Interaction of Hormone-sensitive Lipase with Steroidogenic Acute Regulatory Protein

FACILITATION OF CHOLESTEROL TRANSFER IN ADRENAL*

Received for publication, April 15, 2003, and in revised form, August 14, 2003
Published, JBC Papers in Press, August 18, 2003, DOI 10.1074/jbc.M303934200

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Hormone-sensitive lipase (HSL) is responsible for the neutral cholesteryl ester hydrolase activity in steroidogenic tissues. Through its action, HSL is involved in regulating intracellular cholesterol metabolism and making unesterified cholesterol available for steroid hormone production. Steroidogenic acute regulatory protein (StAR) facilitates the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane and is a critical regulatory step in steroidogenesis. In the current studies we demonstrate a direct interaction of HSL with StAR using in vitro glutathione S-transferase pull-down experiments. The 37-kDa StAR is coimmunoprecipitated with HSL from adrenals of animals treated with ACTH. Deletional mutations show that HSL interacts with the N-terminal as well as a central region of StAR. Coexpression of HSL and StAR in Chinese hamster ovary cells results in higher cholesteryl ester hydrolase activity of HSL. Transient overexpression of HSL in Y1 adrenocortical cells increases mitochondrial cholesterol content under conditions in which StAR is induced. It is proposed that the interaction of HSL with StAR in cytosol increases the hydrolytic activity of HSL and that together HSL and StAR facilitate cholesterol movement from lipid droplets to mitochondria for steroidogenesis.

Neutral cholesteryl ester hydrolase activity can be demonstrated in most cells, including adipose tissue, adrenal, testes, placenta, macrophages, heart, skeletal and smooth muscles; steroidogenic tissues are especially enriched in this activity (1). Several lines of evidence suggest that hormone-sensitive lipase (HSL)1 is responsible for the neutral cholesteryl ester hydrolase activity in steroidogenic tissues. The most direct and convincing evidence comes from HSL knockout mice, where no detectable HSL protein and no neutral cholesteryl ester hydrolase activity are observed in the adrenal (2) or testis (3). It is believed that through its action as a neutral cholesteryl ester hydrolase, HSL is involved in regulating intracellular cholesterol metabolism and, thus, contributing to a variety of pathways in which cells utilize cholesterol.

The primary amino acid sequence of HSL is unrelated to any of the other known mammalian lipases; however, it shares some sequence similarity with liver arylesterase deacetylase within its catalytic domain (4). The C-terminal portion of HSL displays secondary structural homology with that of acetylcholinesterase and several fungal lipases (5) and bacterial brefeldin A esterase (6), consisting of parallel β-sheets flanked by α-helical connections, which has allowed these proteins to be classified as α/β-hydrolases (7). Using limited proteolysis, it has been suggested that HSL is composed of two major structural domains (8, 9). Based on sequence alignment, structural homology with fungal lipases, and mutational analyses, the C-terminal domain has been shown to contain the catalytic triad and other residues important in hydrolytic activity, as well as a 150-amino acid insert that has been termed the regulatory module because several serines located within this region have been shown to be phosphorylated (1, 10). The N-terminal domain in rat HSL constitutes the first 323 amino acids, which are encoded by exons 1–4, and displays no sequence or structural similarity with any other known proteins (8, 9).

We (11) and others (12) have shown that HSL interacts specifically with intracellular proteins in adipose tissue. The interaction of HSL with adipocyte lipid-binding protein (ALBP) occurs through amino acid residues within the N-terminal domain; the physical interaction of ALBP with HSL increases the hydrolytic activity of HSL and protects HSL from product inhibition by fatty acids (13). In the same way that the interaction of HSL with ALBP might help to facilitate the trafficking of fatty acids in adipose cells, HSL might interact with specific cholesterol carrier proteins in the adrenal and, thus, facilitate intracellular cholesterol trafficking to mitochondria for steroidogenesis.

The steroidogenic acute regulatory protein (StAR) is synthesized as a 37-kDa protein that is targeted to and processed in mitochondria to a 30-kDa mature protein that facilitates the movement of cholesterol from the outer mitochondrial membrane to the cholesterol side chain cleavage enzyme (CYP11A1) on the inner mitochondrial membrane (14, 15). Identification of mutations in humans (16) and mouse knockout experiments (17) have implicated StAR as a critical regulatory step in steroidogenesis. StAR has been shown to possess sterol transfer activity in vitro (18, 19). The crystal structure of StAR shows that it has a classic lipid transporter-like structure (20, 21),

* This work was supported by research grants from the Research Service of the Department of Veterans Affairs and by Grant DK 46942 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Division of Endocrinology, S-025, Stanford University, Stanford, CA 94305-5103. E-mail: fbk@stanford.edu.
‡ The abbreviations used are: HSL, hormone-sensitive lipase; ALBP, adipocyte lipid-binding protein; βtCaMP, dibutyryl cAMP; CHO, Chinese hamster ovary; CMV, cytomegalovirus; GST, glutathione S-transferase; HDLα, human high density lipoprotein; LDL, low density lipoprotein; StAR, steroidogenic acute regulatory protein; START domain, StAR-related lipid transfer domain; TGH, triacylglycerol hydrolase.
which is characterized by internal hydrophobic cavities, clefs, or tunnels.

In this paper, we demonstrate a direct interaction of HSL with StAR in both in vitro and in vivo physiological conditions. HSL interacts with the N-terminal as well as a central region of StAR. Coexpression of HSL and StAR in CHO cells results in higher hydrolytic activity of HSL. Transient overexpression of HSL in Y1 adrenocortical cells increases mitochondrial cholesterol content under conditions in which StAR is induced. It is proposed that the interaction of HSL with StAR in cytoplasm increases the hydrolytic activity of HSL and that together HSL and StAR facilitate cholesterol movement from stored lipid droplets to mitochondria for steriogenesis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Reagents were obtained from the following sources. Bovine serum albumin (fraction V) was from Intercogen, Inc., Purchase, NY. Fetal bovine serum was from Gemini Bio-Products, Inc., Calabassas, CA. Coon’s F-12/Dulbecco’s modified Eagle’s media and Lipofectin reagent were from Invitrogen. ECL Western blotting detection reagents were from Amersham Biosciences. Protein assay reagent was from Bio-Rad. Nicotinolose paper was from Schleicher & Schuell. The TNT® Transcription/Translation System was from Promega, Madison, WI. Sf9 cells, 5/21 cells, TNM-FH insect medium, baculovirus transfer vector pOChLT-A, and BaculoGold™-linearized baculovirus DNA were from Pharmingen. Nickel-nitrilotricarboxylic acid-agarose was from Qiagen, Valencia, CA. The QuickChange mutagenesis kit was from Stratagene, La Jolla, CA. Organic solvents were from J. T. Baker, Phillipsburg, NJ. Thin layer chromatography (TLC) plates were from Whatman. Allopapainoprotein E-free human high density lipoprotein (HDL₃) was isolated and characterized as described previously (22). Deletional and Mutational Constructs of StAR—PCR was used to produce deletional constructs from the C terminus as well as N terminus of StAR (a kind gift from Dr. J. Strauss, III, University of Pennsylvania). For the StAR C-terminal deletional constructs, the 5′-primer was the 5′-CTGGTACCGTACTGGCCTGC, the 3′-primers were: StAR 1–225, 5′-CAT GCC; StAR 1–155, 5′-GAT GAT

In vitro Protein-Protein Interaction—After sequence confirmation of the StAR mutants, the StAR constructs were in vitro translated with [35S]methionine using the TNT® Transcription/Translation System. GST-HSL and GST protein were produced as described previously (11). GST-HSL or GST alone was incubated with glutathione-agarose beads in reaction buffer (20 mM Tris, pH 8.0, 0.15 M NaCl, 0.5% Nonidet P-40). After a 1-h incubation at room temperature, the beads were washed three times in buffer B and then incubated with [35S]methionine-labeled StAR proteins. After a 1-h incubation at room temperature, the beads were washed five times in buffer B, and proteins that bound to the beads were eluted in SDS-PAGE sample buffer, separated on SDS in 10% PAGE, and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and Immunoblotting—Male Sprague-Dawley rats (8 weeks old) were injected with saline or ACTH (25 μg) and the adrenals harvested 1 and 2 h after the injection. Adrenals were homogenized in TES buffer (20 mM Tris-HCL, 1 mM EDTA and 8% sucrose, with 2 μg/ml leupeptin, and okadaic acid) and centrifuged at 10,000 × g for 15 min. The supernatant was used for immunoprecipitation and protein determination. Immunoprecipitation of HSL was performed as described (11). A 250-μg aliquot was precleared with protein A beads and then incubated with an immunomatrix consisting of rabbit polyclonal anti-HSL/fusion protein IgG and protein A. After overnight incubation at 4 °C, the immune complex was centrifuged at 10,000 × g for 15 min and washed twice in phosphate-buffered saline with a 50% bovine serum albumin and then twice in phosphate-buffered saline. The pellet was resuspended in SDS-PAGE loading buffer (0.063 M Tris-HCL, pH 6.8, with 1% 2-mercaptoethanol, 1% SDS, and 13% (v/v) glycerol), boiled for 5 min, and electrophoresed on 15% SDS-PAGE, transferred to nitrocellulose paper, and immunoblotted with rabbit anti-StAR IgG (a kind gift from Dr. J. Strauss, III).

Cell Culture and Transfection—CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37 °C under 5% CO₂. For transient transfection experiments, cells were subcultured at a density of 2 × 10⁶ cells/well in six well plates the day prior to incubation with 0.75 μg of pCMV-StAR or StAR mutants, 0.75 μg of pCMV-GST, and 0.5 μg of pcDNA3-HSL, and 0.5 μg of Lipofectin reagent following the procedure from the manufacturer. Cells were harvested 40 h after transfection for measurement of HSL activity. Y1-BS1 adrenocortical cells were grown in F-10 medium supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO₂. For transient transfection experiments, cells were subcultured at a density of 1 × 10⁶ cells/plate in 10-cm tissue culture plates the day prior to transfection. Each plate was transfected with either 5 μg of vector alone, pcDNS-HSL, or pBK-CMV-TGH (a kind gift from Dr. Dennis Vance, University of Alberta, Canada) using Lipofectin reagent. 24 h after transfection, cells were incubated in medium containing lipoprotein-deficient serum supplemented with 500 μg of protein/mel HDL₃ and 2.5 mM Be₂CAMP for 24 h. Cells were then changed to serum-free medium with 0.2% bovine serum albumin overnight and stimulated with 2.5 mM Be₂CAMP in the presence of 100 μM aminoglutethimide for 2 h before harvesting in TBS buffer.

Mitochondria Preparation—Mitochondria of Y1-BS1 cells were prepared as described (23) with some modifications. Harvested Y1-BS1 cells were centrifuged at 200 × g for 10 min and then washed once with phosphate-buffered saline. The cell pellet was resuspended in a hypotonic buffer (10 mM Tris-HCL, pH 7.5, with 10 mM KCl, 0.5 mM EDTA) and cells were allowed to swell for 10 min at 4 °C. After the incubation, cells were gently broken with several passes in a loose fitting glass-Teflon homogenizer. Phosphate buffer and sucrose were added to final concentrations of 50 and 100 mM, respectively, to maintain an isotonic suspension. Aliquots of the homogenate were removed for total cholesterol and protein determinations. To prepare the mitochondrial fraction, the homogenates were centrifuged at 600 × g for 10 min at 4 °C, and the supernatants were again centrifuged at 10,000 × g for 15 min at 4 °C. The pellet containing the mitochondrial fraction was resuspended in mitochondrial stabilizing buffer (10 mM potassium phosphate, pH 7.2, 20 mM KCl, 15 mM triethanolamine hydrochloride, 0.1 mM EDTA, and 250 mM sucrose).

Measurement of Cholesterol Content—Cholesterol ester and free cholesterol content were measured using the Infinity™ cholesterol measurement kit from Sigma after their separation by TLC. For TLC, lipids from isolated mitochondrial fractions were extracted by the method of Bligh and Dyer (24). After centrifugation, the lower organic solvent phase was transferred, air dried, and dissolved in 50 μl of toluene. An aliquot was then applied to 20 × 20 cm TLC plates and developed sequentially with chloroform/methanol/water 60:40:10 (v/v/v) to 1 cm and then hexane/ether-acetic acid 85:15:2 (v/v/v) to 13 cm. Cholesterol and cholesterol ester standards were applied on a separate lane on the TLC plates. The cholesterol and cholesterol ester spots were visualized by brief exposure to iodine vapor, eluted, and measured enzymatically as described previously (25).

Other Measurements—Measurement of HSL activity was performed using a cholesterol 14C-labeled emulsion as described previously (25). Protein concentration was assayed using Bio-Rad protein assay reagent. Recombinant GST-HSL was produced in baculovirus as described previously (11).

StAR and HSL

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**RESULTS**

**HSL Interacts with StAR in Vitro**—To determine whether HSL might interact with StAR, full-length StAR that was \(^{[35}S\)methionine-labeled by *in vitro* translation was incubated with either rat GST-HSL or with GST alone. The mixture was incubated with glutathione-agarose beads, and the proteins that bound to the beads were washed, eluted, and separated on SDS-PAGE, and then visualized as described under “Experimental Procedures.”

![Image](https://example.com/image1)

**Fig. 1. Interaction of HSL with StAR in vitro.** Full-length StAR was \(^{[35}S\)methionine labeled by *in vitro* translation (lane 1) and then incubated with either rat GST-HSL (lane 3) or with GST alone (lane 2). The mixture was incubated with glutathione-agarose beads, and the proteins that bound to the beads were washed, eluted, and separated on SDS-PAGE, and then visualized as described under “Experimental Procedures.”

**Statistical Analysis**—Data are expressed as the mean ± S.E. Statistical analyses were performed by unpaired two-tailed Student’s *t* test using InStat (GraphPad Software, San Diego) software for Macintosh.

**HSL Interacts with StAR in Vivo**—To document that the interaction of HSL with StAR occurs *in vivo*, male rats were injected with ACTH to induce StAR expression or with saline. The adrenals were harvested 1 and 2 h after the injection, and HSL was immunoprecipitated from extracts of the adrenals with anti-HSL antibodies. The immunoprecipitated complexes were separated on SDS-PAGE and immunoblotted with anti-StAR antibodies. As shown in Fig. 2, anti-StAR antibodies recognize StAR in extracts of control adrenals as a 30-kDa form, which is cleaved in the mitochondria and which is cleaved in the mitochondria, can participate in the interaction with HSL. In view of the ability of the 30-kDa StAR to mediate its interaction with HSL, a series of C-terminal deletions, StAR 1–225, StAR 1–205, StAR 1–155, StAR 1–100, and StAR 1–62, was generated, and the interactions of these *in vitro* translated products with HSL were examined (Fig. 3). Interestingly, although none of the constructs interacted with GST alone, all constructs were specifically pulled down by GST-HSL, suggesting that the N-terminal 62 amino acids of StAR, which contains the signal that targets StAR to the mitochondria, can participate in the interaction with HSL. In view of the ability of the 30-kDa StAR to mediate its interaction with HSL, it is apparent that regions in addition to the N terminus of the protein must also be involved in the interaction with HSL. Therefore, a series of N-terminal deletions of StAR was generated and their ability to interact with HSL tested (Fig. 4). Deleting the first 62 amino acids of StAR did not affect its ability to be pulled down by GST-HSL, suggesting that the N-terminal 62 amino acids of StAR, which contains the signal that targets StAR to the mitochondria, can participate in the interaction with HSL. In view of the ability of the 30-kDa StAR to mediate its interaction with HSL, it is apparent that regions in addition to the N terminus of the protein must also be involved in the interaction with HSL. Therefore, a series of N-terminal deletions of StAR was generated and their ability to interact with HSL tested (Fig. 4). Deleting the first 62 amino acids of StAR did not affect its ability to be pulled down by GST-HSL, suggesting that the N terminus is not the only region participating in the interaction with HSL. Deleting the first 155 amino acids of StAR also did not affect its ability to be pulled down by GST-HSL; however, N-terminal deletions of the first 205 and 225 amino acids did eliminate the interaction. When C- and N-terminal deletions were combined (Fig. 5), StAR 100–241 and StAR 100–221 still retained their ability to bind to HSL; however, StAR 100–182 did not. Thus, it appears that sites within the first 62-amino acid N terminus and within the region between amino acids 182 and 205/221 are able to mediate the interaction of StAR with HSL.
Star Increases HSL Hydrolytic Activity—The interaction of ALBP with HSL is reported to increase the hydrolytic activity of the enzyme (13). The ability of ALBP to increase substrate hydrolysis by HSL is not due entirely to the binding and sequestration of fatty acids by ALBP but is dependent on the physical interaction of ALBP with HSL, suggesting that the protein-protein interaction causes a conformational change or steric effect on the enzyme. Although Star is not known to possess fatty acid binding properties, the fact that Star does interact with HSL raised the possibility that the interaction of Star with HSL might modulate HSL hydrolytic activity. To explore this possibility, HSL was expressed in CHO cells, and hydrolytic activity against cholesteryl ester was determined after coexpression of Star or vector alone (Fig. 6). The amount of HSL expressed under these experimental conditions was similar, as determined by immunoblot (data not shown). Coexpression of HSL and Star resulted in ~75% increased hydrolysis (p < 0.001) of cholesteryl ester substrate. Thus, as observed with ALBP, the interaction of Star with HSL can modulate the hydrolytic activity of the enzyme.

To investigate whether there is a correlation between HSL-Star protein-protein interaction in vitro and HSL function, various deletion mutants of Star were coexpressed with HSL in CHO cells, and hydrolytic activity against cholesteryl ester was determined (Fig. 7). Deletions of the first 100 or 155 amino acids of Star, constructs that bind HSL, stimulated hydrolytic activity against cholesteryl ester (p < 0.05) to an extent similar to that of full-length Star (1–286). Deletions of the first 205 amino acids of Star, which does not bind HSL, also did not stimulate HSL activity. However, small constructs that do bind HSL (Star 100–221 and 100–241) also had no effect on HSL activity. Interestingly, a construct in which amino acids 181–221 were deleted from

Fig. 3. Interaction of HSL with C-terminal Star deletions. C-terminal truncations of Star were generated as described under “Experimental Procedures.” A, pcDNA3-Star 1–225 (lanes 1–3) and pcDNA3-Star 1–205 (lanes 4–6) were in vitro translated with [35S]methionine using the TNT Transcription/Translation System and incubated with GST (lanes 2 and 5) or GST-HSL (lanes 3 and 6) and with glutathione-agarose beads. Proteins that bound to the beads were eluted, separated on 10% SDS-PAGE, and visualized. B, pcDNA3-Star 1–155 was in vitro translated and incubated with GST (lane 2) or GST-HSL (lane 3). C, pcDNA3-Star 1–100 (lanes 1, 3, and 4) and pcDNA3-Star 1–62 (lanes 2, 5, and 6) were in vitro translated and incubated with GST (lanes 3 and 5) or GST-HSL (lanes 4 and 6).

Fig. 4. Interaction of HSL with N-terminal Star deletions. N-terminal truncations of Star were generated as described under “Experimental Procedures.” A, pcDNA3-Star 62–286 was in vitro translated and incubated with GST (lane 2) or GST-HSL (lane 3). B, pcDNA3-Star 155–286 (lanes 1, 4, and 5), pcDNA3-Star 205–286 (lanes 2, 6, and 7), and pcDNA3-Star 225–286 (lanes 3, 8, and 9) were in vitro translated and incubated with GST (lanes 4, 6, and 8) or GST-HSL (lanes 5, 7, and 9) as described in Fig. 3.

Fig. 5. Interaction of HSL with combined N- and C-terminal Star deletions. Star 100–241 (lanes 1, 4, and 5), Star 100–221 (lanes 2, 6, and 7), and Star 100–182 (lanes 3, 8, and 9) were in vitro translated and incubated with either GST (lanes 4, 6, and 8) or GST-HSL (lanes 5, 7, and 9) as described in Fig. 3.
StAR expression increased dramatically during cholesteryl ester measurement by immunoblotting (Fig. 8A and mitochondrial cholesterol content determined. As described under “Experimental Procedures.” Lanes 1 and 2, basal; lanes 3 and 4, after loading with HDL and Bt2cAMP for 24 h; lanes 5 and 6, serum starvation overnight; lanes 7 and 8, Bt2cAMP for 2 h. B, mitochondrial cholesterol content. Mitochondria were isolated from cell extracts and cholesterol content determined as described under “Experimental Procedures.” Results are the mean ± S.E. of four independent experiments. * indicates p < 0.05.

Fig. 7. Effects of StAR truncations on HSL hydrolytic activity. CHO cells were cotransfected with pcDNA3-HSL, pCMV-StAR, or pCMV (vector alone), and pCMV-β-galactosidase. Cells were harvested 40 h after transfection for measurement of HSL, cholesteryl ester hydrolase activity. Results are the mean ± S.E. of triplicate samples and are representative of three independent experiments. * indicates p < 0.05.

Fig. 6. Effects of StAR on HSL hydrolytic activity. CHO cells were cotransfected with pcDNA3-HSL, pCMV-StAR, or pCMV (vector alone), and pCMV-β-galactosidase. Cells were harvested 40 h after transfection for measurement of HSL cholesterol ester hydrolase activity. Results are the mean ± S.E. of triplicate samples and are representative of five independent experiments. * indicates p < 0.001.

StAR also failed to stimulate HSL hydrolytic activity. Thus, it appears that the simple binding of StAR to HSL is insufficient to explain the effects on hydrolytic activity.

**HSL Augments Mitochondrial Cholesterol Content in the Presence of StAR**—To explore the functional significance of the interaction of HSL with StAR and to test the hypothesis that the interaction of HSL with StAR might facilitate intracellular cholesterol trafficking to mitochondria for steroidogenesis in adrenal cells, we transfected Y1-BS1 adrenocortical cells with HSL or, as a control, triacylglycerol hydrolase. Triacylglycerol hydrolase is a neutral cytosolic lipase that has been reported to possess triacylglycerol (26) as well as cholesteryl ester (27) hydrolytic activity; however, triacylglycerol hydrolase does not interact with StAR in in vitro pull-down experiments (data not shown). After transfection, cells were loaded with cholesteryl esters by incubation with HDL₃ (500 µg protein/ml) in the presence of Bt2cAMP for 24 h. Cells were then cultured in serum-free medium for 16 h to lower the expression of StAR to basal levels. Following this, cells were treated with or without 2.5 mM Bt2cAMP for 2 h to induce StAR expression. To prevent conversion of cholesteryl into pregnenolone, a specific inhibitor of CYP11A1, aminoglutethimide, was also included in the incubation. Cells were then harvested, the mitochondria isolated, and mitochondrial cholesterol content determined. As documented by immunoblotting (Fig. 8A), there were only very low levels of StAR protein detected basally in Y1-BS1 cells, but StAR expression increased dramatically during cholesteryl ester loading with HDL and Bt2cAMP. Serum starvation for 16 h lowered StAR to background levels, and subsequent treatment with Bt2cAMP for 2 h again induced StAR expression. Mitochondrial cholesterol content (Fig. 8B) in Y1-BS1 cells transfected with vector alone increased (p < 0.05) with Bt2cAMP treatment and the induction of StAR, consistent with the critical role of StAR in cholesterol transfer into mitochondria. Overexpression of HSL in Y1-BS1 cells in the absence of Bt2cAMP treatment did not alter mitochondrial cholesterol content. However, mitochondrial cholesterol content was augmented by overexpression of HSL and treatment with Bt2cAMP above that observed in cells transfected with vector alone and treated with Bt2cAMP (p < 0.01). In contrast, this augmentation in mitochondrial cholesterol content was not seen when cells were treated with Bt2cAMP and transfected with triacylglycerol hydrolase. These results suggest a functional significance of the interaction of HSL with StAR to facilitate cholesterol movement to mitochondria.

**DISCUSSION**

Interactions of HSL with other proteins could potentially influence several cellular processes in the adrenal. Studies (11, 12) have shown that HSL interacts specifically with intracellular proteins in adipose tissue. Demonstration of a direct interaction of HSL with ALBP (also known as aP2), a member of the family of intracellular lipid-binding proteins that bind fatty acids, retinoids, and other hydrophobic ligands, led to the proposal that HSL and ALBP constitute a “lipolytic complex” in adipose cells. The physical interaction of ALBP with HSL has
been shown to increase the hydrolytic activity of HSL and to protect HSL from product inhibition by fatty acids (13).

In the current studies we have demonstrated a direct molecular interaction of HSL with STAR, which has a functional consequence of increasing the efficiency of cholesteryl ester hydrolysis and the trafficking of intracellular cholesterol among subcellular compartments. The molecular interaction of HSL with STAR was first shown by in vitro pull-down experiments. Making use of deletional mutations of STAR, it appears that sites within the first 62-amino acid N terminus and sites within the region between amino acids 182 and 205/225 are able to mediate the interaction of STAR with HSL. The 62-amino acid N terminus is present in the cytosolic form (37 kDa) of STAR and contains the signal peptide that targets STAR to mitochondria and is normally cleaved when the protein is processed in mitochondria to its mature 30-kDa form (14, 15). Amino acids 182–205/225 of STAR include the β- and β’-sheets, along with the Ω-loop connecting them, and are located at one end of the U-shaped incomplete β-barrel forming a tunnel in the protein (20). Based on the crystal structure of the STAR-related lipid transfer (START) domain of MLN64 (StarD3), the Ω-loop connecting the β- and β’-sheets is located on the exofacial surface and potentially available for interactions with other proteins such as HSL. In addition to demonstrating an interaction of HSL and STAR in vitro, the proteins were shown to interact in vivo in adrenal tissue by demonstrating the coimmunoprecipitation of STAR with HSL. Immunoprecipitation showed that both the full-length 37-kDa and the processed 30-kDa STAR interacted with HSL. It is likely that the interaction of the 30-kDa STAR, which contains amino acids 182–205/225 as a recognition domain, does not occur physiologically within the adrenal because the 30-kDa STAR is found only within mitochondria (28), and HSL is not found within mitochondria (2). Thus, the interaction of the 30-kDa STAR with HSL probably results from the release of mitochondrial contents during tissue homogenization. In contrast, the 37-kDa full-length STAR is expressed in cytosol along with HSL and contains two regions, amino acids 1–62 and 182–205/225, which can mediate the interaction.

One of the functional consequences of the interaction of STAR with HSL is an increase in the efficiency of the cholesteryl ester hydrolytic activity of HSL. The basis for this increase in hydrolytic activity is unclear, but the observation appears similar to experiments examining the functional interaction of ALBP with HSL. ALBP induces an increase in HSL hydrolytic activity that is caused by the ability of ALBP to interact with HSL and the ability of ALBP to bind fatty acid (13). Even though STAR does not possess fatty acid binding properties and the quantity of fatty acids released during the hydrolysis of cholesteryl esters would not be expected to be sufficient to have detrimental effects, the physical interaction of STAR with HSL might either alter the conformation of the enzyme allowing substrate to access the catalytic site more efficiently or prevent fatty acids from inhibiting the enzyme through a conformational change or steric inhibition. However, there is not a direct correlation between HSL-STAR protein-protein interaction in vitro and HSL hydrolytic activity. Although deletions of the N terminus, which resulted in 131- and 186-amino acid STAR proteins that can bind to HSL via sites within the 182–205/225 domain, could stimulate hydrolytic activity, expression of small portions of STAR (81 and 121 amino acids) which can bind HSL through the 182–205/225 region did not stimulate hydrolytic activity. Moreover, expression of a 246-amino acid STAR construct that could bind HSL via the N terminus, but which lacked amino acids 182–205/225, did not stimulate hydrolytic activity. Therefore, amino acids 182–205/225 appear to be necessary for stimulation of HSL hydrolytic activity, but other regions of STAR also seem to be required.

The rate-limiting step in steroidogenesis appears to be the transfer of unesterified cholesterol from the outer mitochondrial leaflet to the inner leaflet where CYP11A1 is located (14, 15, 29). This intramitochondrial movement of cholesterol involves STAR and other proteins such as the peripheral-type benzodiazepine receptor. For steroidogenesis to continue, cholesterol must be replenished on the outer mitochondrial leaflet. Under most circumstances, the movement of cholesterol to the outer mitochondrial leaflet is not rate-limiting because there are a number of different sources of cholesterol. The mechanisms controlling the movement of cholesterol to the mitochondria have not been well studied. Cholesterol trafficking in cells is a complex process that is not fully understood and appears to involve a number of different mechanisms. Cholesterol movement into and out of the plasma membrane, which contains >80% of cellular cholesterol in most cells, can involve vesicular and nonvesicular pathways (31–33). These pathways appear to depend on specialized lipid rafts and associated proteins. The delivery of cholesterol via LDL receptor-mediated endocytosis involves the hydrolysis of lipoprotein cholesteryl esters by acid lipase within late endosomes and lysosomes and then the movement of the unesterified cholesterol out of these organelles. Proteins such as NPC1 (Niemann-Pick Type C protein 1) appear to be important in this process, yet the mechanisms involved are not understood (34). NPC1 appears to be important in trafficking LDL cholesterol for steroidogenesis, particularly under conditions in which the substrate for steri
dogenesis is primarily supported by LDL cholesterol, but does not appear to be involved in trafficking other pathways for cholesterol delivery for steroidogenesis (35). MLN64 (StarD3), a member of the START domain family, has recently been reported to facilitate the movement of lysosomal cholesterol to mitochondria for steroidogenesis (36).

A second and potentially major functional consequence of the interaction of STAR with HSL is suggested by our experiments demonstrating that the overexpression of HSL in the presence of STAR results in an augmentation of the movement of cholesterol into mitochondria in adrenal cells. Importantly, overexpression of a different lipase, which showed no interaction with STAR in vitro, failed to augment cholesterol movement into mitochondria above that seen with STAR alone. These results support the hypothesis that STAR and HSL form a functional complex in the adrenal to facilitate cholesterol movement to mitochondria. The close physical association of STAR with HSL within this complex would enable unesterified cholesterol released during HSL-mediated hydrolysis of cholesteryl esters to be efficiently and rapidly sequestered by newly synthesized STAR for transport first to the outer leaflet and then to the interior of mitochondria for steroidogenesis. In this way the interaction of HSL with a STAR would constitute the initial step in facilitating the trafficking of intracellular cholesterol out of the lipid droplet. However, in view of the recent discovery of additional members of the START domain superfamily (37), some of which (StarD4 and StarD5) are variably, but ubiquitously, expressed in all tissues examined, it is possible that the finding of an interaction of HSL with STAR (StarD1) might not represent the only physiologically relevant interaction. Rather, HSL might interact physiologically with either StarD4 or StarD5 or another related family member. Additional experiments will be required to explore these possibilities.

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Interaction of Hormone-sensitive Lipase with Steroidogeneic Acute Regulatory Protein: FACILITATION OF CHOLESTEROL TRANSFER IN ADRENAL
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doi: 10.1074/jbc.M303934200 originally published online August 18, 2003

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