THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 276, No. 27, Issue of July 6, pp. 24674–24679, 2001
Printed in U.S.A.

Acyl-CoA Synthetase Isoforms 1, 4, and 5 Are Present in Different Subcellular Membranes in Rat Liver and Can Be Inhibited Independently*

Received for publication, March 6, 2001, and in revised form, April 17, 2001
Published, JBC Papers in Press, April 23, 2001, DOI 10.1074/jbc.M102036200

Tal M. Lewin‡‖, Ji-Hyeon Kim‡‖, Deborah A. Granger‡, Jean E. Vance§, and Rosalind A. Coleman‡¶

From the ‡Departments of Nutrition and Pediatrics, University of North Carolina, Chapel Hill, North Carolina 27599 and the §Department of Medicine and Canadian Institutes for Health Research Group on Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

Inhibition studies have suggested that acyl-CoA synthetase (ACS, EC 6.2.1.3) isoforms might regulate the use of acyl-CoAs by different metabolic pathways. In order to determine whether the subcellular locations differed for each of the three ACSs present in liver and whether these isoforms were regulated independently, non-cross-reacting peptide antibodies were raised against ACS1, ACS4, and ACS5. ACS1 was identified in endoplasmic reticulum, mitochondria-associated membrane (MAM), and cytosol, but not in mitochondria. ACS4 was present primarily in MAM, and the 76-kDa ACS5 protein was located in mitochondrial membrane. Consistent with these locations, *N*-ethylmaleimide, an inhibitor of ACS4, inhibited ACS activity 47% in MAM and 28% in endoplasmic reticulum. Troglitazone, a second ACS4 inhibitor, inhibited ACS activity <10% in microsomes and mitochondria and 45% in MAM. Triacsin C, a competitive inhibitor of both ACS1 and ACS4, inhibited ACS activity similarly in endoplasmic reticulum, MAM, and mitochondria, suggesting that a hitherto unidentified triacsin-sensitive ACS is present in mitochondria. ACS1, ACS4, and ACS5 were regulated independently by fasting and re-feeding. Fasting rats for 48 h resulted in a decrease in ACS4 protein, and an increase in ACS5. Re-feeding normal chow or a high sucrose diet for 24 h after a 48-h fast increased both ACS1 and ACS4 protein expression 1.5–2.0-fold, consistent with inhibition studies. These results suggest that ACS1 and ACS4 may be linked to triacylglycerol synthesis. Taken together, the data suggest that acyl-CoAs may be functionally channeled to specific metabolic pathways through different ACS isoforms in unique subcellular locations.

The first step in long chain fatty acid use in mammals requires the ligation of fatty acid with coenzyme A (CoA). This reaction, catalyzed by acyl-CoA synthetase (ACS,[1] EC 6.2.1.3), produces acyl-CoAs, which are primary substrates for energy use via β-oxidation and for the synthesis of triacylglycerol, phospholipids, cholesterol esters, and sphingomyelin, and are the source of signaling molecules like ceramide, diacylglycerol, and arachidonic acid (1, 2). Acyl-CoAs up-regulate uncoupling protein in brown fat and key enzymes of glycolysis, gluconeogenesis, and β-oxidation; are essential for vesicle trafficking; and play a critical role in the transport of fatty acids into cells by making transport unidirectional. Protein esterification with myristate and palmitate anchors proteins to specific membranes and enables them to function correctly (3). Thus, acyl-CoAs participate in a large number of cellular reactions that involve lipid synthesis, energy metabolism, and regulation, but how acyl-CoAs are partitioned or directed toward these diverse synthetic, degradative, and signaling pathways is not understood.

Currently, five different rat ACS cDNAs have been cloned, each the product of a different gene (4–8). Rat ACS1–5 share a common structural architecture and are further classified into two subfamilies based on amino acid identity and fatty acid preference (4–8). ACS1, ACS2, and ACS5 make up one subfamily with about 60% homology to one another, and ACS3 and ACS4 make up a second subfamily with about 70% homology to each other and 30% similarity to ACS1. Within each subfamily, the ACS isoforms differ in their mRNA size, tissue distribution, and transcriptional regulation. ACS1, ACS4, and ACS5 are all expressed in liver.

Studies with the ACS inhibitors triacsin and troglitazone suggested that long chain acyl-CoAs are functionally channeled toward specific metabolic fates. In most of these studies, *de novo* glycerolipid synthesis was severely inhibited, whereas phospholipid reacylation and ketone bodies formation was less impaired. Inhibition of fatty acid incorporation into cholesterol esters varied with cell type, being completely blocked in human fibroblasts and only moderately decreased in hepatocytes. For example, in hepatocytes, triacsin C did not alter oxidation of pre-labeled intracellular lipid, but did inhibit triacylglycerol synthesis 40% and 70% in hepatocytes isolated from starved and fed rats, respectively (9). Additionally, in hepatocytes isolated from fasted rats, troglitazone blocked incorporation of oleate into triacylglycerol, but not into phospholipid (10). In addition, troglitazone inhibited ketone body production. In human fibroblasts, triacsin C blocked the incorporation of $[^3H]$glycerol into phospholipid by 80% and the incorporation into triacylglycerol by 99%, indicating severely impaired acy-

* This work was supported by GlaxoSmithKline, by Grants HD 56598 (to R. A. C.) and HD 08431 (to T. M. L.) from the National Institutes of Health, and by a grant from the North Carolina Institute of Nutrition. 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: CB 7400, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-7213; Fax: 919-966-7216; E-mail: rcoleman@unc.edu.

‖ These authors contributed equally to this work.

[1] The abbreviations used are: ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; GPAT, glycerol-3-phosphate acyltransferase; NEM, *N*-ethylmaleimide; MAM, mitochondria-associated membrane; PEMT, phosphatidylethanolamine methyltransferase.

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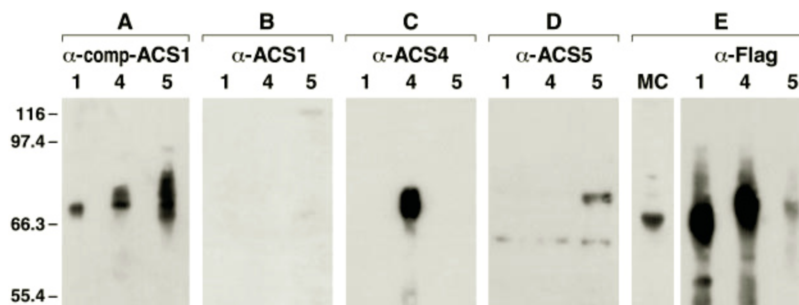

FIG. 1. **Specificity of ACS1, ACS4, and ACS5 antibodies.** Purified recombinant ACS1, ACS4, or ACS5, each with a C-terminal Flag epitope (5 μg of total protein), were analyzed by Western blot with antibodies raised against full-length ACS1 (*A*) or unique peptides from ACS1 (*B*), ACS4 (*C*), ACS5 (*D*), or the Flag epitope (*E*). The *MC lane* represents liver microsomes blotted with ACS1 peptide antibody. Protein antibody complexes were visualized by chemiluminescent detection of horseradish peroxidase linked to goat anti-rabbit or anti-mouse IgG. The molecular mass values of recombinant ACS1, ACS4, and ACS5 were 68, 74, and 76 kDa, respectively, on this gel system.

lation of glycerol 3-phosphate, lysophosphatidic acid, and diacylglycerol via the *de novo* synthetic pathway from glycerol 3-phosphate (11). Incorporation of [^{14}C]oleate into triacylglycerol was also blocked 95%, consistent with impaired acylation via the *de novo* pathway; however, incorporation into phospholipids was not impaired, suggesting that separate pools of acyl-CoAs exist and that the reacylation pathway is functionally separate from *de novo* glycerolipid synthesis. Taken as a whole, these studies suggest that there are functionally independent acyl-CoA pools within cells, and that acyl-CoAs might be channeled toward specific fates rather than being freely available for all possible enzymatic reactions.

Yeast provide clear evidence for functionally different ACS-linked pathways. In *Candida lipolytica*, studies using ACS mutants indicate that ACS I activates exogenous fatty acids for glycerolipid synthesis and ACS II activates them for β-oxidation (12). In *Saccharomyces cerevisiae,* the ACS proteins Faa1p and Faa4p account for 99% of yeast C14-CoA and C16-CoA activity (13) and activate exogenously derived fatty acids destined for phospholipid synthesis (14). Faa4p is specifically needed for myristoylation of protein substrates (15), and Faa2p is required for peroxisomal β-oxidation (16, 17). Yeast ACS isoforms are also differentially inhibited by triacsin C (18). From these data, Gordon's group (17) concluded that there are differences in location or accessibility of those acyl-CoAs that are derived from endogenous synthesis and those acyl-CoAs formed from exogenously provided fatty acids. Thus, genetic studies in yeast link specific ACS isoforms to different pathways that use acyl-CoAs.

We examined ACS1, ACS4, and ACS5 in rat liver, which contains a variety of pathways that use acyl-CoAs, in order to determine whether the subcellular locations, inhibition by specific inhibitors, and nutritional regulation might link the different ACS isoforms with different metabolic pathways. Our data indicate that ACS1, ACS4, and ACS5 are present in different subcellular membranes; that ACS activity is inhibited by triacsin C, troglitazone, and NEM to varying degrees in these subcellular fractions; and that nutritional changes regulate each ACS isoform independently.

EXPERIMENTAL PROCEDURES

Materials—[2-^{3}H]Glycerol and [9,10-^{3}H]palmitate were from Amersham Pharmacia Biotech. Glycerol, palmitoyl-CoA, ATP, and bovine serum albumin (essentially fatty acid-free) were from Sigma. Triacsin C (>95% pure) was from Biomol. Troglitazone was the gift of Dr. Steven Jacobs, GlaxoSmithKline. A polyclonal antibody to rat ACS1 was the gift of Dr. Paul Watkins, Kennedy Krieger Institute.

Animals—Animal protocols were approved by the University of North Carolina (UNC), Chapel Hill, NC and University of Alberta Institutional Animal Care and Use Committees. Male and female (150 g) Harlan Sprague-Dawley rats were housed on a 12-h/12-h light/dark cycle with free access to water. Control animals had free access to Purina rat chow. Fasted animals were sacrificed after being without food for 48 h. Refed rats were sacrificed after being fed Purina rat chow or a high sucrose diet (69.5% sucrose, Dyets, Inc.) for 24 h after a 48-h fast.

Subcellular Fractionation—For subcellular localization experiments, liver cytosol, microsomes, rough and smooth endoplasmic reticulum, mitochondria-associated membrane (MAM), and mitochondria were isolated from male rats by a method (19) modified by Vance (20). Liver total membrane fraction, microsomes, and mitochondria were isolated from female rats by differential centrifugation (21) in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for subcellular localization and nutritional regulation experiments. Fractions were stored in aliquots at −80 °C. Protein concentrations were determined by the BCA method (Pierce) using bovine serum albumin as the standard.

Immunoblotting—Peptides corresponding to regions of rat ACS1, ACS4, and ACS5 that show poor amino acid conservation (ACS1, MEVHELFRYFRMPELIDIR; ACS4, EIHSMQSVEELGSKPENSSI; ACS5, KCGIEMLSLHDAENL) were synthesized, purified, and coupled to keyhole limpet hemocyanin in the UNC/PMBB Micro Protein Chemistry Facility. Rabbit antibodies to these peptides and to purified mitochondrial GPAT were raised commercially in New Zealand White rabbits (ImmunoDynamics, La Jolla, CA). Antibodies to PEMT2 were made as described previously (22). Proteins were separated on an 8% (or 12% for PEMT2) polyacrylamide gel containing 1% SDS and transferred to a polyvinylidene difluoride membrane (Bio-Rad). For chemiluminescent detection, the immunoreactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-rabbit IgG and PicoWest reagents (Pierce). For quantitation, the polyvinylidene difluoride membrane was incubated with 0.5 μ Ci of ^{125}I-Protein A (ICN), exposed to a phosphor screen, and quantified with the Molecular Dynamics Storm 840 and ImageQuant software.

Enzyme Assays—Diacylglycerol acyltransferase was assayed at 23 °C with 200 μM *sn*-1,2-diolein and 25 μM [^{3}H]palmitoyl-CoA (23), acyl-CoA synthetase was assayed at 37 °C with 5 mM ATP, 250 μM CoA, and 50 μM [^{3}H]palmitate (24), and glycerol 3-phosphate acyltransferase (GPAT) was assayed at 23 °C with 300 μM [^{3}H]glycerol-3-P and 112.5 μM palmitoyl-CoA in the presence or absence of 2 mM *N*-ethylmaleimide to inhibit the microsomal isoform (25). Microsomal GPAT was estimated by subtracting the *N*-ethylmaleimide-resistant activity (mitochondrial GPAT) from the total. All assays measured initial rates. [^{3}H]Palmitoyl-CoA (26) and [^{3}H]glycerol 3-phosphate (27) were synthesized enzymatically.

RESULTS

ACS1, ACS4, and ACS5 Are Located in Different Subcellular Fractions—The relative distribution of ACS activity in rat liver is 7% in peroxisomes, 20% in mitochondria, and 73% in microsomes (28). Since measurements of ACS activity do not distinguish among the different ACS isoforms, we localized each isoform in order to determine whether it is evenly distributed or, instead, located in a specific subcellular membrane.

We raised isoform-specific rabbit antibodies against unique peptides present in ACS1, ACS4, or ACS5 because an antibody raised against purified ACS1 not only recognized purified recombinant ACS1, but also recognized purified recombinant ACS4 and ACS5 (Fig. 1*A*). The cross-reactivity is most likely

TABLE I
DGAT and GPAT activity in liver subcellular fractions

	Specific activity *nmol/min/mg* (n = 3)		
	DGAT	GPAT[a]	Mitochondrial GPAT[b]
Microsomes[c]	1.72 ± 0.22	1.27 ± 0.16	0.06 ± 0.01 (4.7%)[d]
ER1[e]	0.31 ± 0.15	2.35 ± 0.23	0.51 ± 0.19 (22%)[d]
ER2[e]	0.59 ± 0.19	1.41 ± 0.09	0.29 ± 0.02 (20%)[d]
MAM[f]	0.94 ± 0.07	2.05 ± 0.35	0.61 ± 0.32 (30%)[d]
Crude mitochondria[g]	0.38 ± 0.09	1.20 ± 0.07	0.64 ± 0.05 (54%)[d]
Pure mitochondria[f]	0.19 ± 0.13	1.10 ± 0.35	1.00 ± 0.22 (91%)[d]

[a] GPAT specific activity assayed in the absence of NEM.
[b] Remaining GPAT activity following treatment of sample with 2 mM NEM.
[c] 100,000 × g pellet from supernatant fraction after isolation of crude mitochondria.
[d] Percent of GPAT specific activity assayed in the absence of NEM.
[e] Fraction isolated from sucrose gradient.
[f] Fraction isolated from Percoll gradient.
[g] 10,000 × g pellet.

due to the high degree of sequence similarity found among ACS1, ACS4, and ACS5. The specificity of each of our peptide antibodies was verified by immunoblot analysis of each isoform specific antibody against purified recombinant rat ACS1, ACS4, and ACS5, each with a C-terminal Flag epitope (29). Peptide antibodies for ACS4 and ACS5 each recognized only the correct ACS isoform (Fig. 1, *C* and *D*). The ACS1 peptide antibody failed to recognize recombinant ACS1-Flag (Fig. 1*B*) because the initial two amino acids of the N-terminal sequence had been altered to clone the recombinant protein. However, the recombinant ACS1-Flag protein detected by the Flag antibody and the major band in rat liver microsomes detected by the ACS1 peptide antibody migrate to the same position, showing that the ACS1 peptide antibody recognizes native ACS1 (Fig. 1*E*).

Rat liver was fractionated by two different methods. One method used a sucrose gradient to further purify microsomes into ER1 and ER2 fractions that are enriched in rough and smooth ER, respectively (19, 20). A Percoll gradient separated crude mitochondria into MAM and purified mitochondria (19, 20). The second method used only differential centrifugation (21) and produced mitochondria that had very little microsomal contamination and microsomes that primarily contained ER membranes, as well as the MAM fraction. The purity of each fraction was ascertained by enzymatic assays for the ER enzymes DGAT and NEM-sensitive GPAT, and for the mitochondrial enzyme NEM-resistant GPAT and an by immunoblots for mitochondrial GPAT and for PEMT, the MAM marker. These markers showed that the microsome fraction was free of mitochondrial contamination because little NEM-resistant GPAT activity was present (Table I) and no mitochondrial GPAT protein was detected by immunoblotting (Fig. 2*B*). In the ER and MAM fractions purified using gradients, 11–28% of the GPAT activity was resistant to NEM (Table I), indicating that these fractions were somewhat contaminated with mitochondria. No PEMT was detected in the ER and purified mitochondria by immunoblot analysis (Fig. 2*B*), indicating that these fractions were not contaminated with the MAM fraction. Very low DGAT and NEM-sensitive GPAT activities were measured in the purified mitochondria (Table I), indicating little contamination with ER. DGAT activity was enriched in the MAM fraction compared with ER, consistent with previous results (40). The microsome fraction (100,000 × g pellet) also has high DGAT activity, and it contains the MAM fraction, as evidenced by the presence of the MAM-specific marker, PEMT (Fig. 2*B*).

The location of ACS1, ACS4, and ACS5 in rat liver was determined by Western blot analysis of various subcellular fractions. ACS1 (68 kDa) was strongly detected in the microsomal fraction, which comprises rough ER, smooth ER, and MAM (Fig. 2*A*). The molecular mass is smaller than that predicted by the cDNA sequence (78 kDa), but in agreement with the size of the recombinant ACS1 run in the same manner (Fig. 1). A prominent ACS1 band was also detected in the cytosol, but cytosolic ACS specific activity was only 2.6% that of microsomal ACS specific activity (3.5 *versus* 132 nmol/min/mg of protein). Although ACS1 was present in crude mitochondria, which contain the MAM fraction, no ACS1 band was observed in purified mitochondria (Fig. 2*A*), as we reported previously (9). ACS4 (74 kDa) was enriched in the MAM fractions from rat liver (Fig. 2*A*) and Chinese hamster ovary cells (data not shown), and was only very weakly detected in microsomal and mitochondrial fractions. In some preparations, ACS4 was detected as a doublet with a larger 75-kDa protein (data not shown), consistent with alternative start sites as described for the human ACS4 homologue (30). The ACS5-specific antiserum detected a protein of 76 kDa in the mitochondrial fraction (Fig. 2*A*), 73- and 74.5-kDa proteins in ER, and a 74.5-kDa protein in cytosol and MAM fractions (Fig. 2*A*). The 76-kDa protein agrees with the size predicted by the cDNA sequence and migrates to the same position as recombinant ACS5. Taken together, these data indicate that ACS1, ACS4, and ACS5 each have unique distributions in liver subcellular membranes.

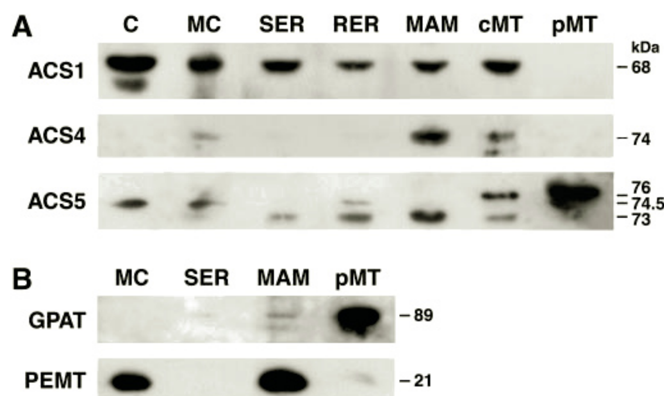

FIG. 2. **Location of ACS1, ACS4, and ACS5 in rat liver subcellular fractions.** Cytosol (*C*), microsomes (*MC*), fractions enriched in smooth endoplasmic reticulum (*SER*), fractions enriched in rough endoplasmic reticulum (*RER*), MAM, crude mitochondria (*cMT*), and pure mitochondria (*pMT*) fractions (100 μg of protein) were analyzed by Western blot with antibodies against ACS1, ACS4, or ACS5 (*A*) and GPAT or PEMT (B) . Molecular mass is indicated on the *right*.

Inhibition of ACS Activity in Liver Subcellular Fractions— Since ACS1, ACS4, and ACS5 were present in different subcellular membranes and the recombinant proteins expressed in *Escherichia coli* were inhibited to different extents by triacsin

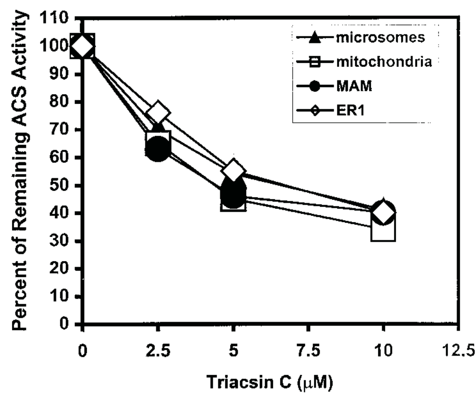

FIG. 3. **Triacsin C inhibits ACS activity in microsomes, smooth ER, mitochondria, and mitochondria-associated membrane fractions.** Microsomes, ER1 (enriched in rough ER), mitochondria, or MAM proteins (0.5 μg) were assayed for ACS activity in the presence of increasing concentrations of triacsin C (0–10 μM). Data are representative of two independent experiments and are presented as percentage of ACS activity remaining after treatment with inhibitor. Control specific activities (100%): microsomes = 109 nmol/min/mg; ER1 = 211 nmol/min/mg; mitochondria = 105 nmol/min/mg; MAM = 345 nmol/min/mg.

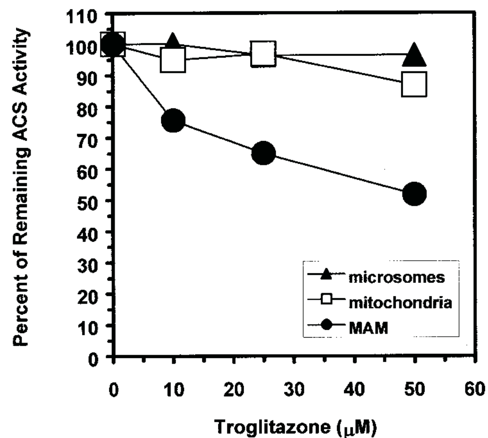

FIG. 4. **Troglitazone specifically inhibits ACS activity in mitochondria-associated membrane.** Microsomes, mitochondria, or MAM proteins (0.5 μg) were assayed for ACS activity in the presence of increasing concentrations of troglitazone (0–50 μM). Data are representative of two independent experiments and are presented as percentage of ACS activity remaining after treatment with inhibitor. Control specific activities (100%): microsomes = 89 nmol/min/mg; mitochondria = 67 nmol/min/mg; MAM = 255 nmol/min/mg.

C, thiazolidinediones, and NEM (29), we hypothesized that these inhibitors would affect ACS activity differently in each fraction. Triacsin C (10 μM), which inhibits purified recombinant ACS1 and ACS4 by 60%, and does not inhibit ACS5 (29), decreased ACS activity in microsomes, mitochondria, and MAM by 60% (Fig. 3). This result is surprising because the only ACS we identified in mitochondria was ACS5, which is resistant to inhibition by triacsin C (29). The mitochondria we assayed were free of contamination by the MAM fraction as determined by the absence of PEMT on an immunoblot (data not shown). Therefore, the observed inhibition suggests that mitochondria contain a hitherto undescribed triacsin-sensitive ACS.

Troglitazone and NEM, two specific ACS4 inhibitors, were more selective inhibitors of ACS activity in liver subcellular fractions. Troglitazone at 10 μM decreased ACS activity 25% in MAM, but had no effect on activity in the microsomal or mitochondrial fractions (Fig. 4). With 50 μM troglitazone, ACS activity was inhibited 45% in MAM, again with little effect on microsomal or mitochondrial ACS activity. NEM, a second

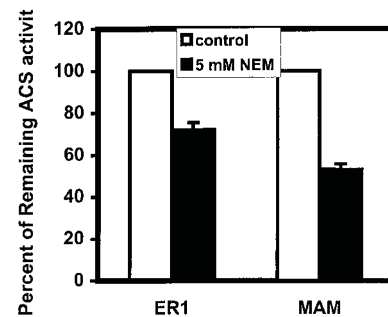

FIG. 5. ***N*-Ethylmaleimide inhibits ACS activity in mitochondria-associated membrane.** ER1 (enriched in rough ER) and MAM fractions (0.5–1.5 μg of protein) were pre-incubated in the absence or presence of 5 mM NEM for 10 min on ice prior to ACS assay. Control specific activities (100%): ER1 = 181 nmol/min/mg; MAM = 397 nmol/min/mg.

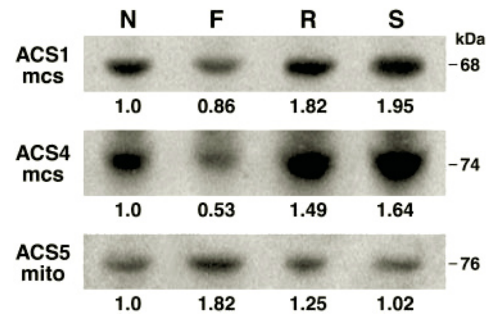

FIG. 6. **ACS1, ACS4, and ACS5 protein expression is regulated differently by fasting and re-feeding.** Liver total microsomes and mitochondria (100 μg of protein) from rats fed normally (*N*) (n = 5), fasted for 48 h (*F*) (n = 6), fasted for 48 h and refed normal chow for 24 h (*R*) (n = 6), and fasted for 48 h and refed a 69% sucrose diet for 24 h (*S*) (n = 4) were analyzed by quantitative Western blot analysis with peptide antibodies against ACS1, ACS4, or ACS5 and ^{125}I-Protein A. The blots were exposed to a phosphor screen for quantitation with a Molecular Dynamics Storm 840 system. The blots shown are representative, and the *numbers below* the bands indicate the -fold change compared with normally fed rats. The mean and S.D. for each are as follows: ACS1, *N* (1 ± 0.05), *F* (0.8 ± 0.05), *R* (1.8 ± 0.1), *S* (1.9 ± 0.02); ACS4, *N* (1 ± 0.08), *F* (0.5 ± 0.07), *R* (1.6 ± 0.15), *S* (2 ± 0.12); ACS5, *N* (1 ± 0.04), *F* (1.8 ± 0.05), *R* (1.2 ± 0.1), *S* (1 ± 0.06).

ACS4 inhibitor, decreased ACS activity 50% in MAM and only 27% in the smooth ER fraction (Fig. 5). These results are consistent with the immunolocalization of ACS4 preferentially to the MAM fraction.

Nutritional Regulation of ACS1, ACS4, and ACS5 in Liver—If ACS1, ACS4, and ACS5 are linked to different metabolic pathways, one might expect that each isoform would be regulated differently under conditions of fasting and re-feeding. ACS activity and protein expression were measured in liver microsomes and mitochondria isolated by differential centrifugation from control rats, from rats fasted for 48 h, and from rats fasted for 48 h and then refed either Purina rat chow or a high sucrose diet for 24 h. The microsomes contained MAM, and the mitochondria were free of contamination by MAM as determined by immunoblot with antibody against PEMT, the MAM-specific marker (data not shown). ACS activity, assayed with palmitate as the fatty acid substrate, was similar in microsomes and in mitochondria isolated from control rats (18.5 ±3.5 and 14.3 ± 2.9, respectively), 48-h fasted rats (21.8 ± 0.9 and 11.9 ± 2.9, respectively), and rats fed Purina rat chow for 24 h after a 48-h fast (15.1 ± 4.5 and 14.2 ± 2.4, respectively).

Although total ACS activity was unchanged by nutritional status, ACS1 protein expression increased 1.8- and 2-fold with Purina chow and high sucrose re-feeding, respectively (Fig. 6). ACS4 protein expression also increased with re-feeding (50%

with Purina chow diet and 64% with sucrose diet). ACS5 protein expression in mitochondria did not appear to be altered by re-feeding. After a 48-h fast, ACS1 protein expression decreased 14%, ACS4 protein decreased 47%, whereas ACS5 protein expression increased 82% in mitochondria (Fig. 6).

DISCUSSION

Long chain acyl-CoA synthetase catalyzes the initial step required for oxidation, elongation, and desaturation of fatty acids; for the synthesis of complex lipids and acylated proteins; and for a variety of signals that regulate cellular metabolism (1, 2). It had been thought that the ACSs synthesize a common pool of acyl-CoAs, which move freely within cell membrane monolayers and have equal access to the numerous metabolic pathways in which they participate. However, genetic studies in yeast link specific ACS isoforms to different pathways that use acyl-CoAs (12–17). In addition, in cultured cells, inhibitors of ACS (triacsin and troglitazone) selectively alter the synthesis and oxidation of cellular lipids, suggesting that the ACS isoforms might be differentially inhibited, and that triacsin-sensitive isoforms might be functionally linked to *de novo* glycerolipid synthesis whereas triacsin-resistant ACS isoforms might be functionally linked to phospholipid reacylation pathways and to β-oxidation (9–11, 31–33). These data, together with our results showing that triacsin C inhibits recombinant ACS1 and ACS4, but not ACS5, and that thiazolidinediones specifically inhibit ACS4 (29), suggested that acyl-CoA entry into specific metabolic pathways could be mediated by the action of individual ACS isoforms.

In this paper, we show that ACS1, ACS4, and ACS5 differ in their subcellular distribution, suggesting that, as in yeast, acyl-CoAs may be channeled toward specific metabolic pathways. Functional channeling could occur if a particular ACS were located in a membrane that contained only a handful of specific pathways that use acyl-CoAs or if each ACS were physically associated with the downstream enzymes in a specific pathway. To examine this question, we investigated the subcellular locations of the three ACSs expressed in rat liver. ACS1, the first cloned and best studied ACS isoform, has been reported to be present in virtually every subcellular fraction. An early study with a polyclonal antibody raised against ACS1 purified from microsomes reported that ACS1 protein was present in rat liver microsomes, peroxisomes, and mitochondria (34). Others have identified ACS1 in GLUT4 vesicles (35) and in plasma membrane (36) from fat cells. ACS activity has been reported in nuclei from rat liver (37), and in cytosol of PC12 neurons (38), but the ACS isoform was not determined in either case. Our studies showed ACS1 protein in the microsomal fraction of rat liver. ACS1 does not appear to contribute to the ACS activity present in mitochondria, since our peptide antibody did not detect ACS1 in purified mitochondria under any nutritional condition, consistent with our previous study (9). Although ACS1 had been identified in rat mitochondria with an antibody raised against an ACS purified from microsomes (34), the antibody may have recognized epitopes on non-ACS1 isoforms present in mitochondrial membranes as we showed in Fig. 1.

We also detected a significant amount of ACS1 protein in rat liver cytosol. Although a cytosolic location is consistent with measurements of oleic acid ACS activity in the cytosol of PC12 cells, ACS activity and ACS1 protein expression are absent in the cytosol from 3T3-L1 adipocytes (36). Since, in liver cytosol, ACS specific activity was barely detectable, our antibody may cross-react with a non-ACS cytosolic protein of the same molecular mass. Alternatively, cytosolic ACS1 may be largely inactive, but become active after it translocates to intracellular membranes as occurs with oleate-activated FadD, the bacterial ACS (39).

The subcellular locations of ACS4 and ACS5 have not been reported previously. Some of the mitochondrial ACS activity is accounted for by ACS5, since we detect a 76-kDa protein (in agreement with the predicted size of ACS5) specifically in mitochondria. The ACS5 peptide antibody also detects 73- and 74.5-kDa proteins in ER and MAM, and a 74.5-kDa protein in cytosol and MAM fractions. Both ACS3 and ACS4 can use alternative start sites (6, 30), but no alternative start site is present in the ACS5 sequence that would yield proteins smaller by 1.5 or 3 kDa. The smaller immunoreactive proteins might represent ACS5 after proteolytic cleavage, or it may be that the ACS5 peptide antibody recognizes a novel ACS isoform with a high degree of sequence similarity.

ACS4 is highly enriched in the MAM fraction, an ER-like membrane that can be found in mitochondria or ER preparations, depending on the cell fractionation method employed. The MAM fraction contains PEMT-2, microsomal triglyceride transfer protein, apoB, and high specific activities of acyl-CoA: cholesterol acyltransferase, DGAT (40), and phosphatidylserine synthase-1 and -2 (41). Vance's group has hypothesized that MAM may be involved in importing lipids into mitochondria (42) or in very low density lipoprotein assembly (40).

Inhibition studies with purified ACS1, ACS4, and ACS5 showed that triacsin C inhibited only ACS1 and ACS4, whereas the thiazolidinediones and NEM were more selective and only inhibited ACS4 (29). Consistent with these inhibition studies and the location of the three isoforms in liver, troglitazone and NEM had their maximum effects in MAM where ACS4 is located. Similarly, triacsin C was a potent inhibitor of ACS activity in ER and MAM, membranes that contain both ACS1 and ACS4. Surprisingly, however, triacsin C inhibited ACS activity in mitochondria to the same extent that it inhibited activity in ER (Fig. 2). Since the triacsin-resistant ACS5 is the only ACS isoform detected in mitochondria, a hitherto unknown triacsin-sensitive ACS may be present in mitochondria. Another possibility is that purified ACS5-Flag, which is not inhibited by triacsin, does not have the same properties as the *in situ* enzyme. We do not believe that this is the case, since the Flag epitope does not interfere with ACS activity (29) and triacsin C is unable to inhibit ACS activity in an *E. coli* membrane fraction containing overexpressed ACS5-Flag (data not shown).

Because MAM may be a specialized site for very low density lipoprotein biosynthesis, the presence of ACS4 in this fraction and its inhibition by triacsin C suggests that ACS4, like ACS1, is linked to triacylglycerol synthesis. Consistent with this hypothesis, we found that ACS4 protein expression decreased 47% after a 48-h fast, and was up-regulated by re-feeding either normal chow (50% increase) or a high sucrose diet (64% increase). The most remarkable finding was the specific inhibition of recombinant ACS4 by thiazolidinediones (29) and the ability of troglitazone to specifically inhibit ACS activity in MAM (Fig. 3). It has been hypothesized that the mechanism by which thiazolidinediones produce their anti-diabetic effects is through their ability to lower plasma fatty acids (43). Further study is needed to determine whether thiazolidinediones are insulin sensitizers, in part, because of their inhibitory effect on ACS4.

In humans, the gene for ACS4 lies on the X chromosome and is part of a large deletion that results in a human disorder that combines Alport syndrome with elliptocytosis, dysmorphic facies, and mental retardation (44). It is not known whether the deleted ACS4 contributes to these problems or whether the disorder is associated with abnormalities in serum lipids. However, since expression of ACS4 is highest in human brain, and human and rat ACS4 show a preference for arachidonate, it has

been suggested that, in brain, ACS4 might be critical for recycling arachidonate into phospholipids that are sources for signaling molecules related to intellect and coordination (30).

Consistent with its microsomal location and the powerful triacsin-mediated inhibition of triacylglycerol synthesis in fibroblasts, hepatocytes, and HepG2 cells (11, 31–33), ACS1 has been linked to triacylglycerol synthesis. ACS1 mRNA is prominent in liver and adipose tissue (4), and in 3T3-L1 cells only after they differentiate into adipocytes (45). ACS1 mRNA expression also increases in adipose tissue and muscle after PPARγ activation (46), and in liver after high dietary fat refeeding (4). The presence of ACS1 in GLUT1 vesicles is hypothesized to be linked to vesicle trafficking, which requires palmitoyl-CoA (35), but ACS1 could, in fact, function to increase fatty entry into cells in response to insulin stimulation. On the other hand, liver ACS1 is regulated by PPARα (46–48), whose activation is usually associated with the up-regulation of enzymes of fatty acid oxidation. Our finding that ACS1 is present in ER, MAM, and possibly peroxisomes[2] provides a potential explanation, if the various physiological processes result in changes in the amount of ACS1 protein present in different subcellular organelles (*e.g.* peroxisomes *versus* ER). In fact, reciprocal differences in ACS activity were observed in mitochondria and microsomes after stimulation by cytokines, but, unfortunately, the ACS isoforms involved were not identified (49).

ACS5 may be linked to the β-oxidation pathway because its protein expression increased 80% after a 48-h fast. This increase in ACS5 protein contrasts with a previous study that reported a 50% decrease in ACS5 mRNA after a fast (8). It is possible that the rate of ACS5 protein turnover is decreased in response to fasting. Although the presence of a triacsin-sensitive ACS linked to β-oxidation is consistent with previous observations that treatment of hepatocytes with triacsin C (10 μM) results in a 30% decrease in acid soluble metabolites (9), because recombinant ACS5 is not sensitive to triacsin (29), there must be a novel triacsin-sensitive ACS in mitochondria.

Many unique eukaryotic enzymes catalyze the same lipid-biosynthetic reaction (50). These enzymes are encoded by different genes and are often located in distinct cellular locations. Our findings show that ACS1, ACS4, and ACS5 are located in different liver subcellular membranes and that nutritional changes regulate each isoform independently, consistent with the hypothesis that eukaryotic systems have redundant lipid-biosynthetic enzymes in order to provide independent regulation of activity and compartmentalization of lipid pools (50).

Acknowledgments—We thank Dr. Paul Watkins for the ACS1 antibody and Dr. David G. Klapper for assistance in synthesizing the peptides for the ACS1, ACS4, and ACS5 antibodies.

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