Mutational Analysis of the Hormone-sensitive Lipase Translocation Reaction in Adipocytes*

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Lipolysis in adipocytes governs the release of fatty acids for the supply of energy to various tissues of the body. This reaction is mediated by hormone-sensitive lipase (HSL), a cytosolic enzyme, and perilipin, which coats the lipid droplet surface in adipocytes. Both HSL and perilipin are substrates for polyphosphorylation by protein kinase A (PKA), and phosphorylation of perilipin is required to induce HSL to translocate from the cytosol to the surface of the lipid droplet, a critical step in the lipolytic reaction (Szatalryd C., Xu, G., Dorward, H., Tansey, J. T., Contreras, J.A, Kimmel, A. R., and Londos, C. (2003) J. Cell Biol. 161, 1093–1103). In the present paper we demonstrate that phosphorylation at one of the two more recently discovered PKA sites within HSL, serines 659 and 660, is also required to effect the translocation reaction. Translocation does not occur when these serine residues are mutated simultaneously to alanines. Also, mutation of the catalytic Ser-423 eliminates HSL translocation, showing that the inactive enzyme does not migrate to the lipid droplet upon PKA activation. Thus, HSL translocation requires the phosphorylation of both HSL and perilipin.

Hormone-sensitive lipase is a broadly expressed enzyme that mediates the hydrolysis of triacylglycerols in adipose cells of animals, leading to the release of fatty acids which are transported in the plasma to supply the energy needs of various tissues (1). This lipolytic process is under strict hormonal and neural regulation, to guarantee an appropriate supply of fatty acids to the peripheral tissues according to the metabolic needs at each moment. Activation of the lipolytic process is mediated by cAMP-dependent protein kinase (PKA), which is acutely stimulated by hormones that elevate cAMP, like catecholamines, corticotropin, glucagon, and others (2).

HSL contains three sites for PKA serine phosphorylation (Ser-563, Ser-659, and Ser-660) (3). Phosphorylation of both HSL and perilipin.

*MATERIALS AND METHODS

Cell Culture—3T3-L1 fibroblasts, from the American Type Culture Collection (ATCC, Manassas, VA), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose (Invitrogen) supplemented with 10% fetal bovine serum (Summit), 100 units/ml penicillin (Biofluids), 100 μg/ml streptomycin (Biofluids), 110 μg/ml sodium pyruvate (Sigma), and 8 μg/ml bovine insulin (Invitrogen). BOSC23 cells, from the ATCC, were maintained in DMEM described above without bovine. Cells were maintained in culture flasks and dishes (Corning) in a 5% CO2 atmosphere at 37 °C. For experiments, cells were split into cell 6-well culture plates with or without glass cover slips (Fisher). Differentiation of 3T3-L1 adipocytes was initiated by culturing the cells with 10 μg/ml insulin (Sigma), 0.5 mm 3-isobutyl-1-methylxanthine (Aldrich), and 10 μm dexamethasone (Sigma) in serum-free medium (DMEM/F-12; Invitrogen) supplemented with 3.5 g/liter glucose, 2 mM glutamine (Biofluids), 100 units/ml penicillin (Biofluids), 100 μg/ml streptomycin (Biofluids), 110 μg/ml sodium pyruvate (Sigma), 8 μg/ml bovine insulin (Invitrogen), 10 μg/ml human transferrin (Sigma), and 1 μg/ml retinoid (Sigma) for 3 days. Cells were subsequently maintained in the serum free medium after 3 days of hormone treatment. For purposes of the present study, this switch to serum-free medium was necessary, because when maintained in serum-replete medium the cells infected with pBabe detached.

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¶ The abbreviations used are: PKA, cAMP-dependent protein kinase or protein kinase A; HSL, hormone-sensitive lipase; wt, wild type; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline.

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from the culture dishes. The cAMP analogs, 8-thiomethyl- and N\(^{\frown}\)-benzoyl-cAMP, were from Sigma.

**Construction of HSL Mutants and Expression in 3T3-L1 Cells**—The C-terminal FLAG-tagged (10) wt HSL with BgIII and SalI sites was made by polymerase chain reaction amplification using Taq polymerase (Invitrogen) against rat wt HSL in pSVL (11). The sequence of the sense oligonucleotide is 5'-GCCGAGATTCATGATTTACGGCACATAATGCACAC-GTCG-3' and that of the antisense oligonucleotide is 5'-CCCGTGAC-TACCTGTCATCTGCCTCTGTATGTCGTCACGGTGTCAGG-GGGGGGG-3'. The BgIII and SalI digestion of this product was cloned into the retroviral pBabe-puro vector (12) digested with SalI. The sequence of FLAG-tagged wt HSL in pBabe was confirmed by polymerase chain reaction-based automated sequencing, using Taq dye deoxy terminator cycle sequencing kit (PerkinElmer Life Sciences). The FLAG-tagged mutated HSLs were cloned into pBabe by switching native HSL from pBabe, cut with BgIII, and EcoRI, with mutants, cut with BgIII and EcoRI, from pSVL (3). The sequences of FLAG-tagged mutants in pBabe were also confirmed.

Bosc23 cells (2 \(\times\) 106 cells/-90 mm dish) were seeded 24 h before transfection. On the day of transfection, the cells were about 80% confluent. After incubating with 25 \(\mu\)g chloroquine (Sigma) for 30 min, 10 \(\mu\)g of pBabe vector or vectors containing FLAG-tagged wt or mutated HSLs were transfected by calcium phosphate precipitation method (Amer- Sham Biosciences) according to the protocol provided by the manufacturer. Fresh medium was replaced 6 h later. The viral supernatants from two 60-mm dishes were collected and filtered through a 0.22- \(\mu\)m filter (Gelman Sciences) after 48 h. Fresh medium was supplemented, and polybrene (Sigma) was added to a final concentration of 6 


g/ml puromycin (Sigma). After 24 h, half of the singly infected 3T3-L1 fibroblasts were re-infected ("doubly infect- ed") with the viral solution obtained from Bosc23 cells described above.

Five rounds of retroviral infections were carried out with each of the HSL-FLAG fusion constructs described in this paper, and in each experiment both single and double infection with the retrovirus were performed. The data shown are representative of the results found with each set of experiments.

**Northern Blot Analysis**—Total RNA was collected from 3T3-L1 fibroblasts at confluence or from 3T3-L1 adipocytes, 5 days after induction of differentiation, by adding TRIzol (Invitrogen) according to the protocol provided by the manufacturer. Ten \(\mu\)g of total RNA were electrophoresed on 1% agarose gels containing formaldehyde, and the RNA was transferred to supercharged Nylon membranes (Schleicher and Schuell). The FLAG-tagged mutated HSLs were cloned into pBabe by switching native HSL from pBabe, cut with BgIII, and EcoRI, from pSVL (3). The sequences of FLAG-tagged mutants in pBabe were also confirmed.

**Immunoblotting**—3T3-L1 fibroblasts at confluence or adipocytes, 5 days after induction of differentiation, were sonicated by a Sonicator (Heat Systems) for 30 s in a hypotonic lysis medium made by polymerase chain reaction amplification using Taq DNA polymerase (PerkinElmer Life Sciences). The FLAG-tagged mutated HSLs were cloned into pBabe by switching native HSL from pBabe, cut with BgIII, and EcoRI, from pSVL (3). The sequences of FLAG-tagged mutants in pBabe were also confirmed.

**HSL Activity Assay**—3T3-L1 fibroblasts and adipocytes were homogenized in 0.25 M sucrose containing 1 mM EDTA, 1 mM dithiothreitol, 20 

\(\mu\)g/ml leupeptin, and 1 mM benzamidine by 10 strokes in a Teflon/glass homogenizer. The homogenate was centrifuged at 1400 \(\times\) g for 10 min. The fat cake was removed, and the infranatant was taken for assay of HSL activity according to Holm et al. (15).

**RESULTS**

**Expression and Activities of FLAG-tagged HSLs**—To examine the regulation of HSL translocation, we introduced native and mutated forms of HSL into 3T3-L1 pre-adipocytes using infection with the pBabe retroviral vector. Serine to alanine mutations were introduced into the three PKA sites, Ser-563, Ser-659, and Ser-660, as well as into the putative AMP kinase site, Ser-655. A double mutation (Ser-659/Ser-660 to Ala) was also produced, since it was found that simultaneous mutation of these two serines eliminated the in vitro PKA-stimulated activation of HSL (3). The expression levels of the ectopic HSL-FLAG proteins and the endogenous HSL are compared in Fig. 1A, which shows that all of the ectopic HSLs, both native and mutated, were expressed at levels three to five times greater than the endogenous HSL.

In preliminary experiments the FLAG epitope was placed at either the N or C terminus of HSL, and it was found that translocation to lipid droplets upon stimulation was detectable only with HSL species carrying the FLAG epitope at the C terminus (data not shown). Accordingly, all mutated HSL con- structs examined in this work were modified with the FLAG epitope at the C terminus.
epitope at the C terminus. Moreover, as noted under “Materials and Methods,” double infection with the pBabe retrovirus was required to achieve sufficient levels of HSL-FLAGs for detection by immunofluorescence. Also, it was necessary to perform such studies no later than 5 days after the initiation of differentiation, since after day 5 the expression of HSL-FLAGs declined precipitously, to the point that they were no longer detectable by IF microscopy (Fig. 1A). The data presented herein are representative of five different experiments in which the 3T3-L1 cells were infected with the various HSL constructs, both singly and doubly, and HSL translocation for each construct was examined from 10 min to 1 h after isoproterenol stimulation. The criterion for HSL translocation was the appearance of smooth, uniform rings of staining at the periphery of the lipid droplets. The constructs we designate as non-translocating failed to show such images in any experiment either shortly after stimulation or after prolonged stimulation.

HSL activities in homogenates of cells expressing ectopic HSL-FLAG contained approximately four to five times greater lipase activity than cells expressing only endogenous HSL, which is in good agreement with the findings of the immunoblotting, and indicates that the ectopic HSL was active. For example, cells expressing ectopic native HSL-FLAG exhibited lipase activity of 289 ± 25 nmol triolein hydrolyzed per h per µl of homogenate infranate versus 72 ± 4 nmol/b/µl of infranate from cells infected with the empty pBabe vector. Surprisingly, despite their increased HSL content and activity neither basal nor stimulated lipolysis in intact cells was increased in cells with the excess ectopic HSL. Indeed, the only change observed was a slight inhibition of lipolytic activity in the cells expressing the HSL-FLAG. Similar data were observed upon stimulation of cells with either non-hydrolyzable cAMP (Table I) analogs or with isoproterenol (data not shown). This failure to find differences among the different types of cells applied to both maximal and submaximal concentrations of the simulating agents.

**Double Mutation of Ser-659 — Ala and Ser-660 — Ala Eliminates HSL Translocation**—When introduced into 3T3-L1 adipocytes, all FLAG-HSL constructs were distributed throughout the cytosol in unstimulated adipocytes (Fig. 2A), although, unlike endogenous HSL which is distributed diffusely in the cytosol (6), the ectopic HSL-FLAGs presented a more punctate distribution; this is especially evident in cells with relatively low levels of expression. Upon stimulation of cells with isoproterenol under conditions known to induce the translocation of native HSL to lipid droplet (6), the constructs, including FLAG-tagged wt HSL and HSL-FLAGs containing single mutations in each of the three PKA sites, translocated to lipid droplets (Fig. 2B), as indicated by the bright, uniform rings of staining at the periphery of lipid droplets. The only species with mutations within PKA sites that failed to translocate was the double Ser-659 → Ala/Ser-660 → Ala mutant, suggesting that phosphorylation of at least one of these sites is required for the translocation of HSL. All constructs were examined in five separate retroviral infections, and the cells were observed from between 10 and 60 min of stimulation. Typically, those species judged to translocate showed evidence of movement to droplets in the majority of cells, showing the typical uniform bright rings of staining at the periphery of lipid droplets.

**Mutation of Ser-656 and Ser-423 Eliminates HSL Translocation** —In addition to those serines phosphorylated by PKA in HSL, we have mutated Ser-656, named the basal phosphorylation site, and Ser-423, that forms part of the catalytic triad of HSL (14).
Ser-565 has been reported to be phosphorylated by AMP-activated kinase (but not by PKA). This site was originally named the basal phosphorylation site, because it was found to be phosphorylated even under conditions were lipolysis was not stimulated (i.e. under basal conditions) (15). Phosphorylation at this site is thought to preclude phosphorylation at Ser-563 by PKA, and it has therefore been proposed that phosphorylation of Ser-565 could have an antilipolytic role. Unexpectedly, mutating Ser-565 into Ala also abolished the translocation of HSL to the lipid droplets (Fig. 2), and in no case was this mutation observed to translocate over the course of five different experiments. To further illustrate the failure of Ser-565 → Ala HSL-FLAG to translocate, we compared the time course of translocation of the endogenous HSL and ser565ala-FLAG in the same population of cells. (Fig. 3). It is readily evident that the endogenous HSL translocated to lipid droplets rapidly and that the mutated FLAG-tagged species did not translocate. Moreover, the data indicate that the expression of excess Ser-565 → Ala HSL does not interfere with the movement of the endogenous lipase. The data are in accord with the findings of Birnbaum and colleagues, who have found that expression of a dominant-negative form of AMP-kinase inhibits lipolysis in 3T3-L1 adipocytes.

Ser-423, together with Asp-703 and His-733, forms the catalytic triad of HSL. Accordingly, mutating Ser-423 abolishes HSL catalytic activity (14). We tested whether catalytic competence is necessary for HSL to translocate to lipid droplet by introducing a FLAG-tagged construct of HSL containing the Ser-423 → Ala mutation. The results show that mutating this active site residue also abolishes the ability of the protein to translocate to the lipid droplets upon stimulation of the cells with isoproterenol (Fig. 4).

DISCUSSION

Acute regulation of lipolysis in the adipose tissue allows mammals and other organisms to adjust the supply of energy substrates to the peripheral tissues according to their metabolic needs at any given time. Until recently, it was believed that the acute regulation of lipolysis relied solely on the phosphorylation/dephosphorylation and subsequent activation/deactivation of HSL in the adipocytes. More recent developments have shown, however, that the process is significantly more complex, and a role for at least one other protein, perilipin A, in the regulation of lipolysis has become apparent (9). The clue to the dramatic changes in the lipolytic rate of intact adipocytes upon exposure to catecholamines (lipolytic) or insulin (antilipolytic) resides in the subcellular distribution of HSL (5, 6), the enzyme responsible for the hydrolysis of the stored triglycerides.

In a separate paper, we demonstrate that perilipin A plays an essential role in both preventing the interaction of HSL with its natural substrate (the lipid droplets) under non-stimulated conditions and allowing activated translocation upon stimulation of cells with catecholamines (9). Thus, we show that phosphorylation of perilipin A at its N-terminal PKA sites is required to allow the translocation of HSL from the cytosol to the surface of the lipid droplets. This finding raised the question of whether phosphorylation of perilipin A sufficed to allow the translocation of HSL or whether phosphorylation of HSL was also required for this phenomenon to occur. To address this issue, in the present work we have introduced mutated forms of HSL, tagged at its C terminus with the FLAG epitope, into 3T3-L1 cells using the pBabe retroviral system. The stable transfectants obtained were used, upon differentiation into adipocytes, to investigate the ability of the different HSL mutants to translocate to the lipid droplets. To achieve sufficient expression of HSL to enable detection of the FLAG-tagged HSL species, double infections with the pBabe retroviral constructs were necessary. Subsequently, HSL tagged in the C terminus with the FLAG epitope could be easily detected in the cells, and translocation of the tagged lipase was readily evident upon stimulation of the cells with catecholamines. We found that single mutations of each of the serines phosphorylated by PKA in HSL have no effect on the ability of HSL to translocate from the cytosol to the lipid storage droplets in 3T3-L1 adipocytes.

\* M. Birnbaum, personal communication.
However, double mutation of the two C-terminal sites, Ser-659 and Ser-660, prevented HSL translocation. This result demonstrates that phosphorylation of at least one of these two sites of HSL is required to induce HSL to change its subcellular location and to therefore activate lipolysis.

We also found that mutation of Ser-423, the catalytic serine, prevented HSL translocation, indicating that only the fully functional enzyme can translocate. This finding is not entirely surprising, since mutations in the active site serine can lead to substantial conformational alterations in lipases, modifying their binding capacity to lipids (16). More surprising was the finding that mutation of Ser-565 also prevented HSL translocation. This site is not phosphorylated by PKA in response to catecholamines stimulation, but rather seems to be constitutively phosphorylated in non-stimulated adipocytes. Phosphorylation of Ser-565 has been proposed to have an antilipolytic role by precluding phosphorylation of Ser-563 by PKA (17). However, the fact that phosphorylation of Ser-563 does not seem to be required neither for HSL activation in vitro (3) nor for the translocation of HSL to the surface of the droplets, the current evidence challenges the proposed antilipolytic role of Ser-565. One possible explanation for the inability of the HSL Ser-565-Ala mutant to translocate could be that phosphorylation of Ser-565 may serve a structural role, and mutating Ser-565 to Ala causes a local structural disturbance that prevents the conformational changes required for the translocation of HSL upon phosphorylation of Ser-659 or Ser-660. Examining model systems in which the relevant protein kinases for this site are ablated could cast light on the role of Ser-565 in lipolysis, if any.

Unfortunately, the lipolysis experiments do not provide any information on the contribution of HSL phosphorylation to the activity of HSL other than its delivery to the lipid droplet substrate, since cellular lipolytic activity was not increased in the presence of excess active HSL. Similar findings have been reported recently by Lucas et al. (18), who demonstrated that the expression of excess active human HSL does not contribute to increased lipolysis in murine adipocytes.

In conclusion, the data presented in the present study, plus those in a parallel paper from this laboratory (9), show that the activation of lipolysis in the adipocytes requires the concerted phosphorylation of both HSL and perilipin. It is clear that HSL translocation is not merely secondary to whatever changes of the droplet surface are rendered upon phosphorylation of perilipin A, as the present paper demonstrates that specific PKA sites within HSL are required to achieve translocation. The precise mechanism by which HSL accumulates at the lipid droplet remains a mystery. Previously, we demonstrated a lack of involvement of cytoskeletal systems in this process, since a variety of cytoskeletal poisons failed to interfere with HSL translocation (6). The simplest scenario would be that the translocation merely reflects an increased affinity of HSL for its substrate, whereas phosphorylation of perilipin A would trigger conformational changes in this protein that would clear the access of HSL to the lipids. This speculation is supported in part by the finding that catalytically inactive HSL, in which the catalytic site serine 423 was mutated, is unable to translocate to lipid droplets, presumably due to a reduced ability to bind to its substrate. However, the possibility of more complex protein-protein interactions between HSL, perilipin, and/or third proteins on the surface of the lipid droplets cannot be discarded and should be the focus of future investigations.

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