Fatty acid transport protein 1 and long-chain acyl coenzyme A synthetase 1 interact in adipocytes

M. Rachel Richards, Jeffrey D. Harp, Daniel S. Ory, and Jean E. Schaffer[1]

Center for Cardiovascular Research and Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110

Abstract The fatty acid transport proteins (FATP) and long-chain acyl coenzyme A synthetase (ACSL) proteins have been shown to play a role in facilitating long-chain fatty acid (LCFA) transport in mammalian cells under physiologic conditions. The involvement of both FATP and ACSL proteins is consistent with the model of vectorial acylation, in which fatty acid transport is coupled to esterification. This study was undertaken to determine whether the functions of these proteins are coordinated through a protein-protein interaction that might serve as a point of regulation for cellular fatty acid transport. We demonstrate for the first time that FATP1 and ACSL1 coimmunoprecipitate in 3T3-L1 adipocytes, indicating that these proteins form an oligomeric complex. The efficiency of FATP1 and ACSL1 coimmunoprecipitation is unaltered by acute insulin treatment, which stimulates fatty acid uptake, or by treatment with isoproterenol, which decreases fatty acid uptake and stimulates lipolysis. Moreover, inhibition of ACSL1 activity in adipocytes impairs fatty acid uptake, suggesting that esterification is essential for fatty acid transport. Together, our findings suggest that a constitutive interaction between FATP1 and ACSL1 contributes to the efficient cellular uptake of LCFAs in adipocytes through vectorial acylation.—Richards, M. R., J. D. Harp, D. S. Ory, and J. E. Schaffer. **Fatty acid transport protein 1 and long-chain acyl coenzyme A synthetase 1 interact in adipocytes. *J. Lipid Res.* 2006.** 47: **665–672.**

Supplementary key words oligomer • fatty acid transport protein • ACSL • lipid import

A central aspect of adipocyte biology is the ability of these specialized cells to efficiently take up and store long-chain fatty acids (LCFAs) in response to nutritional and hormonal cues. In the "fed" state, higher serum levels of insulin stimulate an increase in fatty acid uptake and triglyceride storage in adipose tissue. On the other hand, during "starvation," β-adrenergic receptor agonists, such as epinephrine, stimulate pathways that activate hormone-sensitive lipase in adipocytes, resulting in the hydrolysis of triglycerides and the release of free fatty acids and glycerol. In obesity and the metabolic syndrome, dysregulation of these processes may contribute to increases in serum free fatty acids and the genesis of type 2 diabetes (1, 2).

The movement of LCFAs across the plasma membrane is tightly coupled to thioesterification, a process referred to as vectorial acylation (3). Early evidence for this mechanism came from genetic studies of *Escherichia coli*, in which fatty acid transport requires FadL, an outer membrane LCFA transporter, and FadD, an inner membrane-associated acyl CoA synthetase (4, 5). Vectorial acylation provides cells with an efficient means of rapidly metabolizing incoming fatty acids and contributes to decreasing the intracellular concentration of free fatty acids to favor import. Thioesterification of LCFAs is also an essential initial metabolic step for many downstream metabolic pathways of LCFA use, such as β-oxidation or triglyceride synthesis. The coupled transport and metabolism of LCFAs is analogous to glucose transport in mammalian cells, a process in which GLUT4-mediated translocation of glucose across the plasma membrane is coordinated with rapid phosphorylation by hexokinase.

In mammalian cells, a number of proteins have been suggested to facilitate or regulate fatty acid transport across the plasma membrane, including fatty acid transport protein 1 (FATP1) and long-chain acyl coenzyme A synthetase 1 (ACSL1) (6–8). These proteins were identified in a functional screen for adipocyte proteins that augment cellular fatty acid import (9). The precise function of FATP1, an integral plasma membrane protein, and other FATP family members is not well understood, but it is thought to include both the movement of LCFAs across the plasma membrane and esterification (10–12). ACSL1 catalyzes the addition of a CoA group to the 1-C position over a broad range of LCFA substrates via the formation of transient acyl adenylate intermediates (13). Overexpression of either FATP1 or ACSL1 alone in fibroblasts increases LCFA import, and coexpression of these proteins is synergistic (14). Our previous observation of the

Manuscript received 23 November 2005 and in revised form 14 December 2005.

Published, JLR Papers in Press, December 15, 2005.
DOI 10.1194/jlr.M500514-JLR200

Abbreviations: ACSL, long-chain acyl coenzyme A synthetase; FATP, fatty acid transport protein; HRP, horseradish peroxidase; LCFA, long-chain fatty acid.

[1] To whom correspondence should be addressed.
e-mail: jschaff@wustl.edu

colocalization of FATP1 and ACSL1 by immunofluorescence of plasma membrane lawns from 3T3-L1 adipocytes, which natively express FATP1 and ACSL1, suggested that one way in which the functions of these proteins may be coupled is through a direct physical interaction. Recently, Black and colleagues (11) demonstrated coimmunoprecipitation between Fat1p and Faa1p or Faa4p, yeast orthologs of FATP1 and ACSL1, respectively.

The goal of this study was to determine whether FATP1 and ACSL1 physically interact in adipocytes. Moreover, we sought to determine whether this interaction serves as a point of regulation for LCFA transport. Our results show that these proteins physically interact in mammalian cells in a manner that is likely to be constitutive for vectorial fatty acid uptake.

MATERIALS AND METHODS

Materials

Oleic acid was obtained from Nu-Check Prep, human recombinant insulin from Sigma-Aldrich, protein G-agarose and Protease Complete from Roche Molecular Biochemicals, mouse monoclonal α-HA antibody (HA.11) from Covance, Inc., mouse monoclonal α-Myc antibody (9E10) from Upstate USA, Inc., mouse monoclonal α-FLAG antibody from Sigma, horseradish peroxidase (HRP)-coupled IgGs from Jackson ImmunoResearch Laboratories, Inc., and Renaissance Western blot chemiluminescence reagents from Perkin-Elmer Life Sciences. Antibody to native FATP1 (amino acids 628–640) was generated as described previously (9). Rabbit polyclonal antiserum directed against ACSL1 amino acids 676–689 was generated as described previously (14). Rabbit polyclonal GLUT4 antibody was a gift of M. Birnbaum. [^{14}C]oleic acid was purchased from American Radiolabeled Chemicals. Cell culture reagents were from Invitrogen. 3T3-L1 adipocytes were purchased from the American Type Culture Collection (ATCC CL-173) and were cultured using Sigma and Invitrogen cell culture reagents. All other chemicals were purchased from Sigma.

Cell culture

Plasmid DNA encoding murine FATP1 with an N-terminal HA or Myc epitope tag or murine ACSL1 with an N-terminal Myc or FLAG epitope tag was subcloned into the ΔU3 retroviral expression vector and transiently transfected into 293GPG packaging cells to produce high-titer VSV-G pseudotyped retrovirus, as described previously (15). Subconfluent 3T3-L1 preadipocytes were subsequently transduced with retrovirus to generate 3T3-L1 FATP1nHA or 3T3-L1 ACSL1nMyc cell populations. Culture and differentiation of 3T3-L1 cells to mature adipocytes was induced as described previously (16). Adipocytes were used between days 6 and 8 of differentiation.

Insulin and oleate treatment

3T3-L1 adipocytes were serum-starved for 3 h followed by stimulation with 100 nM insulin and/or 500 μM oleate complexed to BSA at a 2:1 ratio (17) for 20 min, unless noted otherwise.

Immunoprecipitations

All manipulations were at 4°C. Cells were washed in PBS and lysed in TNET (50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100), TNES (50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% SDS), TNE-NP-40 (50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% NP-40), TNET containing 60 mM octylglucoside, or RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.5% SDS). All lysis buffers also contained 1 μM phenylmethylsulfonyl fluoride, 1× Protease Complete, and 100 μM iodoacetamide. Nuclei and unbroken cells were pelleted at 1,000 *g* for 10 min at 4°C. Postnuclear supernatants were precleared in protein G-agarose beads for 20 min followed by incubation with primary antisera for 1 h at the following dilutions: α-HA (1:250), α-Myc (1:100), α-FLAG (1:500). Competing HA, Myc, or FLAG peptide was included in parallel samples at a final concentration of 0.1 mg/ml. Protein G-agarose beads were incubated with samples for an additional 45 min. Beads were washed with lysis buffer followed by PBS. Proteins were eluted by boiling in 2× Laemmli sample buffer.

Western blot analysis

Proteins were separated by 8% SDS-PAGE and transferred to 0.2 μm pore nitrocellulose membranes (Schleicher and Schuell). Primary antibodies were incubated with nitrocellulose blots at the following dilutions: α-HA (1:2,000), α-Myc (1:1,000), α-FLAG (1:2,000), α-FATP (1:1,000), α-ACS (1:1,000), and α-GLUT4 (1:2,500). Detection was performed using HRP-coupled secondary antisera and Renaissance Western blot chemiluminescence reagents (Perkin-Elmer Life Sciences).

Fractionation

Adipocytes were fractionated as described previously (14, 18). Fractions were subsequently resuspended in RIPA buffer containing 1 μM phenylmethylsulfonyl fluoride and 1× Protease Complete, and proteins were quantified by bicinchoninic acid assay (Pierce Chemical Co.).

Lipolysis and glycerol release assay

Mature 3T3-L1 adipocytes were incubated in serum-free medium containing 2% fatty acid-free BSA (Sigma-Aldrich) for 3 h. Cells were then stimulated with 5 or 10 μM isoproterenol (Sigma-Aldrich) in serum-free medium containing 2% fatty acid-free BSA. To measure lipolysis, medium was harvested from cells and glycerol concentrations were measured using a colorimetric assay (Sigma). Glycerol concentrations were normalized to the amount of protein in each sample by bicinchoninic acid assay. Medium was removed before the initiation of fatty acid transport assays.

Fatty acid uptake assay

Fluorescent fatty acid uptake assays were performed as described previously (9). Briefly, after 1 h of serum starvation and short-term stimulation with either 100 nM insulin or 1–10 μM isoproterenol, cells were incubated at 37°C for 1 min with a fatty acid uptake mixture containing 0.6 μM BODIPY-3823 (Molecular Probes) and 20 μM fatty acid-free BSA. Cells were washed three times at 4°C with stop solution (PBS containing 0.1% fatty acid-free BSA and 500 μM phloretin), trypsinized, and pelleted by centrifugation. Cells were resuspended in DMEM containing 1 μM propidium iodide and analyzed by flow cytometry on a Becton Dickinson FACScan.

Esterification assay

3T3-L1 adipocytes were fractionated and membrane microsomes were analyzed as described previously (14). Briefly, 50 μg of protein was incubated with [^{14}C]oleate for 2 min at 35°C in the presence of CoA, ATP, and Mg^{2+}. [^{14}C]oleoyl-CoA was separated from [^{14}C]oleate by heptane extraction, and 1 ml of the aqueous phase was counted by scintillation.

Statistics

All results are expressed as means ± SEM. The statistical significance of differences in mean values was determined by single-factor ANOVA. Data shown are representative of at least three similar experiments.

RESULTS

FATP1 and ACSL1 coimmunoprecipitate in 3T3-L1 adipocytes

To determine whether FATP1 and ACSL1 interact in adipocytes, we assayed for coimmunoprecipitation in differentiated 3T3-L1 cells. Because antisera directed against FATP1 and ACSL1 do not immunoprecipitate these proteins efficiently, we used 3T3-L1 adipocytes expressing either FATP1 with an N-terminal HA epitope tag or ACSL1 with an N-terminal Myc tag. Using antisera to either epitope tag, we immunoprecipitated FATP1nHA or ACSL1nMyc, followed by Western blot analysis with antisera to native ACSL1 or FATP1 to detect the endogenous interacting partner (**Fig. 1**). We observed coimmunoprecipitation between ACSL1 and FATP1, both immunoprecipitating with α-HA antibody followed by Western blot for native ACSL1 and immunoprecipitating with α-Myc antibody followed by Western blot for native FATP1. As expected, competing HA or Myc peptide inhibited immunoprecipitation as well as coimmunoprecipitation. These results indicate that FATP1 and ACSL1 participate in an oligomeric complex in 3T3-L1 adipocytes.

To characterize the nature of the interaction between ACSL1 and FATP1, we assayed for coimmunoprecipitation of FATP1 and ACSL1 when 3T3-L1 FATP1nHA adipocytes were lysed in a variety of detergents. Although immunoprecipitation of FATP1nHA occurred in each detergent, coimmunoprecipitation between ACSL1 and FATP1nHA was diminished (**Fig. 2**, lanes 3, 5) or abrogated (Fig. 2, lane 9) in buffers containing octylglucoside or SDS. Coimmunoprecipitation was most efficient in buffers containing nonionic detergents (Triton X-100 or NP-40) (Fig. 2, lanes 1, 7). Because immunoprecipitation of FATP1nHA was most efficient in 1% Triton X-100, this was used for all subsequent studies. The observation that mild, nonionic detergents are most favorable for coimmunoprecipitation between ACSL1 and FATP1 is consistent with a noncovalent protein-protein interaction.

Previously, we showed that FATP1 forms homodimers and that dimerization plays a functional role in cellular fatty acid import (19). Thus, we tested whether ACSL1 forms an oligomeric complex with more than one molecule of FATP1. We performed coimmunoprecipitation assays in 3T3-L1 adipocytes expressing FATP1nHA, FATP1nMyc, and ACSL1nFLAG. By immunoprecipitating

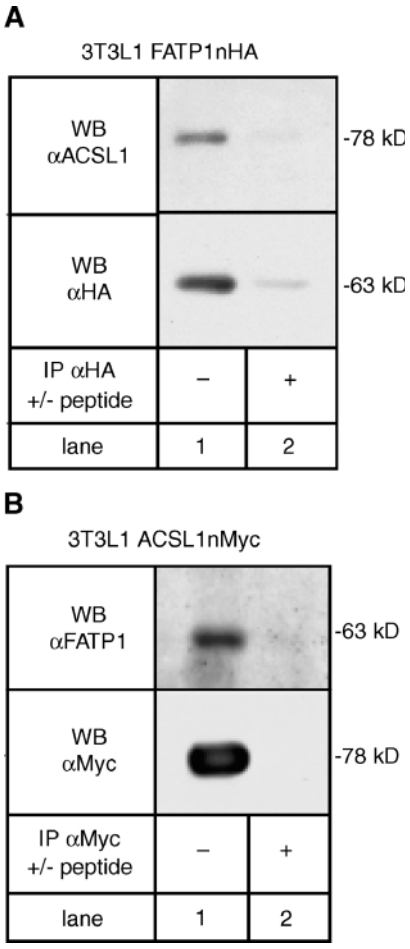

Fig. 1. Fatty acid transport protein 1 (FATP1) and long-chain acyl coenzyme A synthetase 1 (ACSL1) coimmunoprecipitate in 3T3-L1 adipocytes. 3T3-L1 adipocytes expressing either FATP1nHA (A) or ACSL1nMyc (B) were lysed in TNET (see Materials and Methods) and immunoprecipitated (IP) with either α-HA (A) or α-Myc (B) antibody, respectively. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blot (WB), using horseradish peroxidase (HRP)-coupled secondary antibody, and chemiluminescence. HA peptide (A, lane 2) or Myc peptide (B, lane 2) was included as a competitor in control immunoprecipitations. Blots are representative of three independent experiments.

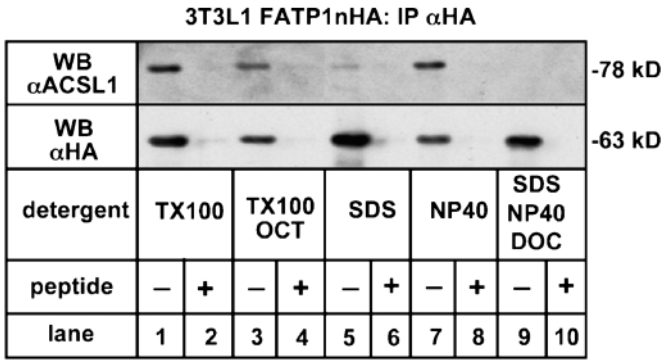

Fig. 2. ACSL1 and FATP1 coimmunoprecipitate in buffers containing nonionic detergents. 3T3-L1 adipocytes expressing FATP1nHA were lysed in buffers containing nonionic detergents [Triton X-100 (TX-100), Triton X-100 + octylglucoside (OCT), NP-40] or ionic detergents [SDS, SDS + NP-40 + deoxycholate (DOC)]. Immunoprecipitations (IP) were performed with α-HA antibody followed by SDS-PAGE, Western blot analysis (WB), using HRP-coupled secondary antibody, and chemiluminescence. Competing HA peptide was included in even-numbered lanes. Blots are representative of three independent experiments.

to FATP1nHA, FATP1nMyc, or ACSL1nFLAG and immunoblotting with α-HA, α-Myc, and α-FLAG antibodies, we observed interactions between all three partners, irrespective of which antibody was used for immunoprecipitation or Western blot (**Fig. 3**). These results suggest a higher order oligomer in 3T3-L1 adipocytes containing two FATP1 molecules and ACSL1.

Interaction between ACSL1 and FATP1 is constitutive

A physical interaction between FATP1 and ACSL1 may be important for coupling LCFA transport across the plasma membrane to subsequent thioesterification (20). Based on this model, we sought to determine whether the interaction between FATP1 and ACSL1 might serve as a point of regulation for LCFA import. Insulin is a lipogenic hormone that promotes fatty acid uptake in adipocytes and the accumulation of triglyceride in adipose tissues. Because FATP1 and ACSL1 participate in fatty acid uptake and metabolism, we examined whether insulin might regulate fatty acid uptake by increasing the interaction between FATP1 and ACSL1. We stimulated 3T3-L1 adipocytes with 100 nM insulin for periods of up to 20 min, a time frame suitable for analyzing posttranslational changes in these proteins. Subcellular fractionation followed by Western blot analysis to GLUT4 protein showed enrichment of GLUT4 protein in the plasma membrane fraction upon insulin treatment, as expected (**Fig. 4A**). Concomitantly, there was a progressive increase in fatty acid uptake, with a 2.5-fold increase in uptake at 5 min and 3.3-fold increased fatty acid uptake at 20 min compared

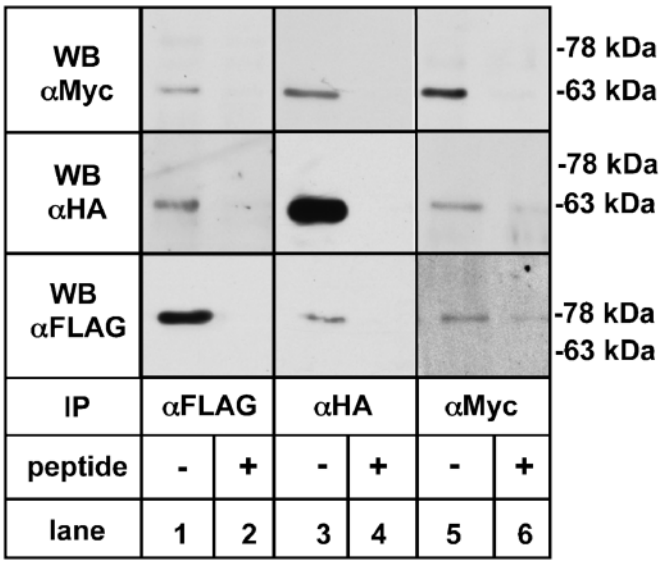

Fig. 3. FATP1 dimers coimmunoprecipitate with ACSL1. 3T3-L1 adipocytes expressing FATP1nHA, FATP1nMyc, and ACSL1nFLAG were lysed in TNET and immunoprecipitated (IP) with α-HA, α-Myc, or α-FLAG. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blot (WB), using HRP-coupled secondary antibody, and chemiluminescence. FLAG peptide (lane 2), HA peptide (lane 4), or Myc peptide (lane 6) was included as a competitor in control immunoprecipitations. Blots are representative of three independent experiments.

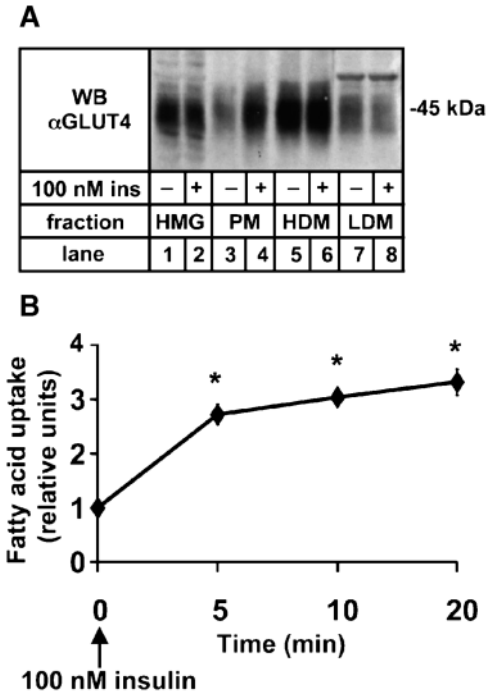

Fig. 4. Acute insulin treatment of adipocytes stimulates fatty acid uptake. A: 3T3-L1 adipocytes were stimulated with 100 nM insulin (ins) for 20 min (even-numbered lanes), homogenized (HMG), and fractionated to yield plasma membrane (PM), high density microsomes (HDM), or low density microsomes (LDM). Protein (30 μg) was analyzed by Western blot (WB) using α-GLUT4 antibody, HRP-coupled secondary antibody, and chemiluminescence. B: After treatment with 100 nM insulin for 5, 10, or 20 min, fatty acid uptake was assayed using BODIPY-3823 and flow cytometry. Data are reported as means ± SEM fold increase in fluorescence for 10^5 cells in each of six samples (three independent experiments). * $P < 0.0001$ compared with untreated cells.

with untreated controls (Fig. 4B). We also observed similar results in 3T3-L1 adipocytes expressing FATP1nHA (data not shown).

To determine whether insulin-mediated increases in fatty acid uptake were mediated through enhanced ACSL1 and FATP1 interaction, we assessed the efficiency of coimmunoprecipitation of these proteins from 3T3-L1 FATP1nHA adipocytes under basal conditions and after treatment with 100 nM insulin for 20 min. After immunoprecipitation of FATP1nHA, Western blot analysis was used to detect coimmunoprecipitated native ACSL1. We detected no significant change in the relative levels of ACSL1 that coimmunoprecipitated with FATP1nHA in response to insulin when normalized to the amount of FATP1nHA that immunoprecipitated by densitometry (**Fig. 5A**, lane 3). We observed similar results in 3T3-L1 adipocytes overexpressing ACSL1nMyc, in which case we immunoprecipitated ACSL1nMyc and performed Western blot analysis using antisera to native FATP1 and α-Myc (data not shown).

Because in the physiological fed state adipocytes are exposed to increased systemic free fatty acid as well as insulin, we also treated 3T3-L1 FATP1nHA adipocytes with

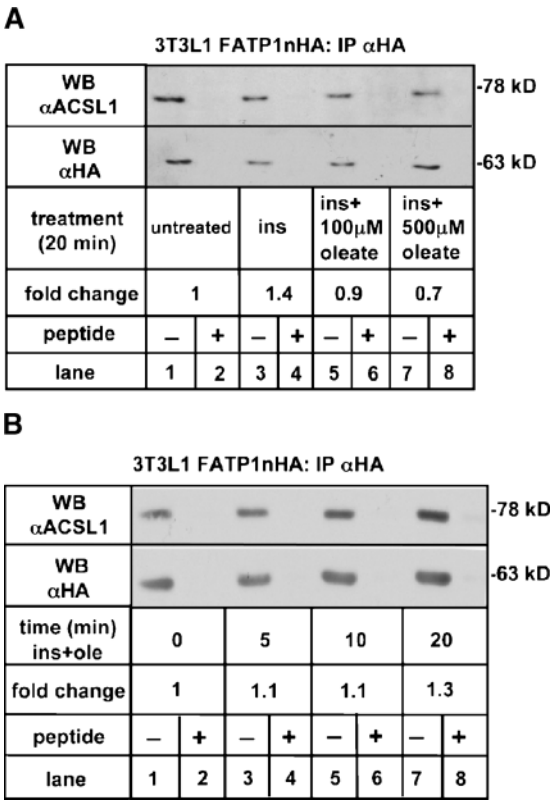

Fig. 5. Efficiency of coimmunoprecipitation is not affected by insulin or oleate. 3T3-L1 adipocytes expressing FATP1nHA were stimulated with insulin (ins) alone (A, lanes 3, 4) or insulin plus oleate (A, lanes 5–8, B) for varying times as indicated (A, B). Cells were lysed in TNET and immunoprecipitated (IP) with α-HA antibody. Immunoprecipitations in even-numbered lanes included competing HA peptide. Immunoprecipitated proteins were analyzed by SDS-PAGE, Western blot analysis (WB), using HRP-coupled secondary antibody, and chemiluminescence. Densitometry was performed to normalize relative levels of coimmunoprecipitating ACSL1 to immunoprecipitating FATP1nHA (fold change). Data are representative of three independent experiments.

100 nM insulin in conjunction with different concentrations of oleate complexed to BSA at a 2:1 ratio. After treatment, we immunoprecipitated FATP1nHA and performed Western blot analysis to endogenous ACSL1 and α-HA. As with insulin alone, we observed no significant changes in normalized levels of coimmunoprecipitated ACSL1 to immunoprecipitated FATP1nHA compared with untreated cells for 5, 10, or 20 min (Fig. 5A, lanes 5 and 7, B). Treatment of 3T3-L1 FATP1nHA adipocytes with 500 μM oleate alone also did not alter the relative levels of ACSL1 and FATP1nHA coimmunoprecipitation (data not shown). Together, these results indicate that although acute insulin treatment stimulates GLUT4 translocation to the plasma membrane and increases cellular fatty acid uptake, the extent of the interaction between ACSL1 and FATP1 remains unchanged under these conditions.

β-Adrenergic stimulation is another physiological stimulus that alters the movement of free fatty acids across the plasma membrane of adipocytes. Isoproterenol has been used as a pharmacological trigger for lipolysis in cultured 3T3-L1 adipocytes and results in the release of glycerol and free fatty acids (21, 22). We sought to determine whether isoproterenol might decrease the levels of ACSL1 and FATP1 coimmunoprecipitation to downregulate fatty acid uptake and enhance fatty acid efflux. Lipolysis was stimulated significantly after 30 and 60 min of incubation of 3T3-L1 FATP1nHA adipocytes with 5 μM isoproterenol in the presence of 2% fatty acid-free BSA as an extracellular acceptor for free fatty acids (**Fig. 6A**). We also observed similar magnitudes of glycerol release in response to 10 μM isoproterenol, indicating that 5 μM isoproterenol is saturating (data not shown). Next, we examined the effects of short-term isoproterenol treatment on fatty acid uptake in 3T3-L1 adipocytes. Because

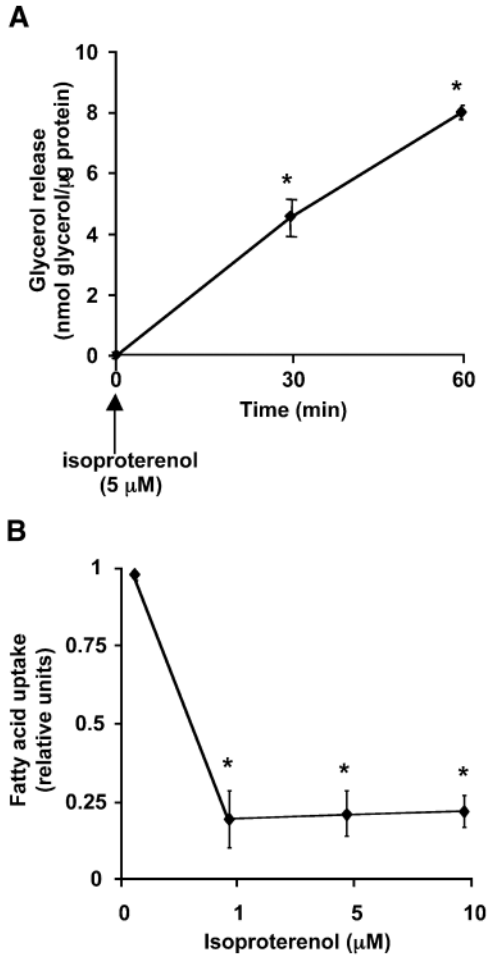

Fig. 6. Isoproterenol treatment stimulates lipolysis and decreases fatty acid uptake. A: 3T3-L1 adipocytes expressing FATP1nHA were stimulated with 5 or 10 μM isoproterenol for 30 or 60 min. The amount of glycerol released into medium for each sample was normalized to cellular protein concentration. Each data point represents the mean value ± SEM from nine samples (three independent experiments each performed in triplicate). * $P < 0.0001$ compared with untreated cells. B: After treatment with 1, 5, or 10 μM isoproterenol for 30 min, fatty acid uptake was assayed using BODIPY-3823 and flow cytometry. Data are reported as average means ± SEM fluorescence from three independent experiments (in each 2×10^5 cells analyzed). * $P < 0.0001$ compared with untreated cells.

hydrolysis of triglyceride and fatty acid efflux is stimulated under lipolytic conditions, we expected fatty acid uptake to be downregulated. Isoproterenol caused a significant decrease in fatty acid uptake after 30 min of treatment at 1, 5, or 10 μM, inducing an 80% decrease in uptake compared with untreated adipocytes (Fig. 6B).

To examine the effects of lipolysis on the interaction between ACSL1 and FATP1, we treated serum-starved 3T3-L1 adipocytes overexpressing FATP1nHA with 5 μM isoproterenol for 30 or 60 min. After stimulation, we immunoprecipitated FATP1nHA and performed Western blot analysis with α-ACSL1 or α-HA antibodies (**Fig. 7**). We observed no significant changes in the ratios of coimmunoprecipitated ACSL1 to immunoprecipitated FATP1nHA after stimulation with isoproterenol compared with untreated cells. These results indicate that in 3T3-L1 FATP1nHA adipocytes, although lipolysis is stimulated in response to isoproterenol and fatty acid uptake is decreased, the association between ACSL1 and FATP1nHA is unaffected under these conditions.

Esterification is critical for fatty acid transport

The unaltered extent of association between FATP1 and ACSL1 in response to various stimuli of fatty acid metabolism indicates that the association may occur in a constitutive manner. This would further support the model of vectorial acylation, which suggests that fatty acid transport is tightly linked to esterification. To further explore this possibility and to characterize the functional significance of the interaction between FATP1 and ACSL1, we hypothesized that fatty acid esterification might be required for fatty acid uptake in adipocytes. To assess this, we treated 3T3-L1 adipocytes with triacsin C, an inhibitor of LCFA esterification activity, followed by fatty acid uptake assays.

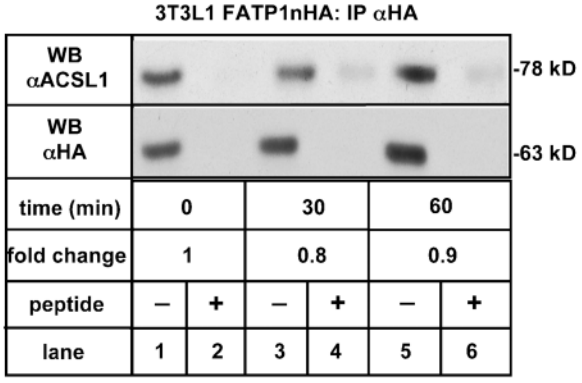

Fig. 7. Isoproterenol treatment does not alter the coimmunoprecipitation of ACSL1 and FATP1. 3T3-L1 adipocytes expressing FATP1nHA were treated with 5 μM isoproterenol. Cells were lysed in TNET and immunoprecipitated (IP) with α-HA antibody. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blot (WB). Even-numbered lanes included competing HA peptide. Densitometry was performed to normalize relative levels of coimmunoprecipitating ACSL1 to immunoprecipitating FATP1nHA (fold change). Blots are representative of three independent experiments.

In a dose-dependent manner, triacsin C treatment appropriately inhibited LCFA esterification activity in 3T3-L1 adipocytes compared with untreated cells (**Fig. 8A**). We observed 75% inhibition after 10 μM treatment and 90%

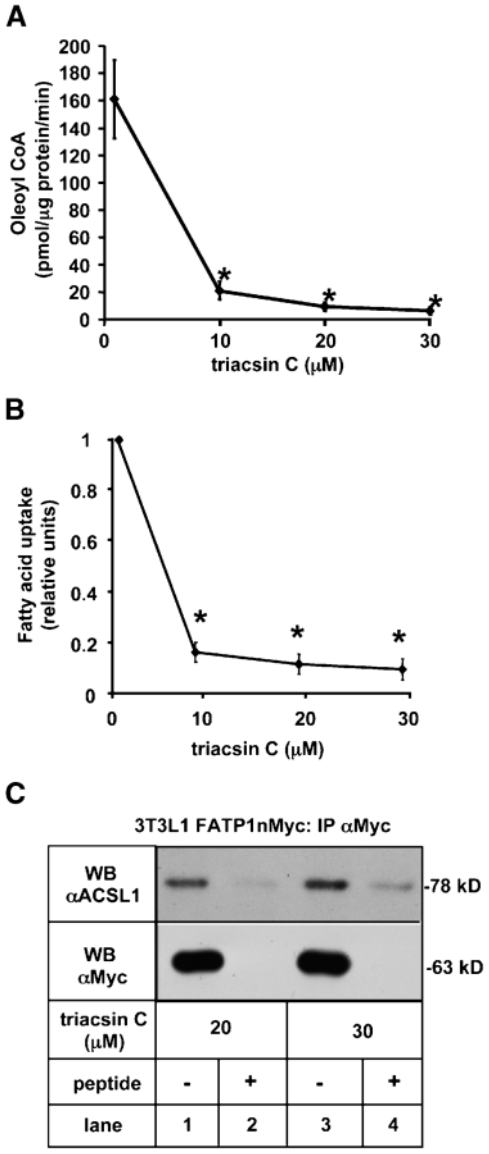

Fig. 8. Triacsin C inhibition of long-chain fatty acid esterification also inhibits fatty acid uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with 10, 20, or 30 μM triacsin C for 15 min. A: After treatment, microsomes were harvested from adipocytes and esterification assays were performed and normalized for microsomal protein. Results are averages ± SEM of four independent experiments in which each sample was assayed in triplicate. * $P <$ 0.0001 compared with untreated cells. B: After treatment, fatty acid uptake was assayed using BODIPY-3823 and flow cytometry. Data are reported as average means ± SEM fluorescence from three independent experiments (in each 10^5 cells analyzed). * $P <$ 0.0001 compared with untreated cells. C: After treatment, 3T3-L1 adipocytes expressing FATP1nMyc were lysed in TNET and immunoprecipitated (IP) with α-Myc antibody. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blot (WB) using α-ACSL1 and α-Myc. Even-numbered lanes included competing Myc peptide. Blots are representative of three independent experiments.

inhibition at 20 and 30 μM triacsin C. To assess the effects of inhibition of esterification on fatty acid uptake, we next performed fatty acid uptake assays on 3T3-L1 adipocytes treated with triacsin C, using a fluorescent analog of palmitate and flow cytometry. We observed significant levels of inhibition of fatty acid uptake at all concentrations of triacsin C compared with untreated cells (Fig. 8B). In addition, the levels of inhibition were very similar to the extent of inhibition observed for esterification activity (84% inhibition after 10 μM treatment and 99.9% inhibition at 20 and 30 μM triacsin C). The extent of coimmunoprecipitation between FATP1nMyc and ACSL1 was not affected by triacsin C treatment (Fig. 8C). These results indicate that fatty acid uptake activity is dependent on esterification activity in 3T3-L1 adipocytes.

DISCUSSION

In mammalian cells, FATP and ACSL proteins participate in the import of LCFAs across the membrane. Our prior studies showed that FATP1 and ACSL1 function coordinately in fatty acid uptake (9, 14) and that these two proteins codistribute at the plasma membrane of 3T3-L1 adipocytes. In this study, we demonstrate for the first time that ACSL1 and FATP1 coimmunoprecipitate in the murine adipocyte, indicating that they participate in a higher order oligomeric complex. These results extend the recent findings of Black and colleagues (11) in which yeast orthologs of these proteins, Fat1p, Faa1p, and Faa4p, were shown to interact physically. Our findings are consistent with the vectorial acylation model of fatty acid uptake in mammalian cells, lending support to the notion that this mechanism is conserved across evolution.

Previously, we published results indicating that FATP1 homodimerization plays a role in fatty acid uptake function (19). In this study, we observed coimmunoprecipitation between differently epitope-tagged forms of FATP1 and ACSL1 in 3T3-L1 adipocytes. These results are consistent with a model in which FATP1 homodimers interact with ACSL1 in an oligomeric complex in adipocytes. Upon expression of tagged forms of both FATP1 and ACSL1, we observed weak coimmunoprecipitation in preadipocytes and no coimmunoprecipitation in Cos7 cells (data not shown), suggesting that the interaction is relatively specific for the adipocyte milieu. Future studies are needed to identify the domains necessary for the formation of protein-protein interactions between FATP1 and ACSL1 and to determine whether these proteins also interact with other proteins demonstrated to play a role in fatty acid permeation (e.g., CD36, $FABP_{pm}$, and FABP).

In addition to the potential efficiency of vectorial acylation as a transport mechanism, we hypothesized that tight coupling of fatty acid uptake and esterification might provide a point for the regulation of fatty acid trafficking in adipocytes through alterations in the degree of physical interaction of these proteins. If the interaction between ACSL1 and FATP1 were regulated, we would expect stimuli that cause increased fatty acid uptake or increased fatty acid efflux to modulate the efficiency of this interaction. However, our coimmunoprecipitation studies show that the degree of interaction between ACSL1 and FATP1 is unchanged in response to insulin, insulin plus oleate, or isoproterenol. Furthermore, stimulation of adipocytes with AICAR, an AMPK agonist that enhances β-oxidation, did not affect the interaction between ACSL1 and FATP1 (data not shown). Although it is beyond the scope of this study to exhaustively test the stimuli of all fatty acid metabolic pathways in adipocytes, our results indicate that stimuli with established effects on fatty acid uptake and efflux do not alter the degree of FATP1-ACSL1 interaction. Our results are most consistent with a model in which ACSL1 and FATP1 interact in a constitutive manner in adipocytes.

The conditions we chose to assay for changes in the FATP1-ACSL1 interaction were sufficiently brief (20 to 30 min) to focus on posttranslational changes, such as alterations in protein-protein interactions. Our controls show that 20 min of insulin stimulation is sufficient to observe the enrichment of GLUT4 protein in plasma membrane fractions and a significant increase in fatty acid uptake, findings consistent with the activation of downstream insulin signaling and metabolic pathways. Although we did not observe changes in FATP1 or ACSL1 subcellular localization by fractionation after 20 min of insulin (data not shown), longer periods of insulin stimulation (60 min) are associated with the translocation of FATP1 from an intracellular perinuclear fraction to the plasma membrane (23). In addition, 30 min of isoproterenol treatment was sufficient to stimulate glycerol release and to downregulate fatty acid uptake in 3T3-L1 adipocytes, indicating that lipolysis had been stimulated. We focused on these relatively shorter periods of stimulation to avoid a potential contribution of newly synthesized protein.

Maintenance of a constitutive interaction between FATP1 and ACSL1 may contribute to the efficient cellular uptake of LCFAs in adipocytes through vectorial acylation. In support of this model, inhibition of ACSL1 activity by biochemical means using triacsin C impairs fatty acid uptake in adipocytes. This result extends previous findings from Black and colleagues (24), who reported that deletion of the yeast homologs of ACSL1 (Faa1p and Faa4p) disrupted fatty acid uptake in yeast. In addition, these results are the first indication that the inhibition of esterification inhibits fatty acid uptake in mammalian cells. Triacsin C may inhibit contributions to total esterification by ACSL1 as well as by FATP1 itself (25).

Our findings suggest that the degree of interaction between FATP1 and ACSL1 may not depend on the nutritional and hormonal milieu but rather on structural properties of these proteins. Our results are most consistent with a working model in which vectorial acylation may contribute to the efficiency of fatty acid uptake. Immediate esterification of imported fatty acids might prevent the intracellular accumulation of potentially toxic molecules. The continual maintenance of coupled transport and esterification may be critical in providing adipocytes with the ability to rapidly and efficiently metabolize

LCFAs in response to external stimuli, such as occur after a high-fat meal. Regulation of fatty acid transport may occur through modulation of the activity of individual proteins through mechanisms such as posttranslational modifications. In addition, other proteins may interact with the FATP1-ACSL1 complex to regulate fatty acid mobilization. Ongoing studies to discern whether such mechanisms contribute to the regulation of fatty acid transport will help to elucidate an essential function of adipocytes that contributes to adipose tissue dysfunction in pathophysiologic states.

This work was supported by grants from the National Institutes of Health (1RO1 DK-54268 and 5T32 HL-07873).

REFERENCES

1. Basu, A., R. Basu, P. Shah, A. Vella, R. A. Rizza, and M. D. Jensen. 2001. Systemic and regional free fatty acid metabolism in type 2 diabetes. *Am. J. Physiol. Endocrinol. Metab.* **280:** E1000–E1006.
2. Jensen, M. D., M. W. Haymond, R. A. Rizza, P. E. Cryer, and J. M. Miles. 1989. Influence of body fat distribution on free fatty acid metabolism in obesity. *J. Clin. Invest.* **83:** 1168–1173.
3. Klein, K., R. Steinberg, B. Fiethen, and P. Overath. 1971. Fatty acid degradation in *Escherichia coli*. An inducible system for the uptake of fatty acids and further characterization of old mutants. *Eur. J. Biochem.* **19:** 442–450.
4. Azizan, A., D. Sherin, C. C. DiRusso, and P. N. Black. 1999. Energetics underlying the process of long-chain fatty acid transport. *Arch. Biochem. Biophys.* **365:** 299–306.
5. Dirusso, C. C., and P. N. Black. 2004. Bacterial long chain fatty acid transport: gateway to a fatty acid-responsive signaling system. *J. Biol. Chem.* **279:** 49563–49566.
6. Abumrad, N., C. Coburn, and A. Ibrahimi. 1999. Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim. Biophys. Acta.* **1441:** 4–13.
7. Glatz, J. F. C., and J. Storch. 2001. Unravelling the significance of cellular fatty acid-binding proteins. *Curr. Opin. Lipidol.* **12:** 267–274.
8. Frohnert, B. I., and D. A. Bernlohr. 2000. Regulation of fatty acid transporters in mammalian cells. *Prog. Lipid Res.* **39:** 83–107.
9. Schaffer, J. E., and H. F. Lodish. 1994. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell.* **79:** 427–436.
10. Coe, N. R., A. J. Smith, B. I. Frohnert, P. A. Watkins, and D. A. Bernlohr. 1999. The fatty acid transport protein (FATP1) is a very long chain acyl-CoA synthetase. *J. Biol. Chem.* **274:** 36300–36304.
11. Zou, Z., F. Tong, N. J. Faergeman, C. Borsting, P. N. Black, and C. C. DiRusso. 2003. Vectorial acylation in *Saccharomyces cerevisiae*. Fat1p and fatty acyl-CoA synthetase are interacting components of a fatty acid import complex. *J. Biol. Chem.* **278:** 16414–16422.
12. Hall, A. M., A. J. Smith, and D. A. Bernlohr. 2003. Characterization of the acyl-CoA synthetase activity of purified murine fatty acid transport protein 1. *J. Biol. Chem.* **278:** 43008–43013.
13. Watkins, P. A. 1997. Fatty acid activation. *Prog. Lipid Res.* **36:** 55–83.
14. Gargiulo, C. E., S. M. Stuhlsatz-Krouper, and J. E. Schaffer. 1999. Localization of adipocyte long-chain fatty acyl-CoA synthetase at the plasma membrane. *J. Lipid Res.* **40:** 881–892.
15. Ory, D. S., B. A. Neugeboren, and R. C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA.* **93:** 11400–11406.
16. Frost, S. C., and M. D. Lane. 1985. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. *J. Biol. Chem.* **260:** 2646–2652.
17. Listenberger, L. L., D. S. Ory, and J. E. Schaffer. 2001. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J. Biol. Chem.* **276:** 14890–14895.
18. Simpson, R. J., K. M. Brindle, and I. D. Campbell. 1983. Centrifugal analysis of undiluted packed human erythrocyte lysates. Studies of the association of glyceraldehyde-phosphate dehydrogenase with the membrane fraction. *Biochim. Biophys. Acta.* **758:** 187–190.
19. Richards, M. R., L. L. Listenberger, A. A. Kelly, S. E. Lewis, D. S. Ory, and J. E. Schaffer. 2003. Oligomerization of the murine fatty acid transport protein 1. *J. Biol. Chem.* **278:** 10477–10483.
20. Schaffer, J. E. 2002. Fatty acid transport: the roads taken. *Am. J. Physiol. Endocrinol. Metab.* **282:** E239–E246.
21. Brasaemle, D. L., D. M. Levin, D. C. Adler-Wailes, and C. Londos. 2000. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim. Biophys. Acta.* **1483:** 251–262.
22. Rosenstock, M., A. S. Greenberg, and A. Rudich. 2001. Distinct long-term regulation of glycerol and non-esterified fatty acid release by insulin and TNF-alpha in 3T3-L1 adipocytes. *Diabetologia.* **44:** 55–62.
23. Stahl, A., J. G. Evans, S. Pattel, D. Hirsch, and H. F. Lodish. 2002. Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev. Cell.* **2:** 477–488.
24. Faergeman, N. J., C. C. DiRusso, A. Elberger, J. Knudsen, and P. N. Black. 1997. Disruption of the *Saccharomyces cerevisiae* homologue to the murine fatty acid transport protein impairs uptake and growth on long-chain fatty acids. *J. Biol. Chem.* **272:** 8531–8538.
25. Ribarik Coe, N., M. A. Simpson, and D. A. Bernlohr. 1999. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J. Lipid Res.* **40:** 967–972.