

Long Chain Acyl-CoA Synthetase 3-mediated Phosphatidylcholine Synthesis Is Required for Assembly of Very Low Density Lipoproteins in Human Hepatoma Huh7 Cells*

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Hepatocytes play a crucial role in regulating lipid metabolism by exporting cholesterol and triglyceride into plasma through secretion of very low density lipoproteins (VLDL). VLDL production is also required for release of hepatitis C virus (HCV) from infected hepatocytes. Here, we show that long chain acyl-CoA synthetase 3 (ACSL3) plays a crucial role in secretion of VLDL and HCV from hepatocytes. In cultured human hepatoma Huh7 cells, ACSL3 is specifically required for incorporation of fatty acids into phosphatidylcholine. In cells receiving small interfering RNA targeting ACSL3, secretion of apolipoprotein B, the major protein component of VLDL, was inhibited and the lipoprotein was rapidly degraded. This inhibition in secretion was completely eliminated when these cells were treated with phosphatidylcholine. Treatment of cells with small interfering RNA targeting ACSL3 also inhibited secretion of HCV from Huh7-derived cells. These results identify ACSL3 as a new enzymatic target to limit VLDL secretion and HCV infection.

Very low density lipoproteins (VLDL)² are a family of spherical particles produced in livers to export triglyceride and cholesterol into plasma (1, 2). Assembly of VLDL begins in the endoplasmic reticulum with synthesis of apolipoprotein B (apoB), a protein that confers structural integrity to VLDL (3). Unlike other secretory proteins, apoB translocates across the membrane to reach the endoplasmic reticulum lumen when lipids are available to associate with the nascent polypeptide chain (4). This association requires the activity of microsomal triglyceride transfer protein (MTP) (5). Once in the endoplasmic reticulum lumen, apoB fuses with lipid droplets that are rich in triglyceride and cholesterol ester, a step also facilitated by MTP (5). The resulting VLDL particles are secreted through exocytosis (2). In the absence of lipid association, apoB is not exported out of cells but is rapidly degraded by proteasomes (4, 6).

VLDL secretion is also critical for the life cycle of hepatitis C virus (HCV). HCV is a single-stranded positive-sense RNA virus of the Flaviviridae family (7). Like other positive-sense RNA viruses, HCV RNA replicates on intracellular membrane vesicles (8, 9). We recently purified the vesicles that contain the HCV replication complex from human hepatoma Huh7 cells and found that these vesicles were enriched in apoB, MTP, and apolipoprotein E, three proteins known to be involved in VLDL assembly (10). The colocalization of HCV replication and VLDL assembly allows the virus to be secreted in complex with VLDL (10). When Huh7 cells that constitutively produce infectious HCV (11) were treated with an MTP inhibitor or siRNA targeting apoB, the secretion of VLDL and HCV were both inhibited (10).

The colocalization of HCV replication and VLDL assembly permits isolation of membrane vesicles that assemble both particles, providing an opportunity to identify novel proteins that participate in VLDL assembly. Proteomic analysis of these membrane vesicles revealed that they contained long chain acyl-CoA synthetase (ACSL) 3 (ACSL3) (10). ACSL3 belongs to a family of enzymes that catalyze the activation of fatty acids by conjugating them with coenzyme A (12), a reaction required for fatty acids to be incorporated into various lipids including triglyceride, cholesterol ester, and phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (13). Although the catalytic activity of ACSL3 is well characterized, its specific physiological role is unknown.

In the current studies we used human hepatoma Huh7-derived cells, which secrete apoB-containing lipoprotein particles with densities similar to those of VLDL (10), to study the role of ACSL3 in VLDL assembly. We report that ACSL3 is specifically required for incorporation of fatty acids into PC, a reaction that is essential for VLDL assembly. We show that secretion of VLDL as well as HCV is inhibited when expression of ACSL3 is reduced by RNA interference (RNAi). These studies identify ACSL3 as a new enzyme required for VLDL assembly and confirm the link between VLDL assembly and HCV production.

EXPERIMENTAL PROCEDURES

Materials—MG-132 and Nonidet P-40 alternative (Nonidet P-40) were obtained from Calbiochem. PC (catalog number 840051), PE (catalog number 841118), phosphatidic acid (PA, catalog number 840857), phosphatidylglycerol (PG, catalog number 840457), and phosphatidylserine (PS, catalog number 840034) were obtained from Avanti. [1-¹⁴C]Oleate (specific activity 50 mCi/mmol) was obtained from PerkinElmer Life Sciences.

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² The abbreviations used are: VLDL, very low density lipoproteins; apoB, apolipoprotein B; HCV, hepatitis C virus; ACSL, long chain acyl-CoA synthetase; MTP, microsomal triglyceride transfer protein; GFP, green fluorescent protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; siRNA, small interfering RNA; RNAi, RNA interference.

ACSL3 Is Required for VLDL Assembly

Coenzyme A, oleoyl-coenzyme A, and triacsin C were obtained from Sigma. Antibody reagents were obtained from the following sources: sheep polyclonal anti-apoB and anti- α 1-antitrypsin from Biodesign International; rabbit polyclonal anti-prohibitin from Abcam; and horseradish peroxidase-conjugated donkey anti-rabbit and anti-sheep IgGs (affinity-purified) from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture—Huh7.5 cells, a mutant line of Huh7 cells that support HCV replication in high efficiency (14), were obtained from Charles Rice (The Rockefeller University, New York). They were cultured and set up in medium A (Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate) supplemented with 10% fetal calf serum. Huh7-GL cells, a line of Huh7 cells that contain a chromosomally integrated genotype 2a HCV cDNA and constitutively produce infectious virus (11), were obtained from Guangxiang Luo (University of Kentucky College of Medicine, Lexington, KY). They were cultured and set up in medium A supplemented with 10% fetal calf serum and 5 μ g/ml blasticidine. Both cells were maintained in monolayer culture at 37 °C and 5% CO₂.

RNA Interference—Duplexes of siRNA were synthesized by Dharmacon Research (Lafayette, CO). The two siRNA sequences targeting human ACSL3 (GenBankTM accession number BC041692) are at nucleotide positions (relative to the codon for the initiating methionine) 810–828 and 1223–1241 for ACSL3a and ACSL3b, respectively. The siRNA sequences targeting human long chain acyl-CoA synthetase 1 (ACSL1) and long chain acyl-CoA synthetase 4 (ACSL4) (GenBankTM accession numbers BC050073 and BC034959, respectively) correspond to the following nucleotide positions relative to the codon for the initiating methionine: ACSL1 (nucleotides 614–632) and ACSL4 (nucleotides 1653–1671). The sequence for the siRNA targeting green fluorescent protein (GFP) was reported previously (15). Huh7.5 and Huh7-GL cells cultured in a 60-mm dish were transfected with 400 pmol of siRNA duplexes using LipofectamineTM RNAiMAX Reagent (Invitrogen) as described by the manufacturer, after which the cells were used for experiments as described in the figure legends.

Real-time PCR—For measurement of intracellular RNA, the protocol was identical to that was previously described by Liang *et al.* (16). Briefly, triplicate samples of first strand cDNA were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA with human 36B4 as an invariant control (16). Relative amounts of mRNAs were calculated using the comparative C_T method. For measurement of extracellular HCV RNA, RNA from culture medium was extracted by QIAamp Viral RNA Mini Kit (Qiagen). First-strand cDNA was synthesized using extracted RNA or various amounts of *in vitro* transcribed HCV RNA. Triplicate samples of cDNA were subjected to real-time PCR quantification using primers specific for HCV RNA. HCV copy numbers were determined by the standard curve generated by the *in vitro* transcribed HCV RNA using a conversion constant assuming that 1 μ g of single-stranded RNA equals to 1.96×10^{11} viral copy numbers (Ambion Technical Bulletin number 165).

Immunoblot Analysis—Cell pellets from duplicate dishes were solubilized in 0.1 ml of buffer A (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Nonidet P-40, and protease inhibitor mix-

ture containing 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, and 2 μ g/ml aprotinin). Cell lysates containing 1 μ g of protein and 40 μ l of culture medium (containing about 20 ng of protein) were subjected to SDS-PAGE followed by immunoblot analysis as previously described (17). The proteins were transferred to Hybond-C extra nitrocellulose filters (Amersham Biosciences), which were incubated with one of the following antibodies: 1:1000 dilution of anti-apoB, 1:1000 dilution of anti- α 1-antitrypsin, and 1:500 dilution of anti-prohibitin. Bound antibodies were visualized with peroxidase-conjugated, affinity-purified donkey anti-rabbit or anti-sheep IgG (1:5000 dilution) using the SuperSignal ECL-HRP Substrate System (Pierce) according to the manufacturer's instructions. Filters were exposed to Kodak X-Omat Blue XB-1 film at room temperature for the indicated times.

ACSL Activity Measurement—ACSL activity was measured as described (18) with some modifications. Cells pulled from four 60-mm dishes were homogenized by passing 25 times through needles (22 gauge) in 100 μ l of buffer B (100 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, 5 mM dithiothreitol, 250 mM sucrose, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, and 2 μ g/ml aprotinin). ACSL activity was assayed by mixing buffer C (100 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, 10 mM ATP, 0.5 mM coenzyme A, 5 mM dithiothreitol, 50 μ M [1-¹⁴C]oleate (0.25 μ Ci)) with cell homogenates containing 50 μ g of protein in a reaction volume of 100 μ l. Following incubation for 10 min at 37 °C, 400 μ l of chloroform/methanol (1:2, v/v) was added to terminate the reaction. After centrifugation at $16,000 \times g$ for 10 min at room temperature, the supernatant was dried, and then dissolved in 60 μ l of chloroform/methanol (1:2; v/v). 30 μ l of the solution was mixed with non-radioactive standard containing 50 nmol of oleoyl-coenzyme A, and analyzed by thin-layer chromatography (TLC) on POLYGRAM[®] SIL G membrane (MACHERY-NAGEL, Germany) in a solvent system of 1-butanol/acetic acid/water (5:2:3, v/v/v). After visualization by exposing the TLC plates to I₂ vapor, bands containing acyl-CoA were excised, and the amount of radioactivity in these bands was determined by scintillation counting.

Lipid Analysis—Following incubation with [1-¹⁴C]oleate as described in the figure legends, cells were harvested and homogenized in 0.5 ml of buffer D (250 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, pH 7.4). Lipids in the homogenates were extracted by 0.5 ml of chloroform/methanol (2:1; v/v), dried, and then dissolved in 120 μ l of chloroform/methanol (1:1; v/v). 60 μ l of the lipid extracts were mixed with non-radioactive phospholipid standards containing 40 μ g of PC and PE, and analyzed by TLC on POLYGRAM SIL G membrane in a solvent system of chloroform/methanol/water (65:25:4, v/v/v). The remaining lipid extracts were mixed with non-radioactive neutral lipid standard containing 30 μ g of cholesterol oleate ester, 10 μ g of triglyceride, and 20 μ g of oleate, and analyzed by TLC on the same membrane in a solvent system of hexane/ethyl ether/acetic acid (80:20:1, v/v/v). After visualization by exposing the TLC plates to I₂ vapor, bands containing triglyceride, cholesterol ester, free oleate, PC, and PE were excised, and the amount of radioactivity in these bands was determined by scintillation counting. The activity of lipid synthesis was determined by the radioactivity found in various classes of lipid normalized by the amount of cellular protein.

Liposome Preparation—Liposome phospholipids were prepared as previously described (19) with some modification. PC, PE, PA, PG, or PS was dissolved in chloroform/methanol (2:1, v/v), dried in glass tubes under a stream of nitrogen, resuspended in Dulbecco's phosphate-buffered saline at pH 7.4 to reach a final concentration of 6 mM, and sonicated at 35% of amplitude with a Branson Digital Sonifier 15 times (10 s each time) on ice. The liposome suspensions were passed through 0.22- μ m pore filters (Millipore, Carrigtwohill, Co. Cork, Ireland) before they were added to culture medium.

HCV Titer—HCV titer was determined exactly as previously described (10).

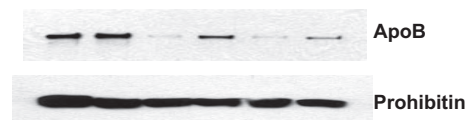
RESULTS

To determine whether ACSL3 is required for VLDL assembly, we transfected Huh7.5 cells with duplexes of siRNA targeting either ACSL3 or GFP, a control mRNA not present in the cells. Following incubation in serum-free medium, cells and culture medium were harvested and the amount of apoB proteins in the cells and medium was analyzed by immunoblot analysis. In control cells receiving the GFP siRNA, apoB was readily detectable by immunoblot analysis, and the amount of apoB did not change when cells were incubated with the proteasome inhibitor MG132 before harvest (Fig. 1A, lanes 1 and 2). In cells transfected with two different ACSL3 siRNAs targeting different regions of ACSL3 mRNA, apoB became barely detectable (Fig. 1A, lanes 3 and 5). The amount of apoB in these cells was significantly elevated when cells were treated with MG132 (Fig. 1A, lanes 4 and 6). The amount of apoB secreted by cells receiving the ACSL3 siRNA was also reduced (Fig. 1B). Secretion of α 1-antitrypsin, another abundant protein produced by hepatocytes (20), was not affected by the knockdown of ACSL3 (Fig. 1B). Quantitative real-time PCR revealed that each ACSL3 siRNA duplex decreased the level of ACSL3 mRNA by >80% but had no effect on the level of apoB mRNA (Fig. 1C). These results indicate that the amount of apoB protein in cells with less expression of ACSL3 is markedly reduced, presumably through accelerated degradation mediated by proteasomes.

We have previously shown that export of HCV from infected cells requires assembly and secretion of VLDL (10). We thus examined whether siRNA targeting ACSL3 would decrease production of infectious HCV particles. For this purpose, we used Huh7-GL cells, a line of Huh7 cells that contain a chromosomally integrated genotype 2a HCV cDNA and constitutively produce infectious virus (11). These cells were transfected with duplexes of siRNA targeting either GFP or ACSL3. Following incubation in serum-free medium, culture medium was harvested and the amount of HCV RNA, HCV titer, and apoB in the medium was measured by real-time PCR, foci formation, and immunoblot analysis, respectively. As expected, transfection of cells with the ACSL3 siRNA blocked the secretion of apoB but not α 1-antitrypsin (Fig. 2A). Treatment of the cells with the ACSL3 siRNA reduced the amount of HCV RNA in the medium and decreased the viral titer by about 60% (Fig. 2B). The reduction in the amount of HCV in the medium is not due to inhibition of viral RNA synthesis because the amount of intracellular HCV RNA was not reduced in cells receiving the ACSL3 siRNA (Fig. 2B). The failure to accumulate a large amount of intracellular HCV RNA in cells

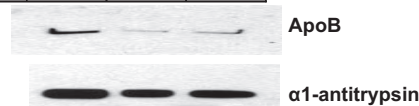
A. Cell Lysate

siRNA	GFP		ACSL3a		ACSL3b	
MG132	-	+	-	+	-	+
Lane	1	2	3	4	5	6



B. Medium

MG132	-		
siRNA	GFP	ACSL3a	ACSL3b
Lane	1	2	3



C.

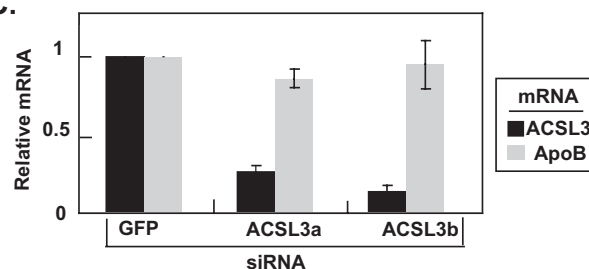


FIGURE 1. RNAi-mediated knockdown of ACSL3 promotes degradation of apoB in Huh7.5 cells. On day 0, Huh7.5 cells were set up at 1×10^5 /60-mm dish. On day 1, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or different regions of ACSL3 (ACSL3a and ACSL3b). A and B, on day 4, cells were washed once with medium A, then switched to medium A in the absence (–) or presence (+) of 10 μ M MG-132. After incubation for 5 h, cells and culture medium were harvested. Aliquots of cell lysates (A) and culture medium (B) were subjected to SDS-PAGE followed by immunoblot analysis with the indicated antibodies. The filters were exposed to film for 10–60 s. C, on day 4, total RNA was prepared from cells and subjected to first strand cDNA synthesis and quantitative real-time PCR analysis. Values shown are relative to the GFP siRNA-transfected control, which was set at 1. Data are reported as mean \pm S.E. from three independent experiments.

treated with the ACSL3 siRNA was to be expected, because the secretion of HCV RNA was inefficient. Even in the control cells, the amount of HCV RNA detected in the medium was less than 5% of that found in the cells. A decrease in this low level of secretion is not expected to generate a significant increase in cellular HCV RNA.

Besides ACSL3, two other long chain acyl-CoA synthetases, ACSL1 and ACSL4 (21), were found to be expressed in Huh7.5 cells (data not shown). However, neither of these two enzymes was found in vesicles involved in VLDL assembly (10). To test whether ACSL3 is specifically required for assembly of VLDL, Huh7.5 cells were transfected with siRNA targeting GFP, ACSL1, ACSL3, or ACSL4. Quantitative real-time PCR revealed that mRNAs for ACSL1, ACSL3, and ACSL4 were each reduced by >80% in the appropriate cells (Fig. 3A). The amount of apoB protein was markedly reduced in cells treated with the ACSL3 siRNA (Fig. 3B, lane 2) despite a compensatory increase of ACSL1 mRNA in these cells (Fig. 3A). The amount of intracellular apoB was not reduced by RNAi-mediated knockdown of ACSL1 or ACSL4 (Fig. 3B, lanes 3 and 4). These results indicate a specific requirement for the ACSL3 isoform in VLDL assembly.

ACSL3 Is Required for VLDL Assembly

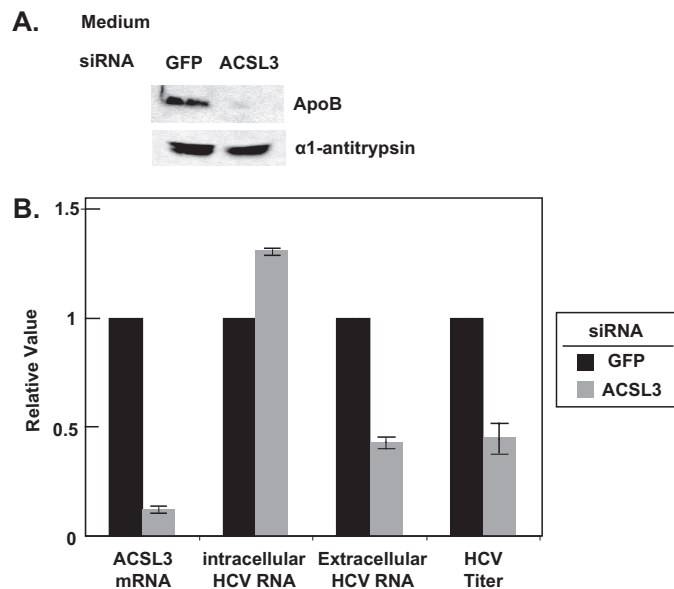


FIGURE 2. Decreased secretion of infectious HCV particles from Huh7-GL cells treated with siRNA targeting ACSL3. On day 0, Huh7-GL cells were set up at 2×10^5 cells/60-mm dish. On day 1, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or ACSL3 (*ACSL3b*) as indicated. On day 4, cells were switched to medium A and incubated for 4 h. Cells and culture medium were then harvested. *A*, aliquots of the culture medium were subjected to SDS-PAGE followed by immunoblot analysis with the indicated antibodies. Filters were exposed for 30 s. *B*, HCV RNA copy number and titer in the medium were determined as described under "Experimental Procedures." ACSL3 mRNA and intracellular HCV RNA were quantified by real-time PCR analysis. Values (mean \pm S.D. of three measurements in the same experiment) are plotted relative to the GFP siRNA-transfected control, which was set at 1. HCV copy number and titer in GFP siRNA-transfected control cells were 1.2×10^5 /ml and 4.3×10^2 foci formation units/ml, respectively. Similar results were obtained in two other independent experiments.

Judging by C_T values of real-time PCR analysis, ACSL4 but not ACSL3 is the predominant ACSL expressed in Huh7.5 cells (data not shown). Thus, knockdown of ACSL3 is not expected to significantly affect total ACSL activity in the cells. To test this hypothesis, we transfected Huh7.5 cells with siRNA targeting ACSL3. As a positive control, we also treated Huh7.5 cells with triacsin C, a known inhibitor of ACSL (22). As shown in Fig. 3C, treatment of cells with triacsin C inhibited ACSL activity by about 95%, whereas transfection of siRNA targeting ACSL3 did not significantly affect ACSL activity in these cells, even though ACSL3 mRNA was reduced by more than 80% (data not shown).

The above results suggest that ACSL3 is specifically required for synthesis of lipids that are attached to apoB. To test this hypothesis, we transfected Huh7.5 cells with duplexes of siRNA targeting either ACSL3 or GFP as a control. Cells were then labeled with [$1\text{-}^{14}\text{C}$]oleate, and the amount of radiolabeled fatty acids incorporated into cholesteryl ester, triglyceride, PC, and PE was determined. The free oleate remained in the cells was also quantified. Compared with control cells treated with the GFP siRNA, incorporation of [$1\text{-}^{14}\text{C}$]oleate into PC in these cells was reduced by about 70% and this change was statistically significant ($p < 0.005$) (Table 1). Incorporation of oleate into other classes of lipids was not significantly affected by the knockdown of ACSL3 (Table 1). Treatment of cells with the ACSL3 siRNA also caused a significant increase in the amount of free [$1\text{-}^{14}\text{C}$]oleate ($p < 0.005$) (Table 1). Unlike ACSL3 RNAi, knockdown of ACSL1 or ACSL4 did not significantly affect

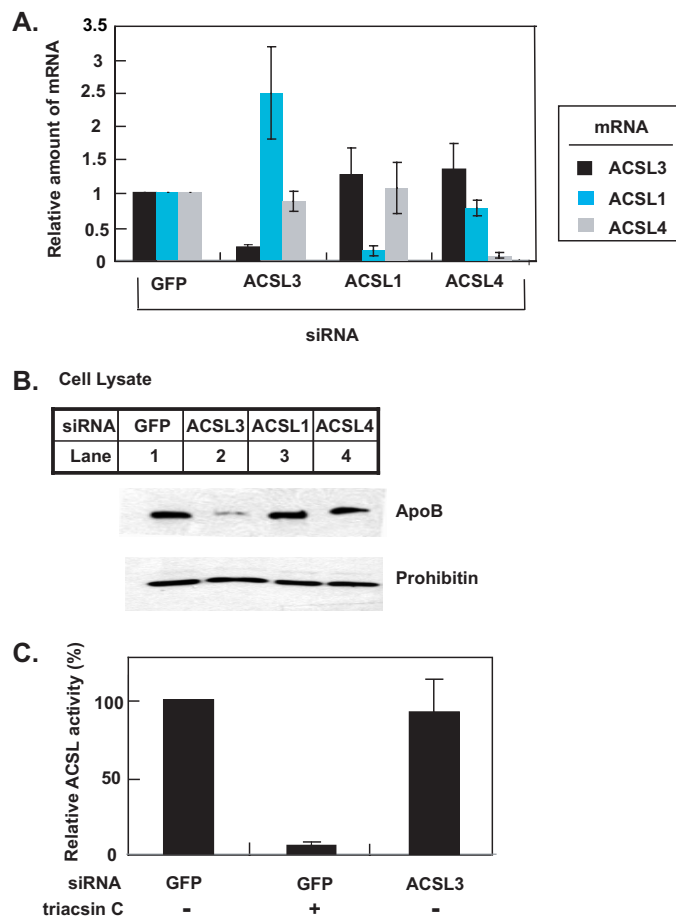


FIGURE 3. RNAi-mediated knockdown of ACSL3 specifically reduces the intracellular amount of apoB in Huh7.5 cells. On day 0, Huh7.5 cells were set up at 1×10^5 /60-mm dish. On day 1, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP, ACSL3 (*ACSL3b*), ACSL1, or ACSL4 as indicated. *A*, on day 4, total RNA was prepared from cells and subjected to first strand cDNA synthesis and quantitative real-time PCR analysis. Values shown are relative to the GFP siRNA-transfected control, which was set at 1. Data are reported as mean \pm S.E. from three independent experiments. *B*, on day 4, cells were washed with medium A before harvesting for preparation of detergent lysates, which were subjected to SDS-PAGE followed by immunoblot analysis with the indicated antibodies. The filters were exposed to film for 10 s. Similar results were observed in two other independent experiments. *C*, on day 4, cells were treated with or without 5 μM triacsin C as indicated. After incubation for 5 h, cells were harvested and ACSL activity in cell homogenates was determined as described under "Experimental Procedures." Triacsin C (5 μM) was included in the assay buffer when we measured the activity in homogenates obtained from cells treated with triacsin C. ACSL activity in GFP siRNA-transfected control cells was 56 ± 16 pmol/min/mg of cellular protein. Values (mean \pm S.D. of three independent experiments) are plotted relative to the GFP siRNA-transfected control, which was set at 1.

incorporation of [$1\text{-}^{14}\text{C}$]oleate into these classes of lipids, including PC (Table 1). These results indicate a preferential requirement for ACSL3 in synthesis of PC in Huh7.5 cells.

To test whether the deficiency in PC synthesis is responsible for the degradation of apoB after ACSL3 siRNA treatment, we determined whether expression of apoB protein in these cells could be restored by exogenously added PC. We added PC in the form of liposomes that have been previously shown to support the growth of a mutant line of Chinese hamster ovary cells deficient in PC synthesis (19). As shown in Fig. 4A, the amount of apoB in cells transfected with the ACSL3 siRNA was markedly reduced as expected (Fig. 4A, lane 4). Addition of 0.1 or 0.3

TABLE 1

Incorporation of oleate into various lipids in Huh7.5 cells transfected with siRNA targeting different ACSL isoform

On day 0, Huh7.5 cells were set up at 1×10^5 /60-mm dish. On day 1, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or each of the ACSL isoform as indicated. On day 4, cells were washed once with Dulbecco's phosphate-buffered saline and incubated in medium A for 1 h. Each dish of cells was then labeled with 1.0 μ Ci of [14 C]oleate (specific activity 50 mCi/mmol) in 2 ml of medium A at 37 °C for 4 h. Cells were washed with Dulbecco's phosphate-buffered saline twice, harvested, and lipids were extracted from the cells. The amount of radioactivity found in cholesteryl ester, triglyceride, PC, PE, and free oleate was determined as described under "Experimental Procedures." Lipid synthesis activity is expressed as nanomole of [14 C]oleate incorporated per mg of cellular protein. Data are reported as mean \pm S.E. from three independent experiments.

Lipids	siRNA			
	GFP	ACSL1	ACSL3	ACSL4
Cholesteryl ester	0.28 \pm 0.09	0.35 \pm 0.07	0.19 \pm 0.05	0.36 \pm 0.10
Triglyceride	7.34 \pm 1.26	7.51 \pm 1.63	6.00 \pm 0.84	7.76 \pm 1.75
PC	2.27 \pm 0.82	2.58 \pm 0.93	0.80 \pm 0.32 ^a	2.41 \pm 1.02
PE	1.09 \pm 0.23	1.12 \pm 0.31	0.78 \pm 0.19	1.24 \pm 0.41
Free oleate	0.35 \pm 0.09	0.31 \pm 0.03	0.63 \pm 0.13 ^a	0.26 \pm 0.04

^a The level of statistical significance (Student's test) between control cells transfected with the GFP siRNA and cells transfected with the indicated ACSL siRNA, $p < 0.005$.

μ M PC to the medium slightly increased the amount of apoB in these cells (Fig. 4A, lanes 5 and 6). Adding 1 μ M PC to the medium, however, completely restored the amount of intracellular apoB to the level that is observed in control cells treated with GFP siRNA (Fig. 4A, lanes 1 and 7). This effect is specific to PC, because exogenously added PE failed to produce the same result (Fig. 4A, lanes 8–10). In contrast to the cells receiving the ACSL3 siRNA, treatment of control cells with 1 μ M PC did not further raise the amount of apoB in these cells (Fig. 4A, lane 2). We thus conclude that the deficiency in PC synthesis resulting from the knockdown of ACSL3 is responsible for the reduction in intracellular apoB proteins.

To examine whether exogenously added PC also restored the secretion of VLDL after ACSL3 siRNA treatment, we performed a similar experiment as shown in Fig. 4A but we also examined the amount of apoB in culture medium. As shown in Fig. 4B, addition of PC not only restored the intracellular amount of apoB but also increased the amount of apoB in culture medium to the extent that is observed in control cells treated with GFP siRNA.

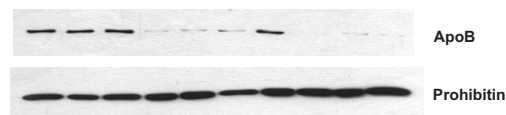
Unlike PC that forms bilayers in liposome, PE has the tendency to form hexagonal phases (23). We thus examined other phospholipids that form bilayers to determine whether they restored VLDL assembly and secretion in cells with reduced expression of ACSL3. The phospholipids that were examined included PA, PG, and PS. As shown in Fig. 4C, treatment with PA or PS did not increase the level of apoB in cells transfected with the ACSL3 siRNA or the amount of apoB secreted by these cells (Fig. 4C, lanes 8 and 10). PG was able to raise the amount of intracellular apoB, but it failed to rescue the secretion of apoB from cells treated with the ACSL3 siRNA (Fig. 4C, lane 9). Only PC restored the amount of intracellular apoB as well as the amount of apoB secreted into culture medium from cells transfected with the ACSL3 siRNA (Fig. 4C, lane 7).

DISCUSSION

In the current studies we identify ACSL3 as a new enzyme required for VLDL assembly in cultured human hepatoma cells. In the absence of ACSL3, VLDL secretion was inhibited and

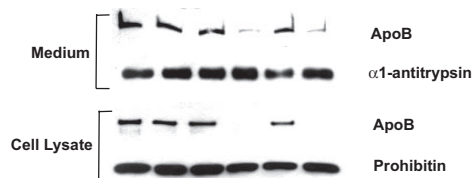
A. Cell Lysate

siRNA	GFP			ACSL3						
PE(μ M)	-	-	1	-	-	-	-	0.1	0.3	1
PC(μ M)	-	1	-	-	0.1	0.3	1	-	-	-
Lane	1	2	3	4	5	6	7	8	9	10



B.

siRNA	GFP			ACSL3		
PC	-	+	-	-	+	-
PE	-	-	+	-	-	+
Lane	1	2	3	4	5	6



C.

siRNA	GFP					ACSL3				
Phospholipids	-	PC	PA	PG	PS	-	PC	PA	PG	PS
Lane	1	2	3	4	5	6	7	8	9	10

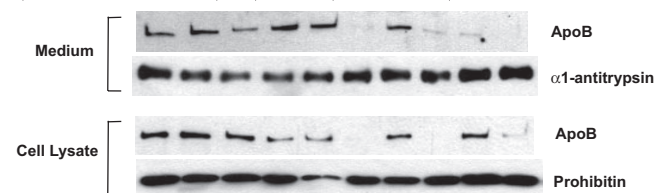


FIGURE 4. PC restores the level of intracellular apoB and the amount of apoB secreted into medium from Huh7.5 cells transfected with the ACSL3 siRNA. On day 0, Huh7.5 cells were set up at 1×10^5 /60-mm dish. On day 1, cells were transfected with 400 pmol/dish of siRNA duplexes targeting GFP or ACSL3 (ACSL3b) as indicated. A, on day 3, cells were switched to medium A supplemented with 10% fetal calf serum and the indicated amount of liposome containing PC or PE. 16 h later on day 4, cells were washed with medium A before harvesting for preparation of detergent lysates, which were subjected to SDS-PAGE followed by immunoblot analysis with the indicated antibodies. The filters were exposed to film for 10 s. B and C, on day 3, cells were switched to medium A supplemented with 10% fetal calf serum and 1 μ M of the indicated phospholipid. 16 h later on day 4, cells were washed with medium A, and then switched to medium A supplemented with 1 μ M of the indicated phospholipid. After incubation for 5 h, cells and culture medium were harvested. Aliquots of cell lysates and culture medium were subjected to SDS-PAGE followed by immunoblot analysis with the indicated antibodies. The filters were exposed to film for 10–60 s. A–C, similar results were observed in at least one other independent experiment.

apoB was rapidly degraded (Fig. 1). This inhibition of VLDL assembly is largely caused by a deficiency in ACSL3-mediated PC synthesis (Table 1), because addition of PC to the culture medium restored the amount of apoB in cells in which ACSL3 was knocked down by RNAi (Fig. 4). Secretion of apoB was also back to normal after these cells were treated with PC (Fig. 4, B and C). Inasmuch as PC is a major component of cellular membrane, treatment with ACSL3 siRNA longer than 5 days was toxic to cells (data not shown). In the current studies we harvested cells 3 days after transfection of the ACSL3 siRNA, at which time cellular toxicity is not apparent.

PC is the major phospholipid located on the surface of VLDL, and its role in VLDL assembly and secretion cannot be filled by other phospholipids (24). In hepatocytes, PC can be synthesized by the CDP-choline pathway or by methylation of PE (25), both

of which are required for efficient secretion of VLDL (26–29). For PC synthesized via methylation of PE, fatty acids are incorporated into PE before PE is converted to PC by methylation. This pathway requires the enzyme phosphatidylethanolamine *N*-methyltransferase, which is mainly expressed in hepatocytes. By real-time PCR analysis, we determined that phosphatidylethanolamine *N*-methyltransferase is also expressed in Huh7.5 cells (data not shown). If phosphatidylethanolamine *N*-methyltransferase in Huh7.5 cells is functional, PC synthesized by methylation of PE is unlikely to depend solely on ACSL3 because incorporation of fatty acids into PE was not significantly affected by the knockdown of ACSL3 (Table 1). Thus, ACSL3-mediated activation of fatty acids appears to be required for the synthesis of diacylglycerol that is the substrate for synthesis of PC via the CDP-choline pathway.

A major finding in the current studies is that in Huh7.5 cells ACSL3 is specifically required for PC synthesis, an activity unable to be replaced by other isoforms of ACSL (Table 1). We also observed an accumulation of free fatty acids in cells with less expression of ACSL3 (Table 1). This result suggests that the pool of fatty acids destined to be incorporated into PC is not activated by other isoforms of ACSL to enter alternative metabolic pathways. These observations are consistent with a previous hypothesis that individual ACSL isoforms might channel fatty acids into distinct metabolic pathways (30, 31). Although the mechanism by which each isoform of ACSL directs the metabolism of fatty acids remains unclear, the unique subcellular localization has been suggested to be important for the specificity of each isoform of the enzyme (21). In this regard, we found that ACSL3 is localized in endoplasmic reticulum membrane vesicles involved in VLDL assembly (10). This localization might be important for hepatocytes to efficiently provide PC that is necessary for the assembly of VLDL. ACSL3 was also reported to reside on cytosolic lipid droplets in Huh7 cells (18, 32). Similar to VLDL, cytosolic lipid droplets are also surrounded by a single layer of phospholipids. It will be interesting to know whether ACSL3-mediated PC synthesis is required for the generation of cytosolic lipid droplets.

Another finding of the current studies is that secretion of HCV from Huh7 cells that constitutively produce infectious viral particles was inhibited when ACSL3 was knocked down by RNAi (Fig. 2). This result agrees well with our previous observations that HCV is secreted together with VLDL (10). Thus, as an alternative to MTP inhibitors, drugs targeting ACSL3 might also be effective in limiting VLDL secretion and HCV infection.

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