and normalized to the intensity of α-tubulin (n = 3 ± S.E.). C, intensity of protein bands corresponding to P-HSLSer-566 was measured and normalized to the intensity of α-tubulin in response to isoproterenol or Fsk/IBMX. (n = 3 ± S.E., p < 0.05).

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**TABLE 1**

Density of HSL labeling on the surface of LDs

Results are shown as mean ± S.D., n = 2 separate experiments, 6–12 cells/treatment.

| Gold labeling/μm² LD surface area | Control | 10 μM isoproterenol | 10 μM Fsk/500 μM IBMX |
|----------------------------------|---------|---------------------|------------------------|
| >1 μm diameter                   | 3.8 ± 4.2 | 6.3 ± 2.6 | 7.8 ± 2.4               |
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Temporal Regulation of Specific HSL Phosphorylation Events—We next examined the kinetics of individual phosphorylation events in response to isoproterenol or Fsk/IBMX treatment. Strikingly, phosphorylation of HSL on Ser-660 was found to consistently occur within seconds of activation and preceded both the phosphorylation of HSL on Ser-563 and the initial phosphorylation of the LD protein perilipin A (detected using an antibody raised against phospho-PKA substrates, consensus sequence RRX(S*/T*)) (Fig. 3, A and B). Phosphorylation of Ser-563 was significantly delayed, first appearing 30–60 s after the onset of activation, and corresponded temporally with maximal phosphorylation of Ser-660 and a significant increase in the phosphorylation of perilipin A (Fig. 3B). The very rapid phosphorylation of Ser-660, preceding other phosphoryla-
tion events, suggests that this is the initial step in the lipolysis pathway. The sequential nature of HSL phosphorylation, and its relation to perilipin A phosphorylation, was consistent regardless of whether lipolysis was activated by receptor-mediated signaling or direct elevation of cAMP. However, the initial phosphorylation of Ser-660 and perilipin A occurred slightly later when cAMP levels were directly elevated by Fsk/IBMX than with the \( \beta_3 \)-adrenergic receptor agonists, suggesting tight coupling of receptor activation and downstream phosphorylation events. Together with the incredibly rapid kinetics of activation, this suggests the presence of a signaling complex directly coupled to the \( \beta_3 \)-adrenergic receptor.

When the net phosphorylation of HSL was examined over 30 min, further differences in regulation were observed. Following receptor-mediated signaling and direct elevation of cAMP, HSL Ser-660 phosphorylation attained maximal levels by 30 s and remained at a high level for the total period of activation. In contrast, the different activation protocols resulted in differences in the net phosphorylation of Ser-563 and Ser-565 after a 30-min activation. Whereas Ser-563 phosphorylation was augmented by Fsk/IBMX treatment relative to isoproterenol (Fig. 3, A and B), the phosphorylation of Ser-565 was significantly lower after a 30-min Fsk/IBMX treatment than a 30-min isoproterenol treatment (Fig. 3C). These results show that the net phosphorylation of HSL on specific serine residues in response to receptor-mediated activation is controlled by both phosphorylation and dephosphorylation events.

**Primary Adipose Tissue**—To determine whether the effect of acute activation of lipolysis on the phosphorylation of HSL is similarly regulated *in vivo*, we performed experiments in mouse adipose tissue explants (Fig. 4). As in the 3T3-L1 adipocytes, we compared the effect over time of activating lipolysis using either isoproterenol or Fsk/IBMX. Consistent with the results in 3T3-L1 adipocytes, we found that HSL was constitutively phosphorylated on Ser-565 only in control explants. Both isoproterenol and Fsk/IBMX induced a robust phosphorylation of both HSL Ser-563 and HSL Ser-660 after 10 min, with a concomi-

tant decrease in the phosphorylation of HSL Ser-565. After 30 min of activation, phosphorylation of HSL Ser-563 was significantly reduced in the isoproterenol-treated samples but was unchanged in cells activated with Fsk/IBMX, confirming the validity of the 3T3-L1 adipocyte model and suggesting that similar regulatory mechanisms were acting *in vivo*.

**Spatial Distribution of Phosphorylated HSL**—As biochemical analysis of HSL phosphorylation upon activation of lipolysis had shown that the two PKA-mediated phosphorylation events could be temporally separated, we subsequently analyzed these events spatially using immunofluorescence microscopy and biochemistry. The localization of phosphorylated forms of HSL was analyzed by immunofluorescence microscopy at times corresponding to early (1 min) and late (10 min) post-activation, times at which all three serines were predicted to be phosphorylated (Fig. 3). Neither P-HSL Ser-563 nor P-HSL Ser-660 was detected in control cells, and P-HSL Ser-565 was detected only in the cytosol, consistent with the basal distribution state of HSL (Fig. 2). After a 1-min activation using either isoproterenol (Fig. 5A) or Fsk/IBMX (results not shown), P-HSL Ser-660 was observed in both the cytosol of 3T3-L1 adipocytes and at the surface of LDs. In contrast, P-HSL Ser-565 was only observed to label lipid droplets, even after short times of activation, and there was no detectable cytosolic pool. At all times P-HSL Ser-565 distribution paralleled that of total HSL. Following activation of the cells for 10 min, all phosphorylated forms of HSL were found to be present on the surface of large perilin-LDs, often in a punctate pattern, and as small puncta distributed through the cytosol. It is important to note that at these times the large increase in the number of micro-LDs observed at 30 min (Fig. 2) had not yet occurred, simplifying the analysis of P-HSL distribution. As all the antibodies were raised in rabbits, we were unable to determine unequivocally that HSL phosphorylated on Ser-565, -563, and/or -660 were present on the same LDs. However, no significant difference between the numbers of LDs labeled for each HSL-phosphorylated species was observed.

To confirm the distribution of phosphorylated forms of HSL, we analyzed cytosol and LD fractions at various times after activation of lipolysis using isoproterenol (Fig. 5B). Consistent with the immunofluorescence microscopy, HSL predominantly fractionated into the cytosol, and a small component was immunoblotted for total HSL. P-HSL Ser-563 and perilipin A, PKA substrates (RR\( \rightarrow \) receptor-mediated signaling and direct elevation of cAMP, respectively, were separated, we subsequently

**FIGURE 4. Phosphorylation of HSL and perilipin A in primary adipose tissue explants**. A, adipose tissue explants were treated with vehicle (C), 10 \( \mu \)M isoproterenol (Isop), or 10 \( \mu \)M forskolin, 500 \( \mu \)M IBMX for 10 or 30 min, and homogenates were immunoblotted for total HSL, P-HSL Ser-563, P-HSL Ser-660, perilipin A, PKA substrates (RR\( \rightarrow \)S*/T*), or \( \beta \)-actin. B, level of phosphorylation was quantified relative to \( \beta \)-actin expression. Results expressed as mean ± S.E., \( n = 5, ^* p < 0.05 \).
LD fraction. The amount of P-HSLSer-563 decreased after 30 min in the LD fraction consistent with the transience of this phosphorylation event in response to isoproterenol activation. Perilipin A was predominantly present in the LD fraction. The low signal detected in the cytosolic fraction was presumed to derive from contamination with LDs. Phosphorylated perilipin A was identified by both the shift in mobility on SDS-PAGE and as a phospho-PKA substrate predominantly in the LD fraction, whereas markers of the membrane compartment (caveolin 1) and the cytosolic fraction (α-tubulin) confirmed that there was little contamination between the different fractions.

Distribution of P-HSLSer-660 following Activation of Lipolysis—The temporal analysis of HSL phosphorylation of Ser-660 had shown that this species was generated within 5 s of isoproterenol activation of lipolysis (Fig. 3), and spatial analysis demonstrated that the phosphorylated form was detected in the cytosol 1 min after activation (Fig. 5). To examine the rapid kinetics of HSLSer-660 phosphorylation and translocation to the LD surface, we analyzed its distribution within 5–10 s of isoproterenol stimulation by immunofluorescence microscopy. In addition, we compared the distribution of P-HSLSer-660 in fixed cells, where there is a significant cytosolic component during early activation, and in cells permeabilized prior to fixation, to deplete the cytosolic pool and examine whether P-HSLSer-660 is associated with membrane structures in the cell. Within 5 s of isoproterenol activation of lipolysis, P-HSLSer-660 was detected in the cytosol of 3T3-L1 adipocytes (Fig. 6A). Upon analysis, P-HSLSer-660 was found to preferentially label at the periphery in most cells, close to the plasma membrane. Little P-HSLSer-660 was detected on the LDs. When cells were permeabilized for 5 s following a 5-s activation with isoproterenol, a very different distribution profile was detected (Fig. 6B). Although P-HSLSer-660 could still be detected close to the plasma membrane in some cells, there was now a significant component detectable on the surface of LDs. In addition, LDs closer to the cell periphery were labeled more strongly than central LDs, and in many cases only the more peripheral regions of larger LDs were

FIGURE 5. Localization of phosphorylated HSL in activated 3T3-L1 adipocytes. A, 3T3-L1 adipocytes were activated using 10 μM isoproterenol for 1 or 10 min and analyzed by indirect immunofluorescence microscopy for P-HSLSer-563, P-HSLSer-660, P-HSLSer-565, and total HSL (red). Cells were co-stained for Bodipy493 (green) and 4′,6-diamidino-2-phenylindole (blue). z-series stacks were collected and rendered into two dimensions. B, 3T3-L1 adipocytes were treated with 10 μM isoproterenol for 0 min (control), 1, 10, and 30 min, and subsequently fractionated into cytosol, LDs, and a P100 membrane fraction. Volume equivalents of each fraction were immunoblotted for total HSL, P-HSLSer-563, P-HSLSer-660, P-HSLSer-565, perilipin A, PKA substrates (RRXX(S/T)), caveolin 1, and α-tubulin.
labeled (see Fig. 6B). These studies show that HSL is phosphorylated on Ser-660 within seconds of activation of lipolysis and that the phosphorylated form accumulates in the peripheral cytosol prior to very rapid translocation and stable association with the LD surface.

**Mutation of the Ser-659/Ser-660 Phosphorylation Site to Ala Reduces the Subsequent Phosphorylation of HSL on the Ser-563 Site**—Previous studies have shown that mutation of the HSL Ser-659/Ser-660 phosphorylation site to alanines prevents the translocation of HSL to the LD (13). We hypothesized that loss of phosphorylation on Ser-659/Ser-660 would therefore prevent or reduce the subsequent phosphorylation of Ser-563. To test this hypothesis, we generated a His-tagged mutant of HSL, His-HSL(S650A,S651A), the murine equivalent to the Ser-659, Ser-660 site in the rat sequence, and expressed it in 3T3-L1 adipocytes in parallel to His-HSL. The cells were subsequently activated using either isoproterenol or Fsk/IBMX for 30 min; the His-tagged proteins were isolated by affinity chromatography, and the relative level of phosphorylation on Ser-651 and the Ser-563 site was analyzed by immunoblotting. Consistent with the hypothesis that HSL(Ser-563) phosphorylation is associated with the prior phosphorylation of HSL on Ser-660, mutation of the latter site resulted in a significant decrease in the level of Ser-563 phosphorylation in response to either isoproterenol or Fsk/IBMX (Fig. 7).

One caveat in the interpretation of these experiments is the possible formation of dimers in 3T3-L1 adipocytes between the expressed His-tagged HSLs and the endogenous protein. Both His-HSL and His-HSL(S650A,S651A) were found to co-purify with endogenous HSL, despite the presence of 8 M urea in the binding buffer, resulting in a low level of Ser-660 phosphorylation observed following affinity chromatography of the mutated protein (Fig. 7C). It is possible that interaction of the His-HSL(S650A,S651A) with endogenous HSL partially compensates in the subsequent phosphorylation of Ser-563. Detailed analysis of the formation of HSL dimers and the impact of this on phosphorylation will be an important future undertaking.

**DISCUSSION**

In this study, we have described for the first time the earliest events regulating the acute activation of lipolysis by signal transduction from the β-adrenergic receptor in adipocytes. These studies provide new insights into fundamental aspects of lipolysis. By examining the phosphorylation of HSL within adipocytes, both spatially and temporally, we have identified several important aspects of HSL regulation. First, we describe the remarkably rapid, sequential phosphorylation of specific serine residues in HSL, at spatially distinct intracellular locales. Second, we provide evidence for the presence of two distinct PKA signaling complexes directly regulating lipolysis. Finally, we describe differences in the mutually exclusive phosphorylation of two serine residues in HSL, targeted by distinct protein kinases, upon activation of lipolysis by signal transduction from the cell surface compared with the direct elevation of intracellular cAMP levels. Together, these data show that the acute stimulation of lipolysis in adipocytes by β-adrenergic receptor

**FIGURE 6. Initial localization of P-HSLSer-660 in fixed or pre-permeabilized 3T3-L1 adipocytes.** A, 3T3-L1 adipocytes directly fixed (Control) or activated using 10 μM isoproterenol (Isop) for 5 s were fixed in 4% paraformaldehyde, and P-HSLSer-660 (red) localization was analyzed by immunofluorescence microscopy. Cells were co-stained for Bodipy493 (green) and 4′,6-diamidino-2-phenylindole (blue). B, 3T3-L1 adipocytes were treated with 10 μM isoproterenol for 5 s, permeabilized for 5 s, and fixed. Cells were labeled for P-HSLSer-660 (red), Bodipy493 (green), and 4′,6-diamidino-2-phenylindole (blue). In both experiments the pixel intensity of P-HSLSer-660 labeling is demonstrated using both a pseudo-colored heat map (low intensity = black, high intensity = white) and by a profile of pixel intensity across a representative cell in the direction indicated. Size bars, 10 μm.

**FIGURE 7. Phosphorylation of His-HSL and His-HSL(S650A,S651A) in 3T3-L1 adipocytes.** A, His-tagged HSL and His-HSL(S650A,S651A) were expressed in 3T3-L1 adipocytes. Whole cell lysates were prepared from control cells (lane 1) and cells in which lipolysis was activated using either 10 μM isoproterenol (lane 2) or a combination of 10 μM Fsk/500 μM IBMX (lane 3) for 30 min. Untransfected (Untrans) 3T3-L1 adipocytes were prepared as a control. His-tagged HSL constructs were resolved at a higher molecular weight than the endogenous HSL on SDS-PAGE. B, His-tagged HSL and His-HSL(S650A,S651A) were expressed in 3T3-L1 adipocytes and the cells were stimulated using either 10 μM isoproterenol (2), or a combination of 10 μM Fsk/500 μM IBMX (3) for 30 min, or untreated (1). His-tagged proteins were isolated by affinity chromatography and immunoblotted for total HSL, P-HSLSer-563, and P-HLSer-660. C, level of P-HLSer-563 and P-HLSer-660 was quantified relative to expressed His-tagged HSL constructs (Fsk/IBMX, n = 4, isoproterenol (Isop), n = 5, ± S.E.).
agonists is controlled by a complex series of phosphorylation and dephosphorylation events, mediated by multiple signaling complexes. Although the later events occur at the surface of the LD itself, we propose that the primary signaling complex initiating lipolysis is located at the cell surface in close proximity to the $\beta_3$-adrenergic receptor (Fig. 8).

The phosphorylation of both HSL and perilipin A on multiple residues in basal adipocytes and during activated lipolysis has been well described (7, 8, 10, 11). However, the exact progression and spatial organization of distinct phosphorylation reactions is not well understood. By using the 3T3-L1 adipocyte model cell system, we have been able to examine the incredibly rapid kinetics and spatial organization of early phosphorylation of HSL, studies that would be difficult to accomplish in vivo or in primary adipocytes. Using explants of primary mouse adipose tissue, we show that identical phosphorylation responses are observed upon activation of lipolysis by $\beta_3$-adrenergic receptor signal transduction compared with the direct elevation of intracellular cAMP levels, as shown in 3T3-L1 adipocytes, confirming the relevancy of this system to primary adipose tissue. The results of these analyses suggest the presence of a highly organized signaling platform generating maximal phosphorylation and association with LDs within seconds rather than minutes. Our observation that P-HSL$^{Ser-660}$ initially appeared in the peripheral cytosol of the cell prior to its association with the LD, within the shortest time frame that we could reasonably measure (5 s), leads us to hypothesize that this phosphorylation event is mediated by a pool of PKA present at the cell surface, possibly in a complex with the $\beta_3$-adrenergic receptor (Fig. 8). The appearance of both PKA-phosphorylated perilipin A and P-HSL$^{Ser-563}$ on the LD strongly suggests that either a second LD-associated pool of PKA is activated at the LD surface or that activated PKA is also capable of translocating to the LD surface.

The initial phosphorylation of the LD scaffold/adaptor protein perilipin A was found to occur many seconds later than the phosphorylation of HSL on Ser-660, but it preceded the phosphorylation of HSL on Ser-563. As the maximal phosphorylation of HSL$^{Ser-563}$ closely follows the maximal phosphorylation of perilipin A, it is possible that the latter is required to stabilize HSL$^{Ser-563}$ at the LD surface. At present, it is unclear whether phosphorylation of Ser-563 occurs on nonphosphorylated HSL (Fig. 8A) or whether a phosphatase on the LD surface catalyzes the dephosphorylation of the HSL$^{Ser-563}$ site prior to phosphorylation on the Ser-563 (Fig. 8B). The low steady state level of Ser-563 phosphorylation of HSL in activated cells suggests that unlike Ser-660 phosphorylation, Ser-563 phosphorylation is not a requirement for translocation. At present, the functional significance of HSL$^{Ser-563}$ phosphorylation remains unknown.

Cyclic AMP is a key signaling molecule in many different signal transduction pathways in numerous cell types. Specificity in cAMP signaling is achieved by anchoring PKA to the appropriate target compartment via an interaction with protein kinase A-anchoring proteins (23). Preventing PKA anchoring in adipocytes inhibits isoproterenol-stimulated lipolysis showing that correct localization is a requirement for signal transduction (24). In adipocytes, D-AKAP1 has been proposed to anchor PKA to the LD surface (25) and could therefore mediate phosphorylation of perilipin A and P-HSL$^{Ser-563}$. The ability of protein kinase A-anchoring proteins to form complexes with multiple components of a signaling pathway, including kinases, phosphatases, and phosphodiesterases (reviewed in Refs. 26, 27) suggests that a signaling platform is likely to exist on the LD surface. Identifying the components and regulation of this complex will be an important future endeavor.

Determining the molecular composition of the different lipolytic signaling complexes in adipocytes, and the mechanism by which agonist-mediated activation of lipolysis is controlled by protein kinases and phosphatases, is crucial to understanding lipolysis. Furthermore, the relationship between HSL translocation and the activity of a second major LD-associated lipase, adipocyte TG lipase, is also of primary importance. Adipocyte TG lipase catalyzes the first hydrolytic reaction of lipolysis resulting in the generation of diglyceride from TG (28). As dig-
Lysceride is the major intracellular substrate of HSL, and it has been shown that HSL requires an intact catalytic binding site to translocate to the LD (13), it is intriguing to hypothesize that the activity of adipocyte TG lipase is required to stabilize the interaction of phosphorylated HSL with the LD by providing the substrate (diglyceride). In conclusion, we have shown that phosphorylation of key lipolytic proteins in 3T3-L1 adipocytes is subject to regulation both spatially and temporally, demonstrating previously unknown layers of complexity in the regulation of lipolysis at the cellular level.

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