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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 (Sztalryd 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 serines 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),[1] 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 HSL *in vitro* with exogenous PKA causes a modest (2-fold) activation of HSL, and mututational analysis has revealed that phosphorylation of Ser-659 or Ser-660, but not Ser-563, are required for this modest activation of HSL *in vitro* (3). However, this meager increase in the specific activity of HSL cannot account for the 30–100-fold increases in lipolysis observed upon elevation of PKA activity in intact cells. Rather, we have proposed that PKA-mediated translocation of HSL from the cytosol to the surface of lipid droplets more likely explains the large cellular response to lipolytic stimuli (4–6). Furthermore, while PKA-mediated lipolysis has long been thought to be attributed solely to HSL, more recent developments point to a role for the perilipins in lipolytic activation. Perilipin A is an adipocyte protein that coats the lipid storage droplet (7) and contains six sites for PKA phosphorylation (8), most of which are phosphorylated upon lipolytic stimulation. In a separate paper (9), we demonstrate that the interaction of HSL with the intracellular neutral lipid droplets is critically regulated by the presence of perilipin. Furthermore, we demonstrate that fully phosphorylatable perilipin A is required to induce HSL to translocate from the cytosol to the surface of the lipid droplets, a key process for the activation of lipolysis.

In the present study we examine the role of the various PKA sites within HSL in the translocation reaction in 3T3-L1 adipocytes and identify the sites that are required for HSL to associate with the lipid droplet. We further demonstrate that the mutation of the catalytic serine (Ser-423) abrogates the translocation phenomenon, showing that the enzyme must be catalytically competent to undergo translocation.

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), 2 mM glutamine (Biofluids), 100 units/ml penicillin (Biofluids), 100 μg/ml streptomycin (Biofluids), 110 μg/ml sodium pyruvate (Sigma), and 8 μg/ml biotin (Invitrogen). Bosc23 cells, from the ATCC, were maintained in DMEM described above without biotin. Cells were maintained in culture flasks and dishes (Corning) in a 5% CO_2 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 biotin (Invitrogen), 10 μg/ml human transferrin (Sigma), and 1 mg/ml fetuin from fetal calf serum (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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[1] 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.

from the culture dishes. The cAMP analogs, 8-thiomethyl- and N^6-benzoyl-cAMP, were from Sigma.

Construction of HSL Mutants and Expression in 3T3-L1 Cells—The C-terminal FLAG-tagged (10) wt HSL with *Bgl*II and *Sal*I sites was made by polymerase chain reaction amplification using *Taq* polymerase (Invitrogen) against rat wt HSL in pSVL (11). The sequence of the sense oligonycleotide is 5′-CGCAGATCTATGGATTTACGCACAATGACACAGTCG-3′ and that of the antisense oligonucleotide is 5′-CGCGTCGACTCACTTGTCATCGTCGTCCTTGTAGTCGGTCAGCGGTGCAGCAGGGGGGGG-3′. The *Bgl*II and *Sal*I digestion of this product was cloned into the retroviral pBabe-puro vector (12) digested with *Sal*I. 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 *Bam*HI and EcoRI, with mutants, cut with *Bgl*II and EcoR I, from pSVL (3). The sequences of FLAG-tagged mutants in pBabe were also confirmed.

Bosc23 cells (2 × 106 cells/60-mm dish) were seeded 24 h before transfection. On the day of transfection, cells were about 80% confluent. After incubating with 25 μM chloroquine (Sigma) for 30 min, 10 μg of pBabe vector or vectors containing FLAG-tagged wt or mutated HSLs were transfected by calcium phosphate precipitation method (Amersham 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 0.45-μm filter (Gelman Sciences) after 48 h. Fresh medium was supplemented, and polybrene (Sigma) was added to a final concentration of 6 μg/ml. 3T3-L1 cells on 100-mm dishes were incubated with the viral solution for 24 h. Selection was carried out by splitting the infected 3T3-L1 cells in the DMEM containing 3 μg/ml puromycin (Sigma). After 24 h, half of the singly infected 3T3-L1 fibroblasts were re-infected ("doubly infected") 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 round of double retroviral infection.

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 μg of total RNA were electrophoresed on 1% agarose gels containing formaldehyde, and the RNA was transferred to supercharged Nylon membranes (Schleicher and Schuell) by the Turboblotter system (Schleicher and Schuell). Northern blot hybridization was carried out by incubating ^{32}P-labeled rat HSL cDNA with the membrane.

Immunoblotting—3T3-L1 fibroblasts at confluence or adipocytes, 5 days after induction of differentiation, were sonicated with a Sonicator Ultrasonic Processor (Heat Systems) for 30 s in a hypotonic lysis medium containing 10 mM Tris, pH 7.4, 1 mM EDTA, 10 mM sodium fluoride, 20 μg/ml leupeptin, 1 mM benzamidine, and 100 μM [4-(2-amineothy)benzenesulfluoride] hydrochloride. Proteins from aliquots of the crude sonicates were separated on 10% polyacrylamide SDS-PAGE, 1 mM dithiothreitol gels and transferred to a nitrocellulose membrane (Micron Separations). The membrane was blocked with 5% milk and incubated with either affinity-purified rabbit anti-mouse HSL immunoglobulin (6) or mouse anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.) for 1 h. After washing, the membrane was incubated with alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch), sheep horseradish peroxidase-linked anti-mouse whole phosphatase color development method (Bio-Rad), or by enhanced chemiluminescence (PerkinElmer Life Sciences), respectively.

DNA Assay—Sonicated whole cell homogenates were mixed a solution containing 50 mM Na_2HPO_4, pH 7.4, 2 mM EDTA, and 0.02% sodium azide. Standards were prepared by diluting calf thymus double-stranded DNA in the phosphate buffer ranging from 0 to 1500 ng/ml. Samples and standards were incubated with 15 μg/ml bisbenzamide (Molecular Probes) in phosphate buffer containing 2 M NaCl for 10 min. Fluorescence was detected by a LS50B luminescence spectrometer (PerkinElmer Life Sciences) (356 nm excitation wavelength and at 458 nm emission wavelength).

Immunofluorescence Microscopy—Five days after the induction of differentiation, 3T3-L1 adipocytes on coverslips were incubated with 10 μM isoproterenol in the serum-free medium, described above, containing 3% fatty acid free bovine albumin (ICN Biochemicals) at 37 °C. After 20 min of incubation, cells were fixed by 3% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS; Biofluids) for 1 h. Subsequently, cells were permeabilized, quenched, and blocked in PBS containing 1 mg/ml saponin (Sigma), 10 mM glycine (ICN Biochemicals), and 1.25 mg/ml goat IgG (Jackson ImmunoResearch) for another hour. Immunostainning was carried out by incubating cells with affinity-purified rabbit anti-mouse HSL (6) or mouse anti-FLAG M2 monoclonal antibody (Kodak) for overnight. After three washes with 0.1% saponin in PBS, Oregon green goat anti-mouse (Molecular Probes) or anti-rabbit IgG (Molecular Probes), respectively, was incubated with cells for 1 h. Cells were washed three times and mounted with 10 mg/ml *p*-paraphenylenediamine in 10% PBS and 90% glycerol. The signal was detected with a Zeiss LSM 510 Laser Scanning confocal microscope.

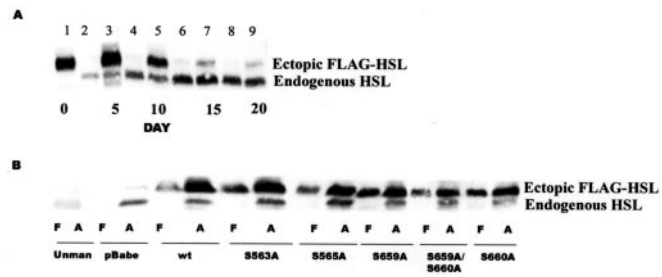

FIG. 1. **Expression of endogenous and Ectopic HSL in 3T3-L1 adipocytes: ectopic HSL-FLAG peaks at day 5 of differentiation.** 3T3-L1 adipocytes transfected with the HSL-FLAG constructs and harvested for immunoblot analysis at day 5 of differentiation for *B* and harvested on the days indicated for *A*. *A*, *upper row* represents cells expressing HSL-FLAG showing the HSL-FLAG band and lower row are unmanipulated cells showing the native HSL band. *Lanes 2/3*, *4/5*, *6/7*, and *8/9* were harvested, respectively, on days 5, 10, 15, and 20 after initiation of differentiation. The peak of expression of HSL-FLAG at day 5 was typical of all FLAG-tagged constructs used in this study. Cellular extracts were processed for immunoblotting as described under "Materials and Methods." The blots were developed with affinity-purified anti-HSL. Loading of gel was normalized for DNA content; for each lane the amount of protein was equivalent to 2500 ng of DNA. *F* indicates fibroblasts, and *A* shows differentiated adipocytes.

Glycerol Assay—3T3-L1 adipocytes on 24-well plates, 5 days after induction of differentiation, were incubated with 200 μl of PBS containing 3% fatty acid-free bovine albumin (ICN Biochemicals) with or without 10 μM isoproterenol for indicated periods of time. 50 μl of the samples were removed from the cells, and the amount of glycerol released from the cells was determined radiometrically based on the microtiter plate method as described (6).

HSL Activity Assay—3T3-L1 fibroblasts and adipocytes were homogenized in 0.25 M sucrose containing 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, and 1 mM benzamidine by 10 strokes in a Teflon/glass homogenizer. The homogenate was centrifuged at 1400 × *g* for 10 min. The fat cake was removed, and the infranatant was taken for assay of HSL activity according to Holm *et al.* (13).

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. 1*A*, 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 constructs examined in this work were modified with the FLAG

TABLE I
Overexpression of HSL-FLAG does not increase cellular lipolysis in 3T3-L1 adipocytes

The table shows lipolysis, expressed in nmol of glycerol released per h per well upon stimulation with a serial dilutions of a combination of 8-thomethyl-cAMP and N^6-benzoyl-cAMP. The stock solution contained 4.4 mM 8-thiomethyl-cAMP and 8.8 mM N^6-benzoyl-cAMP; at their highest concentrations (top row) these compounds were at 250 and 500 μM, respectively. The column labeled pBabe represents cells infected with the empty pBabe vector without an HSL insert, and the column labeled wt-HSL-FLAG represents cells expressing ectopic HSL-FLAG as shown in Fig. 1 ('HSL-FLAG'). Values shown are μM ± S.E. of glycerol determinations from 6 different wells (n = 6).

CAMP analog concentrations	pBabe	wt HSL-FLAG
500/250	137 ± 12	102 ± 3
250/125	88 ± 5	76 ± 2
125/62.5	86 ± 11	62 ± 3
62.5/32	55 ± 2	43 ± 2
32/16	46 ± 1	29 ± 1
16/8	36 ± 1	22 ± 1
8/4	31 ± 2	29 ± 1

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. 1*A*). 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/h/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 inhbition 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. 2*A*), 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. 2*B*), 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.

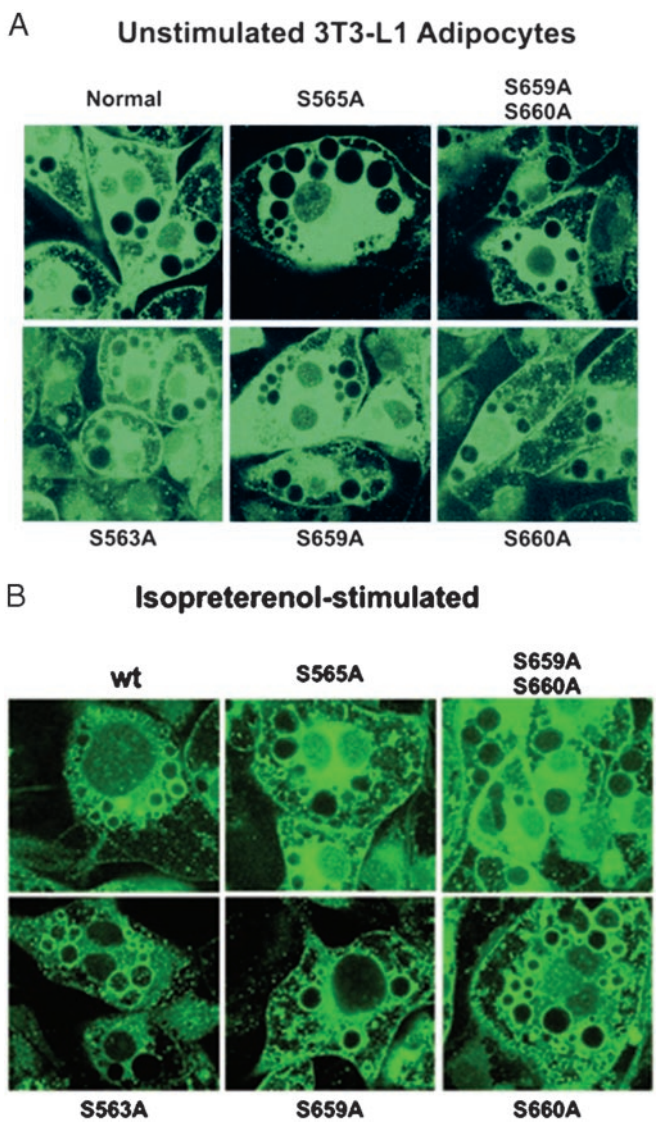

FIG. 2. **Double mutation of PKA site serines 659 and 660 abrogates the HSL translocation reaction.** Fusion constructs of FLAG and the HSL species indicated were introduced into 3T3-L1 preadipocytes, which were stimulated to differentiate into adipocyte as described under "Materials and Methods." The panels labeled wt represent cells expressing FLAG-tagged wt HSL, and the other panels expressed the indicated mutated HSL constructs fused to the FLAG epitope. Five days after induction of differentiation, cells were immunostained with anti-FLAG antibodies and viewed under a Zeiss confocal microscope. *A*, unstimulated cells. *B*, cells stimulated for 10 min with 10 μM isoproterenol.

Mutation of Ser-565 and Ser-423 Eliminates HSL Translocation—In addition to those serines phosphorylated by PKA in HSL, we have mutated Ser-565, named the *basal phosphorylation site*, and Ser-423, that forms part of the catalytic triad of HSL (14).

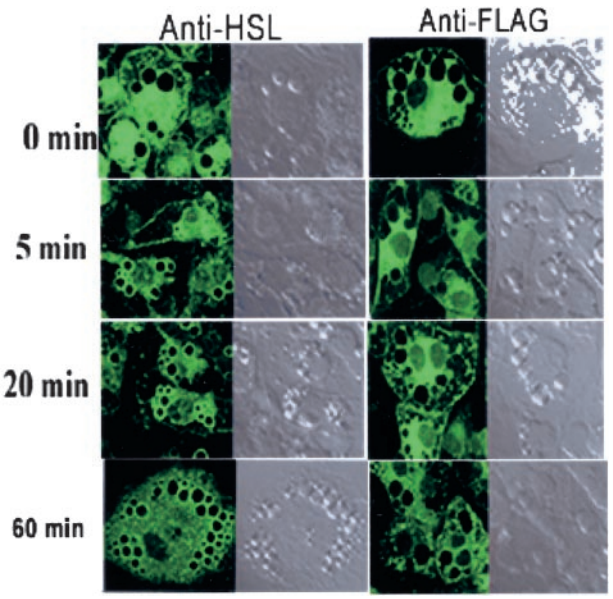

FIG. 3. **Comparison of translocation of native HSL and Ser-565 → Ala FLAG-tagged HSL.** Cells were infected to express Ser565Ala HSL-FLAG and stimulated for the indicated time periods with 10 μM isoproterenol. Cells in the *left column* were stained with anti-HSL antibody and in the *right column* with anti-FLAG antibody.

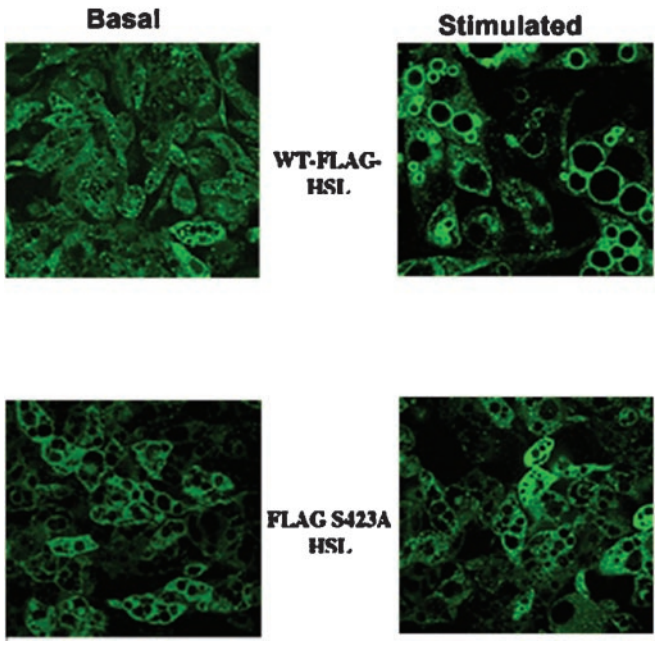

FIG. 4. **Catalytically inactive FLAG-Ser-423 → Ala HSL fails to translocate to lipid droplets upon stimulation.** *Top row*, cells infected with the empty pBabe vector and immunostained with anti-HSL antibody. *Bottom row*, cells infected with a construct that encoded HSL-FLAG bearing the Ser-423 → Ala mutation and immunotstained with anti-FLAG antibody. Ser-423 is the catalytic site serine in HSL and Ser-423 → Ala is catalytically inactive.

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 phoshorylation 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,[2] 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 cathecholamines (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.

[2] 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 accumates 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 HLS 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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REFERENCES

1. Holm, C., Osterlund, T., Laurell, H., and Contreras, J. A. (2000) *Annu. Rev. Nutr.* **20,** 365–393
2. Honnor, R. C., Dhillon, G. S., and Londos, C. (1985) *J. Biol. Chem.* **260,** 15130–15138
3. Anthonsen, M. W., Ronnstrand, L., Wernstedt, C., Degerman, E., and Holm, C. (1998) *J. Biol. Chem.* **273,** 215–221
4. Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R., and Rondinone, C. M. (1999) *Ann. N. Y. Acad. Sci.* **892,** 155–168
5. Egan, J. J., Greenberg, A. S., Chang, M. K., Wek, S. A., Moos, M. C., Jr., and Londos, C. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8537–8541
6. Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C., and Londos, C. (1999) *Biochim. Biophys. Acta* **1483,** 251–262
7. Greenberg, A. S., Egan, J. J., Wek, S. A., Garty, N. B., Blanchette-Mackie, E. J., and Londos, C. (1991) *J. Biol. Chem.* **266,** 11341–11346
8. Greenberg, A. S., Egan, J. J., Wek, S. A., Moos, M. C., Jr., Londos, C., and Kimmel, A. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 12035–12039
9. Sztalryd, C., Xu, G., Dorward, H., Tansey, J. T., Contreras, J. A., Kimmel, A. R., and Londos, C. (2002) *J. Cell Biol.* **161,** 1093–1103
10. Hopp, T. P. (2002) *Biotechnology* **6,** 1204–1210
11. Holm, C., Belfrage, P., Osterlund, T., Davis, R. C., Schotz, M. C., and Langin, D. (1994) *Protein Eng.* **7,** 537–541
12. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18,** 3587–3596
13. Holm, C., Contreras, J. A., Verger, R., and Schotz, M. C. (1997) *Methods Enzymol.* **284,** 272–284
14. Holm, C., Davis, R. C., Osterlund, T., Schotz, M. C., and Fredrikson, G. (1994) *FEBS Lett.* **344,** 234–238
15. Stralfors, P., Bjorgell, P., and Belfrage, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3317–3321
16. Peters, G. H., Svendsen, A., Langberg, H., Vind, J., Patkar, S. A., Toxvaerd, S., and Kinnunen, P. K. (1998) *Biochemistry* **37,** 12375–12383
17. Garton, A. J., Campbell, D. G., Carling, D., Hardie, D. G., Colbran, R. J., and Yeaman, S. J. (1989) *Eur. J. Biochem.* **179,** 249–254
18. Lucas, S., Tavernier, G., Tiraby, C., Mairal, A., and Langin, D. (2003) *J. Lipid Res.* **44,** 154–163