FUNCTIONS OF RAT ACYL-COA SYNTHETASES IN BACTERIA AND MAMMALIAN CELLS

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Long chain acyl-CoA synthetase (ACSL) activates long chain fatty acids in the initial step of lipid metabolism, and provides substrates for both anabolic and catabolic pathways. Dysregulation of fatty acid partitioning between these opposing pathways results in cellular lipid accumulation, which may cause obesity and lipotoxicity. We hypothesized that ACSL isoforms play a critical role in fatty acid channeling into different metabolic pathways and that regulation of ACSL may change the fatty acid channeling. This dissertation describes three independent projects that examined the function of rat ACSL isoforms in lipid metabolism and their acute regulation by phosphorylation.

Using an *E. coli* model with its endogenous ACSL mutated (*fadD*), we demonstrated that although all rat ACSL isoforms partially rescued fatty acid transport, β-oxidation, and phospholipid synthesis, only ACSL5 supported growth on oleate as the sole energy and carbon source. This study supported the hypothesis that ACSL isoforms are functionally different and prompted the subsequent study on function of ACSL1 in lipid metabolism.
Using an adenovirus that carries an ACSL1 cDNA, we overexpressed rat ACSL1 in primary hepatocytes and examined the effects on metabolic pathways. Consistent with our hypothesis that individual ACSLs partition fatty acid, we found that ACSL1 overexpression increased oleate incorporation into diacylglycerol and phospholipids, but not triacylglycerol, and decreased cholesterol ester synthesis. Together with the finding from a previous study in our lab with ACSL5 overexpression, these data provided direct evidence of different fatty acid channeling by ACSLs. We then studied the potential acute phosphorylation regulation of ACSL1 and ACSL5 in 3T3-L1 adipocytes and hepatocytes. We identified phosphorylated threonine 192 on ACSL5, and detected ACSL5 and ACSL1 with phosphorylated serine residues. These data were the first to show that ACSL could be phosphorylated, and that phosphorylation could occur on distinct residues among different ACSL isoforms. The importance of this dissertation also lies in directing future research on mechanisms of specific role of individual ACSL isoforms and potential clinical implication for obesity and its related metabolic diseases.
To my parents, Jianping Li and Meilan Li,

to my brother, Bozhao Li,

to my husband, Lei Ma,

for their endless love and encouragement.
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<tbody>
<tr>
<td>ACAT</td>
<td>acyl-CoA:cholesterol acyltransferase</td>
</tr>
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<td>ACBP</td>
<td>acyl-CoA binding protein</td>
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<tr>
<td>ACS</td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>ACSL</td>
<td>long-chain acyl-CoA synthetase</td>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropin</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ASM</td>
<td>acid-soluble metabolites</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CE</td>
<td>cholesterol ester</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase 1</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FACS</td>
<td>fatty acyl-CoA synthetase</td>
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<tr>
<td>FATP1</td>
<td>fatty acid transport protein 1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GPAT</td>
<td>\textit{sn}-glycerol-3-phosphate acyltransferase</td>
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<td>Abbreviation</td>
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<tr>
<td>HNF-4α</td>
<td>hepatic nuclear factor 4α</td>
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<tr>
<td>HSL</td>
<td>hormone sensitive lipase</td>
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<tr>
<td>LC-CoA</td>
<td>long-chain acyl-CoA</td>
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<tr>
<td>LCFA</td>
<td>long-chain fatty acid</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>MAM</td>
<td>mitochondrial-associated membrane</td>
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<tr>
<td>MEM</td>
<td>minimal essential medium</td>
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<tr>
<td>MOI</td>
<td>multiplicities of infection</td>
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<tr>
<td>NEAA</td>
<td>nonessential amino acids</td>
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<tr>
<td>PAT</td>
<td>palmitoyl acyltransferase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PC</td>
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<td>phosphatidylethanolamine</td>
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<td>phosphatidylinositol</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR-responsive element (PPRE)</td>
</tr>
<tr>
<td>PS</td>
<td>phosphoserine</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<td>Acronym</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinedione</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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CHAPTER I

INTRODUCTION

1.1 Lipid homeostasis and fatty acid partitioning

In cells, a system of metabolic and signaling pathways maintains lipid homeostasis and normal cell function. Free fatty acids, delivered from exogenous transport, *de novo* synthesis, or released from hydrolysis of triacylglycerol (TAG) and phospholipids (PL), can enter multiple pathways, primarily oxidative or biosynthetic pathways. Dysregulation of fatty acid partitioning between these two opposing pathways results in cellular TAG accumulation. In adipose tissue, TAG accumulation causes an increase of both adipocyte size and numbers, which develops to obesity. In non-adipose tissues with limited capacity for lipid storage, TAG accumulation causes cellular dysfunction or cell death, often referred as lipotoxicity and lipoapoptosis (1,2). Lipotoxicity in skeletal muscle, liver, pancreas and heart, is believed to be associated with insulin resistance, non-alcoholic steatohepatitis (NASH), impaired glucose-stimulated insulin secretion, and cardiomyopathy (1,2). Obesity and its related diseases such as diabetes, hypertension and cardiovascular diseases, often called metabolic syndrome, produce an alarming health challenge (3). Studying how fatty acids are directed into different pathways will provide an understanding of the etiology of obesity and related diseases, and hence provide potential targets for treatment.
This dissertation research is focused on the role of long-chain acyl-CoA synthetases (ACSL) in channeling fatty acid among specific pathways of lipid metabolism.

1.2 Fatty acid activation by acyl-CoA synthetase to acyl-CoA

**Scheme 1.1** provides an overview of ACSL providing long chain acyl-CoAs for multiple downstream pathways. This section will review the role of ACSL in fatty acid transport and activation, as well as additional functions of acyl-CoAs in cells.

1.2.1 *Activation of fatty acids for downstream lipid metabolism*

In the initial step of lipid metabolism, long chain fatty acids are converted to acyl-CoA thioesters by long chain acyl-CoA synthetases (ACSL) before entering downstream pathways (4). ACSL activates fatty acids in a two-step reaction 1) the formation of a fatty acyl-AMP intermediate molecule with pyrophosphate release and 2) the formation of fatty acyl-CoA with the release of AMP (5). The hydrolysis of pyrophosphate renders this reaction irreversible.

1) fatty acid + ATP $\rightarrow$ fatty acyl-AMP + pyrophosphate

2) fatty acyl-AMP + CoA $\rightarrow$ fatty acyl-CoA + AMP

The products of this reaction, long-chain acyl-CoAs (LC-CoAs), with high energy thioester bonds, are substrates for both catabolic pathways to provide energy via β-oxidation, and anabolic pathways to synthesize complex lipids such as triacylglycerol (TAG),
diacylglycerol (DAG), PL and cholesterol ester (CE) (Scheme 1.1). During β-oxidation, LC-CoAs are converted to acyl-carnitines by mitochondrial carnitine palmitoyltransferase 1 (CPT1) and translocated into mitochondria for further degradation. During the de novo synthesis of glycerolipids, acyl-CoAs are transferred to glycerol-3-phosphate by mitochondrial and microsomal sn-glycerol-3-phosphate acyltransferases (GPAT) to synthesize lysophosphatidic acid (LPA). Balance between these two opposing lipid metabolic pathways is determined by nutritional and physiological conditions. ACSL functions at this branch point by providing substrates for diverse pathways. Identification of ACSL in all organisms for which genomic sequence information is available testify to the essential nature of this enzyme (6).

1.2.2 Long chain acyl-CoA synthetase in fatty acid transport

In addition to activating fatty acids for metabolic pathways, ACSL might participate in fatty acids import by metabolically trapping them as CoA thioesters after transport into the cell (7,8). This so called “vectorial acylation” process, is proposed to describe the role of FadD, the ACSL in Escherichia coli (E. coli), in activating exogenous long-chain fatty acids (LCFAs) concomitant with transport across the inner membrane (9). It has been proposed that FadD moves to the inner membrane, where it retrieves and activates LCFAs into membrane impermeable acyl-CoAs, thus rendering LCFA transport unidirectional (10,11). In fact, import of [3H]oleate is abolished in a ΔfadD strain, and specific mutations in FadD not only reduce or abolish enzyme activity but likewise reduce or abolish the import of
Scheme 1.1. Long chain fatty acids, from exogenous or endogenous sources, are converted to long chain acyl-CoAs before entering multiple downstream pathways. Acyl-CoAs are substrates for lipid metabolism in the two opposing pathways, anabolic for complex lipid synthesis such as TAG, PL and cholesterol ester, and catabolic for β-oxidation to provide energy. Balance between these two primary lipid metabolic fates is determined by enzyme activity, nutritional status, and physiological conditions. Disturbance of this balance is linked to lipid accumulation and lipotoxicity, common to obesity and insulin resistance. ACSL also provides acyl-CoAs for other cellular processes, such as allosteric regulation of enzyme activity, protein acylation, membrane fusion and signal transduction. Acyl-CoAs can be synthesized in nuclei or transported to nuclei, where they might serve as ligands for transcription factors such as PPARα and HNF-4α and elicit a broad range of effects. ACSL, long-chain acyl-CoA synthetase; DAG, diacylglycerol; LPA, lysophosphatidic acid; PA, phosphatidic acid; PL, phospholipids; TAG, triacylglycerol.
exogenous LCFAs, suggesting a specific coupling between LCFA activation and import (12). Biochemical studies with isolated plasma membrane vesicles from *E. coli* directly demonstrate that long chain fatty acid uptake occur only when vesicles contain the enzyme and its substrates CoASH and ATP (13). The importance of ACSL in fatty acid uptake is also shown in yeast. Fatty acid uptake is impaired in a *Saccharomyces cerevisiae* strain with mutations in Faa1 and Faa4, two yeast ACSLs (14). The growth of this mutated strain is completely eliminated when endogenous fatty acid synthase is inhibited with cerulenin. These studies suggest that esterification of fatty acid is coupled with transport.

Much evidence suggests that ACSL also plays a role in fatty acid transport in mammalian cells. ACSL1 is one of the two adipocyte proteins that facilitate the import of fatty acid in a functional screen study using an expression cloning approach (15). When overexpressed in NIH fibroblasts, ACSL1 increases fatty acid uptake 8-fold, and has synergistic effects with fatty acid transport protein 1 (FATP1) (16). In fact, FATP1 exhibits acyl-CoA synthetase activity with a broad substrate specificity. It is proposed to enhance fatty acid uptake by generating membrane impermeable acyl-CoAs (17-19). Moreover, overexpressed ACSL1 and FATP1 coimmunoprecipitate in 3T3-L1 adipocytes, indicating that a physical interaction between ACSL1 and FATP1 might exist (20). In 3T3-L1 adipocytes, fatty acid uptake is inhibited 84% by 10 µM triacsin C, an inhibitor of acyl-CoA synthetase activity (21), indicating that fatty acid uptake is dependent on vectorial acylation
in 3T3-L1 adipocytes. When another isoform, ACSL5, is overexpressed in McArdle RH-7777 rat hepatoma cells, exogenous fatty acid uptake is also increased 30% (22), despite the intracellular location of ACSL5 on mitochondria and endoplasmic reticulum (ER), suggesting that metabolism driven by ACSL may also contribute to fatty acid uptake.

1.2.3 Additional functions of long chain acyl-CoAs

In addition to being substrates for lipid metabolic pathways, LC-CoAs also play an important role in a broad range of intracellular processes (23-25) (Scheme 1.1). LC-CoAs regulate enzymes allosterically. For example, LC-CoAs inhibit hexokinase (26) and pyruvate dehydrogenase (23) in glucose metabolism, and inhibit acetyl-CoA carboxylase and ACSL in lipid metabolism (23). In pancreatic β-cells, LC-CoA esters activate ATP-sensitive K⁺ (K_{ATP}) channels (25,27), modulate intracellular Ca^{2+}, activate atypical PKC isoforms (23,28), and directly stimulate exocytosis resulting in insulin secretion (29-31).

The covalent attachment of fatty acids to proteins (acylation) is a widespread post-translational modification, which mediates protein targeting, vesicle budding or fusing and signal transducing (32). One of the most common acylations, palmitoylation occurs on many important signaling proteins such as the G protein coupled receptor, some Gα subunits and Ras protein (33). Palmitoylation also provides mechanisms to regulate rafts and/or caveolae targeting and protein-protein interaction, thus affecting a wide variety of signaling pathways (33). In integral membrane proteins such as the G protein coupled receptors,
palmitoylation occurs on cysteines that are either close to the transmembrane domain/cytoplasmic domain boundary or located in the cytoplasmic domain (33). In peripheral membrane proteins such as Src family and Ga subunits, palmitoylation occurs at a cysteine near myristoylation sites and this dual acylation is required for stable membrane association (33,34). Peripheral membrane proteins can also be palmitoylated at a cysteine several amino acids upstream from the prenylation site, a cysteine in the C-terminal amino acid motif, the CAAX box (C = cysteine, A = aliphatic residue, and X = any amino acid) (33). Although protein palmitoylation by palmitoyl acyltransferases (PAT) has been found in fractions including plasma membrane, Golgi and mitochondria, the sources of the palmitoyl-CoA is unknown. Further studies need to be done on whether acyl-CoA binding proteins (ACBP) send acyl-CoAs to subcellular PATs, or specific ACSL isoforms associate with PAT in different organelles.

Additionally, studies from bacteria, yeast, and mammalian cells also imply that acyl-CoAs might be natural ligands for transcription factors and hence play a broader role in affecting gene expression (23,24,35). In *E. coli*, binding of LC-CoAs to the transcription factor FadR causes significant conformational changes in both the acyl-CoA binding domain and the DNA binding domain. The acyl-CoA binding renders FadR unable to bind cognate operators and allows transcription of target genes (36,37). In mammalian cells, both unsaturated and saturated LC-CoAs bind PPARα with high affinity and elicit conformational changes in PPARα, which increases co-activator recruitment (38,39). Acyl-CoAs may also
act as ligands for hepatic nuclear factor 4α (HNF-4α) and regulate its transcriptional activity as a function of their chain length and degree of unsaturation (40). In fact, significant amounts of LC-CoA are present in rat liver nuclei (~ 0.32 nmol/g wet rat liver) (41). The high performance liquid chromatography profile of nuclear acyl-CoAs is distinct in that the nuclear acyl-CoA pool contains predominantly C18:3 and C16:1 acyl-CoAs, whereas the total liver acyl-CoA pool contains predominantly C20:4, C18:2, C18:1, and C16:0 acyl-CoAs (41). Since ACS activity is present in rat liver nuclei (42) and adipocyte nuclei (43), and ACSL1 protein is found in adipocyte nuclei fractions (16), specific ACSL isoform(s) might contribute to the formation of distinct nuclear acyl-CoA pool for gene expression regulation.

1.3 Long chain acyl-CoA synthetase isoforms

Scheme 1.2 shows the conserved domains of ACSL family in *E. coli*, yeast, rodent and human. This section covers the information on the structure, enzyme kinetics, expression pattern, location, and regulation of ACSL isoforms.

1.3.1 Structure

Acyl-CoA synthetases (ACS) have been classified by their preferences for short, medium, long, and very long chain fatty acids (44). Long-chain ACS (ACSL) activates fatty acids of 12 to 22 carbons. Since the cloning of rat ACSL1 in 1990 (45), four additional ACSL proteins, ACSL3 (46), ACSL4 (47), ACSL5 (48) and ACSL6 (49), were identified, each the product of a separate gene. The five rat ACSLs form two subfamilies.
Sequence alignment (ClustalW alignment http://www.ebi.ac.uk/clustalw/) shows two highly conserved regions in ACSL isoforms from bacteria to human, a putative ATP-AMP signature motif and a FACS (fatty acyl-CoA synthetase) signature motif. The ATP/AMP signature motif contains two highly conserved sequence elements, and is common to all members of the adenylate-forming family. The more highly conserved FACS signature motif, only shared by ACSL isoforms, contains several hydrophobic residues (leucine, isoleucine or valine), which might comprise part of fatty acid binding pocket (7).

ACS1, 5 and 6 (originally ACS2, (50)) share ~60% amino acid identity with each other, but less than 30% with ACSL3 and ACSL4. The latter two share 63% amino acid identity (51). These five ACSL isoforms have been identified and characterized in mouse and humans (44,50,52). In addition to the diversity of isoforms from individual genes, splice variants of mRNAs and proteins of ACSL occur in human and rodents (50). Some splice variants such as human ACSL3 encode transcripts with varying untranslated regions, but are translated as the same proteins (50). Some splice variants encode a longer transcript such as human...
ACSL4, which encodes a protein with 41 more amino acids at the N terminus (53,54). We also identified splice variants of rat ACSL6 that have 12 different amino acids, as a result of alternative exon use (51).

Multiple sequence alignments of ACSL identified two highly conserved regions in ACSL isoforms from bacteria to human, a putative ATP-AMP signature motif and a fatty acyl-CoA signature motif (FACS signature motif) (Scheme 1.2). The ATP-AMP signature motif contains two highly conserved sequence elements, common to all adenylate-forming enzymes, including firefly luciferase (7,55). This ATP-AMP motif might contribute to the binding of ATP and/or to the formation of the adenylated enzyme intermediate because ATP is the one substrate common to all members (12). The FACS signature motif, only shared by ACSL isoforms, contains several hydrophobic residues and is predicted to form the fatty acid binding pocket (7). Site-directed mutation in these motifs in FadD, the ACSL of E. coli, further supports the idea that these sites are important for catalytic activity (12,55). For example, fadD<sup>E361A</sup> in the ATP-AMP signature results in an enzyme with no measurable activity in E. coli (12). Seven of 18 site-directed mutations in the FACS signature motif lower the affinity for fatty acids and alter fatty acid chain length specificity (55). Using affinity-labeled LCFA [<sup>3</sup>H]<sub>9</sub>-p-azidophenoxynonanoic acid, a peptide sequence <sup>422</sup>PDATDEIIK<sup>430</sup> is defined as the fatty acid binding site in FadD. This site overlaps the FACS signature motif, suggesting that this is part of the fatty acid binding domain (56).
Due to the difficulty in purifying membrane proteins, no NMR or X-ray crystal structure data are available for mammalian ACSLs. However, a distantly related ACSL from the thermophilic bacterium *Thermus thermophilus* HB8, ttLC-FACS, has been crystallized, which provides insight into the catalytic mechanism for bacterial ACS (57). The crystal structure from ttLC-FACS suggests that ttLC-FACS forms a homodimer with interactions at the N terminus via intermolecular salt bridges and a hydrogen bond. It is proposed that mammalian ACSLs might also form homodimers because those residues that form the bonds are highly conserved (57). The crystal structure of ttLC-FACS also suggests that a fatty acid-binding tunnel exists at the N terminus. In order to study the importance of this tunnel for substrate specificity, we generated rat ACSL4 mutations at residues that correspond to those in the fatty acid-binding tunnel based on ttLC-FACS sequence alignments. The mutation of 410Glycine (G410L) and 291serine (S291Y), which are located at the entry and termination of the potential fatty acid-binding tunnel, respectively, resulted in an inactive ACSL4 or ACSL4 with diminished affinity for C20:5 and C22:6 (58). These data suggested that specific residues around the proposed tunnel contributed to substrate specificity and catalytic activity.

1.3.2 Substrate preferences and enzyme kinetics

Although ACSL isoforms catalyze the activation of fatty acids by the same reaction, they differ in their substrate preferences and enzyme kinetics. Despite a wide range of fatty acid substrates, the ratios of C16:0 ACS activity to C12:0 ACS activity are 0.76 and 1.03 for
ACSL1 and ACSL6, respectively (49). ACSL3 uses C12:0 and C14:0 most efficiently among saturated fatty acids and C20:4 (arachidonic acid) and C20:5 (eicosapentaenoic acid) among unsaturated fatty acids (46). ACSL4 has a marked preference for C20:4 ($K_m = 15 \, \mu M$) and C20:5 ($K_m = 12 \, \mu M$), compared to C16:0 (palmitic acid) ($K_m = 100 \, \mu M$) (47). ACSL1 and ACSL5 activate shorter-length fatty acids (16–18 carbons) with nearly equal efficiencies, ACSL3 and ACSL4 activate C20:4 (n-6) and C20:5 (n-3) with the greatest efficiency, and ACSL6 preferentially promotes C22:6 (docosahexaenoic acid) metabolism in neuronal cells (59,60). Purified recombinant ACSL1, 3, 4, 5 and 6 have similar apparent $K_m$ values for CoA and palmitic acid, but different $K_m$ values for ATP. The ATP affinity of ACSL4 is approximately 19-, 13-, 20-, 44-, and 360-fold higher than that of ACSL1, ACSL3, ACSL5, ACSL6_v1 and ACSL6_v2, respectively (21,51). ACSL1 is also more sensitive to heating and inhibition by NEM, which modifies sulfhydryl groups (21,51). A direct competition assay to compare the fatty acid preferences shows differences among ACSL3, 4, and 6, the three main ACSL isoforms in brain (Appendix 6.2, Figure 6.3). ACSL3 prefers C18:3 to C16:0 and C22:6, and ACSL4 prefers all the polyunsaturated fatty acids (PUFA) tested, especially C20:4 and C20:5. The 12 amino acids difference between ACSL6_v1 and ACSL6_v2 renders preference of C18:2 and C18:3 to C16:0 by variant 1 and preference of C22:6 and C18:3 to C16:0 by variant 2.

1.3.3 Tissue and developmental expression pattern and subcellular location

ACSL isoforms are expressed to various degrees in different tissues. When rat ACSL1
was first cloned, its mRNA was only found in liver, heart, and adipose tissue, not in lung, small intestine, or brain (45). In a more extensive study using quantitative real-time PCR, we found that ACSL1 mRNA was highest in brown adipose tissue, gonadal adipose tissue, liver and heart, low in skeletal muscles and very low in brain (61). ACSL3 mRNA is expressed at highest level in brain and high level in testis, but at low levels in all other tissues examined (46,61). The highest expression of ACSL4 is in adrenal gland and other steroidogenic organs (47). ACSL4 is expressed at a relatively high level in liver and brain, and at markedly lower levels in other tissues including adipose tissues, skeletal muscle, heart, testis, but not in small intestine (47,61). In addition, in human brain, ACSL4 is specifically expressed in neurons but not in glial cells (54). ACSL5 is expressed most abundantly in small intestine, liver, and brown adipose tissue, but relatively low in lung, adrenal gland, and white adipose tissue (48,61). ACSL6 mRNA is most abundant in brain, and to a lesser extent, in gastrocnemius and soleus muscles, but is low in heart and brown adipose tissue and undetectable in white adipose tissues and liver (49,61). Distinct tissue distribution and enzyme characteristics of each ACSL isoform suggest that ACSL isoforms contribute to specific lipid metabolic pathways in different tissues. For example, abundant expression of ACSL1 in adipocytes might provide acyl-CoAs for TAG synthesis and storage, and high level of PUFA preferring ACSL3 and ACSL6 in brain might provide acyl-CoAs for PL synthesis.

ACSL isoforms also display unique development expression patterns. In rat
reaggregated brain cells, a model for developing brain, from day 7 to day 49, ACSL3 and ACSL6 mRNA increase 3-fold and 7-fold, respectively, whereas ACSL1 mRNA remain low and unchanged (62). In PC12 neuron cells that use fatty acid primarily for PL synthesis, before differentiation the mRNA levels of ACSL4 and ACSL5 are 3- and 5-fold of those of ACSL1, 3, and 6, respectively (63). After PC12 cell differentiation, ACSL4, ACSL5 and ACSL6 mRNAs are increased by 63%, 110% and 27%, respectively, whereas levels of ACSL1 and ACSL3 mRNAs are unchanged (63). Additionally, during the development of rat brain, ACSL3 mRNA is high during early postnatal period (5-15 d) and decreases from day 20 to adulthood. In contrast, ACSL6 mRNA increases gradually from postnatal day 15 and reaches a maximum level in the adult. ACS1 mRNA levels display no change during brain development (46). The unique developmental patterns of ACSL isoforms suggest that they play different tissue-specific roles during development.

Additionally, ACSL isoforms have specific subcellular distributions. Using fractionation techniques, it has been shown that in rat liver, ACSL1 is located in ER, mitochondrial-associated membrane (MAM) and cytosol, but not in mitochondria (64). ACSL4, a peripheral membrane protein, is abundant in MAM and is the only ACSL isoform present in peroxisomes in rat liver (65). In human brain alternative splicing encodes a longer transcript, which produces a brain-specific ACSL4 isoform with 41 additional amino-terminal, hydrophobic amino acids (54). Consistent with predicted location in ER, immunohistochemical staining shows location of this splicing variant of ACSL4 in the outer
nuclear membrane (54). ACSL5 is the only isoform identified on the mitochondria outer membrane in liver (64,66) and is also located on the ER when overexpressed in McArdle rat hepatoma cells (22). In 3T3-L1 adipocytes, ACSL1 is enriched in ER and plasma membrane (16). Distinct subcellular locations of ACSL isoforms might link them to specific metabolic features of each organelle. For example, ACSL1 and ACSL4 in MAM may provide acyl-CoA for glycerolipid synthesis, and ACSL4 in peroxisomes may channel fatty acids for peroxisomal oxidation.

### 1.3.4 Physiological regulation

In addition to the specific pattern during brain development and cell differentiation as mentioned above, expression of ACSL isoforms differs under varying physiological conditions. In rat liver, both ACSL1 and ACSL5 mRNA are increased by a high sucrose diet, but a high fat diet increases only ACSL1 mRNA (45,48). At the protein level, fasting for 48 h decreases ACSL4 protein by 47% in rat liver, but increases ACSL5 protein by 82%, and refeeding rats after fasting results in a significant increase of both ACSL1 and ACSL4, but not ACSL5 (64). In liver from genetically obese (ob/ob) mice, which have leptin deficiency and display severe obesity and insulin resistance, ACS activity is lower in mitochondria but higher in ER when compared with control mice (67). Although ACS activity in these organelles is comprised of more than one isoform, more activity in ER suggests increased substrate availability for esterification. Streptozotocin-induced insulin-dependent diabetes (4 wk) and fasting for 24 h both repress ACSL6 mRNA in rat
heart, with no significant effects on the other ACSL isoforms (68). Conversely, insulin increases the mRNA levels of ACSL1, ACSL3, and ACSL6, with no effect on those of ACSL4 or ACSL5 in rat cardiomyocytes (68). Additionally, adrenocorticotropin (ACTH) increases the protein level of ACSL4 in rat adrenal gland and in adrenocortical Y1 cells after 30 min incubation and 15 min stimulation, respectively (69). In MA-10 Leydig cells, triacsin (ACSL4 inhibitor) blocks 8Br-cAMP stimulated progesterone production, indicating that ACSL4 is essential in steroidogenesis (69).

1.3.5 Regulation associated with pathological conditions

ACSL isoforms have been found to change under certain pathological conditions related to tumorgenesis. Human ACSL4 mRNA and protein levels are increased in colon adenocarcinomas, particularly colon epithelium and crypts, compared with adjacent normal tissues from the same patients (70). Upregulation of ACSL4 is also present in many colon carcinoma cell lines (70). An in vitro study with Int407 cells, an intestinal epithelial cell line, shows that the tumor promoter PMA increases ACSL4 mRNA (71). ACSL5 mRNA is also upregulated in human colorectal cancer tissues (72) and in endometrioid adenocarcinomas (73). Moreover, ACSL1 and ACSL4 are increased in terminal ileum and colon of inflammatory bowel disease patients, whereas other ACSL isoforms remain unchanged (74). Although the causality between the change of ACSL isoforms and these diseases is not established, these observations suggest that ACSL isoforms are linked to metabolic changes or demand under these pathological conditions. For example, the
upregulation of C20:4-preferring ACSL4 in colon adenocarcinoma might decrease the cellular level of unesterified C20:4 and thereby prevent apoptosis, which promotes carcinogenesis (70). The increase of ACSL1 and ACSL4 in terminal ileum and colon of inflammatory bowel disease patients might provide fatty acyl-CoAs for PL synthesis, which can serve as precursors for inflammatory mediators, or support membrane integrity and epithelial barrier function of the affected intestine (74).

In humans, a 2-Mb deletion of Xq22.3 containing ACSL4 and four other genes is linked to the AMME contiguous gene deletion syndrome, characterized by Alport syndrome, midface hypoplasia, mental retardation and elliptocytosis (75,76). Two point mutations in ACSL4 have been identified in patients with nonspecific X-linked mental retardation (54). One results in the substitution of the conserved 529Arg with serine in the FACS signature motif, the other one causes a premature stop mutation and a shortened protein without the FACS signature motif. Both mutations result in non-functional proteins because arachidonoyl-CoA (C20:4) synthetase activity decreases 88% in lymphoblastoid cells from these patients (54). The nervous system has the highest concentration of PUFA (especially C:22:6), which are primarily incorporated into the sn-2 position of PLs and are critical for normal neurodevelopment (59). Since ACSL4 prefers C20:4 and C22:5 and other PUFA, a mutation in ACSL4 might impair PL synthesis and brain development. Alternatively, mutation of ACSL4 might increase the availability of C20:4 and C22:6 and promote C20:4/C22:6-induced apoptosis and excessive loss of brain cells (59,70).
1.4 Fatty acid partitioning by acyl-CoA synthetase

The fundamental question arising from the observations that ACSL isoforms have different enzyme kinetics, unique locations and regulations is why there is more than one ACSL isoform and what the function of each is. Based on the evidence described above and below, we believe that ACSL plays a critical role in fatty acid channeling. That is, different ACSL isoforms provide fatty acyl-CoAs to specific downstream metabolic pathways, and regulation of different isoforms may change the fatty acid partitioning (4,44,66). Functionally independent acyl-CoA pools might exist which are channeled towards specific fates instead of being freely available for all downstream pathways (Scheme 1.3).

1.4.1 Sensitivity to ACSL inhibitors – differentiation of ACSL isoforms

Although ACSL isoforms have overlapping fatty acid substrates, to some extent, they can be differentiated by competitive inhibitors such as triacsin C and thiazolidinediones (TZD). Triacsin C is the most potent inhibitor among triacsins (A, B, C, and D), fungal-derived alkenyl-N-hydroxytriazenes, which inhibit ACS activity by competing with LCFA for binding to active sites (77,78). Triacsin C strongly inhibits purified ACSL1, 3, and 4, with apparent Ki values of 4 µM, 5.5 µM and 7.5 µM, respectively, but has little effect on purified ACSL5 or ACSL6 (21,51). If the hypothesis of fatty acid channeling by ACSL is true, then pathways that are linked to triacsin C sensitive ACSL isoforms would be inhibited by triacsin C. In fact, several studies showed that triacsin C selectively inhibits
Scheme 1.3.  *Proposed model of fatty acid channeling by long chain acyl-CoA synthetases (ACSL).* Exogenous LCFA released from albumin can enter cells either through diffusion or through facilitated transport. Fatty acid transport proteins include FATP family members, CD36/FAT, and in some cases, ACSL. The acyl-CoA synthetase activity of FATP and ACSL may contribute to LCFA uptake by trapping LCFA in the form of LC-CoA upon transport. Imported exogenous LCFA and endogenous LCFA derived from de novo synthesis or from lipolysis of cell lipids associate with FABP, and are transferred to ACSL isoforms. LCFA is activated by ACSL to LC-CoA, which enters further metabolism, or is bound by ACBP. ACSL isoforms located in different subcellular organelles create independent LC-CoA pools and channel LC-CoAs into specific downstream pathways, probably via protein-protein interactions with local acyltransferases or with ACBP. ACSL in the endoplasmic reticulum may provide acyl-CoA to erGPAT for TAG and PL synthesis, or to ACAT for CE synthesis. ACSL in mitochondria may provide separate LC-CoA pools to mtGPAT for TAG synthesis, or to CPT1 for β-oxidation. ACSL in peroxisome can provide LC-CoA for peroxisomal oxidation. ACAT, acyl-CoA: cholesterol acyltransferase; ACBP, acyl-CoA binding protein; ACSL, long-chain acyl-CoA synthetase; CE, cholesterol ester, CPT1, carnitine palmitoyltransferase 1; FABP, fatty acid binding protein; erGPAT, endoplasmic reticulum sn-glycerol-3-phosphate acyltransferase; mtGPAT, mitochondrial sn-glycerol-3-phosphate acyltransferase; LC-CoA, long-chain acyl-CoA; LCFA, long chain fatty acid.
only some lipid metabolic pathways. For example, when human fibroblasts are incubated with 10 µM triacsin C, oleate (C18:1) incorporation into both TAG and CE decreases 91%, while incorporation into PL is not impaired, indicating that triacsin C sensitive ACSL isoforms provide acyl-CoAs for TAG and CE synthesis but not for PL synthesis (79). Differential inhibition is also observed in hepatocytes isolated from starved or fed rats. Triacsin C (10 µM) inhibits oleate (1 mM) incorporation into TAG 70% in hepatocytes from fed rats, but it has little effect on oleate incorporation into CE, PL, or β-oxidation (80). In hepatocytes from 24-h-starved rat, triacsin C inhibits oleate (1 mM) incorporation into TAG and CE 40% and 44%, respectively, and into PL around 15% (80). Since ACSL1, 3, 4, 5, and an unidentified triacsin C sensitive mitochondrial ACSL are present in rat liver (61,64), these data suggest that in rat hepatocytes, ACSL1, 3, 4 might provide acyl-CoAs for TAG and CE synthesis, but not for β-oxidation. Different ACSL isoforms also respond differently to inhibition from TZDs. At 10 µM TZDs inhibit purified ACSL4 85-95% and purified ACSL5 less than 20%, but do not change activities of ACSL1, 3, or 6 (21,51). Since TZDs decrease TAG synthesis without affecting PL synthesis in rat primary hepatocytes (81), this highly specific inhibition of ACSL4 by T2D suggests that ACSL4 is linked to TAG synthesis. Identification of more specific inhibitors that can exclusively inhibit individual isoforms would help us to understand the mechanisms of fatty acid channeling.

1.4.2 Different functions of yeast ACSLs
At least four yeast ACSL isoforms (Faa1p-4) have been cloned and characterized, with distinct substrate preferences and functions (82). Faa1p prefers C12:0-C16:0, with C14:0 and C15:0 having the highest activities (83). Faa2p uses a wide range of fatty acids, preferring C9:0-C13:0 whereas Faa3p has a preference for C16 and C18 fatty acids with a cis-double bond at C-9-C-10 (83). Mutation experiments showed that activation of exogenous fatty acid occurs through Faa1p and Faa4p, primarily Faa1p (14). When Faa1pΔ and Faa1pΔFaa4pΔ mutant strains are incubated with exogenous oleate, intracellular oleoyl-CoA levels are reduced to < 10% of wild-type levels (14). Fatty acid incorporation into PLs are reduced dramatically in these strains and the cells cannot grow when endogenous fatty acid synthesis is inhibited, suggesting Faa1p and Faa4p mediate exogenous fatty acid uptake and metabolism (52,82). Faap2 has been identified as a peroxisomal enzyme and proposed to use endogenous medium-chain fatty acids such as C12:0 for β-oxidation in peroxisomes (84). In a yeast strain containing a mutant myristoyl-CoA:protein N-myristoyltransferase and thus having a reduced affinity for myristoyl-CoA, Faap4 is the only ACSL that is induced during the growth phase. Deletion of Faap4, but not the other three Faap isoforms, severely impairs the colony-forming potential of this yeast strain during stationary phase, indicating that Faap4 is critical in providing sufficient N-myristoylation for cellular proteins during stationary phase (85).

1.4.3 Gain-of-function and loss-of-function models

More direct evidence of fatty acid partitioning by ACSL comes from experiments that
overexpress individual ACSL isoforms. When McArdle-RH7777 rat hepatoma cells are infected with adenovirus carrying rat ACSL5 (Ad-ACSL5), overexpression of ACSL5 increases acyl-CoA synthetase specific activity 2-fold and alters fatty acid partitioning into different pathways (22). Ad-ACSL5 increases exogenous C18:1 (500 µM) incorporation into cellular DAG, TAG, and oxidation products 2-fold, 42% and 13%, respectively, without affecting synthesis of PL or CE or medium TAG (22). Overexpression of ACSL5 also increases glycerol incorporation into cellular TAG 28% but decreases its incorporation into PL 22%, suggesting that ACSL5 provides acyl-CoAs for TAG synthesis through both de novo and reacylation pathways, but away from de novo PL synthesis (22). Further, ACSL5 overexpression does not affect acetate incorporation into any of these pathways, indicating that ACSL5 exclusively partitions exogenously derived fatty acids toward TAG synthesis.

In a ACSL1 heart-specific transgenic mice line that has 11-fold increase of ACSL1 protein, heart TAG content increases 12-fold with broad acyl-chain distribution (C14 to C18) (86). These mice also display a 50% increase in choline glycerophospholipids and a 15% increase in ethanolamine glycerophospholipids, but no difference in heart CE content or β-oxidation (86). The marked accumulation of TAG increases left-ventricular mass developing at 3 weeks of age, concurrent with reduction of systolic function. These mice also display loss of cardiac myocytes and fibrosis replacement, and die prematurely with a dilated cardiomyopathy (86), suggesting that lipid accumulation from unbalanced metabolism can cause lipotoxicity in the heart, thus linking obesity and development of
cardiovascular diseases. This in vivo overexpression model supports the idea that ACSL1 facilitates exogenous fatty acid uptake and metabolism towards specific pathways, and the idea that individual ACSL might be a potential therapeutic target for diseases such as obesity and related metabolic complications.

Additionally, in a study to examine whether the ACS component of FATP1 functions in partitioning fatty acid to acyl-CoA pools for various metabolic pathways, FATP1 upregulates de novo TAG synthesis while down-regulating de novo sphingomyelin synthesis in growing 293 cells (87).

On the other hand, loss of function of specific ACSL isoforms suggests that they are not functionally exchangeable. As described in Chapter 1.3.5, mutations of ACSL4 in humans are linked to nonspecific X-linked mental retardation, despite the presence of other ACSL isoforms in brain, suggesting that the role of ACSL4 cannot be replaced. Heterozygous female mice (ACSL4 +/-) have reduced frequency of pregnancy, a high frequency of in utero embryonic death, and abnormal uterine morphology during development (88). Although a few hemizygous male mice (ACSL4 --/Y) appear normal and fertile, no homozygous female mice (ACSL4 --/-- ) are born (88), suggesting that ACSL4 deficiency can be lethal in female mice. Female ACSL4 +/- mice also have a marked increase in uterine prostaglandins such as PGE2, suggesting that ACSL4 deficiency alters C20:4 metabolism and thus, the production of eicosanoids.
With all the evidence supporting the hypothesis of ACSL in fatty acid channeling, the challenges we are faced with are to define the individual role of ACSL isoforms in lipid metabolism.

1.5 Does ACSL1 channel fatty acid towards triacylglycerol synthesis in liver?

The isoform ACSL1 has been studied most extensively among rat ACSL isoforms. Several lines of evidence suggest that ACSL1 might be associated with TAG synthesis, particularly in adipocytes. However, its function in liver has remained unclear. Studies with overexpression of ACSL1 or knockdown in liver are lacking.

1.5.1 Potential role of ACSL1 in triacylglycerol synthesis

ACSL1 is abundant in tissues with high capacity of TAG synthesis, such as liver and adipose tissue (45). In adipocytes, in addition to plasma membrane and ER, ACSL 1 is present on the surface of lipid droplets (89).

During the differentiation of 3T3-L1 preadipocytes, microsomal ACS activity increases 100-fold, together with increased activity of three other enzymes in TAG synthesis, GPAT, 1-acylglycerol-3-phosphate acyltransferase, and diacylglycerol acyltransferase (DGAT) (90). Concomitant with TAG accumulation, ACSL1 mRNA increases ~160-fold, whereas other isoforms remain unchanged (48,63), indicating that the increase of ACS activity is mainly due to the increase of ACSL1. Moreover, ACSL1 expression is increased by the lipogenic
hormone, insulin, but decreased by the lipolytic cytokine, tumor necrosis factor α (91).

ACSL1 expression is regulated in parallel with the change of TAG synthesis in animal models. In both genetic and acquired obesity, ACSL1 mRNA level and ACS activity increases in adipose tissue and liver (67,92,93). For example, in ventromedial hypothalamus – lesioned rats, a model for acquired obesity, ACSL1 mRNA increases in adipose tissue before the onset of obesity, suggesting that the rapid induction of ACSL1 and other genes contributes to fat accumulation at an early stage (94). In addition, PPARγ agonists, which promote adipogenesis, increase ACSL1 mRNA 7-fold in rat epididymal and omental adipose tissues (95). Conversely, ACSL1 mRNA decreases markedly in primary adipocytes isolated from combined epididymal and retroperitoneal fat depots of rats that have been starved for 1 d or 3 d (96).

This evidence, in addition to the observation that overexpression of ACSL1 in NIH 3T3 fibroblasts and mouse heart increases TAG synthesis, suggests that ACSL1 is linked to lipogenic pathways in these cells and tissues.

### 1.5.2 PPARα regulates ACSL1 via PPRE

The function of ACSL1 in liver, however, is more complicated and controversial. In hepatocytes, ACSL1 is mainly located in ER, the site for TAG synthesis, but is not found in mitochondria, the site for fatty acid β-oxidation (64,80). Refeeding rats with a high fat or
high sucrose diet after fasting increases hepatic ACSL1 mRNA 7-fold (45) and microsomal ACSL1 protein 89% (80), suggesting that ACSL1 might be linked to TAG synthesis in liver. However, PPARα ligands such as fenofibrate, which stimulate genes related to fatty acid β-oxidation, induce a large increase in both ACSL1 mRNA and ACS activity in liver (97,98). Regulation by PPARα is mediated by a PPAR-responsive element (PPRE) in the promoter of Acsl1 (99). In vitro studies also show that fibrates increase ACSL1 mRNA level in a dose-dependence pattern in AML-12 mouse hepatocytes and Fa 32 rat hepatocytes (95). Overexpression of hepatocyte nuclear factor 4α (HNF-4α) in human hepatoma cells increases ACSL1 and PPARα, as well as some enzymes related to fatty acid oxidation (100). Taken together, ACSL1 mRNA in liver can be upregulated under conditions when β-oxidation is increased.

1.6 Potential acute regulation of long chain acyl-CoA synthetase

1.6.1 Indirect evidence of acute regulation

As the enzyme that catalyzes the requisite initial step for cellular fatty acid metabolism, ACSL could be an important point for regulation in the control of lipid metabolism. Previous studies provide indirect evidence that ACS activity is regulated acutely. For example, microsomal ACS activity increases 2-fold in isolated rat adipocytes after incubating with insulin for only 2 minutes (101). Lipolytic factors such as norepinephrine, ACTH and glucagon decrease ACS activity concomitantly with lipolysis, whereas insulin or β-adrenoceptor blocker propranolol reverses the norepinephrine effect rapidly, resulting in
restoration of ACS activity back to control within 5 min (102). The dose-response curve for
inactivation of ACSL by noradrenaline is similar to the curve for stimulation of lipolysis
(102), indicating ACSL in adipocytes may be regulated in a manner similar to hormone
sensitive lipase (HSL). Dibutyryl cAMP decreases ACS activity by 62%, suggesting that
cAMP-protein kinase A-phosphorylation may play a role in the acute regulation (102).

Despite the evidence above suggesting that ACS activity may be acutely regulated,
another study shows that insulin has no effect on ACS activity in the perfused rat liver (103).
Moreover, the observations of acute regulation were made years before the different ACSL
isoforms were cloned. So we do not know which isoform(s) is acutely regulated, if true.
So far, little has been reported on acute regulation of individual ACSL isoforms. In one
study with rat primary adipocytes, a 15 min incubation with insulin increases ACSL specific
activity in the plasma membrane and vesicular fractions, with unchanged amount of
associated ACSL1 protein (43), indicating that acute regulation by insulin might change ACS
activity by mechanisms other than protein expression. However, since the activity of
individual ACSL isoforms was not differentiated, we do not know whether the activity
change was contributed by ACSL1 or other isoforms present in these fractions.

**1.6.2 Potential ACSL1 phosphorylation sites prediction**

The Netphos program (http://www.cbs.dtu.dk/services/NetPhos/) predicts 24 potential
phosphorylation sites on rat ACSL1, including 9 serine, 7 threonine, and 8 tyrosine residues.
Among them are two potential serine sites with high prediction scores for protein kinase A (PKA), S74 (0.823) and S411 (0.986) (Scheme 1.4). Sequence comparison between the sequence of rat ACSL1 with mouse and human ACSL1 (ClustalW Alignment http://www.ebi.ac.uk/clustalw/) shows that these two serine residues and surrounding amino acids are highly conserved. Therefore, if phosphorylation occurs on these serine residues, it is possible that the same mechanism might exist in rodents and human. Additionally, S74 is conserved in rat ACSL6, and S411 is conserved in ACSL5 and ACSL6, with identical surrounding sequences in the subfamily of ACSL1, 5, and 6, suggesting that these two serine sites are important for ACSL function.
Scheme 1.4. Potential phosphorylation sites for protein kinase A (PKA) on long chain acyl-CoA synthetase 1. The Netphos program (http://www.cbs.dtu.dk/services/NetPhos/) predicts two potential PKA phosphorylation sites in rat ACSL1. The two sites, Ser 74 and Ser 411 have high predicted scores, and are conserved among rodent and human ACSL1, with highly conserved surrounding amino acids (ClustalW alignment http://www.ebi.ac.uk/clustalw/). The scheme shows the approximate positions of the potential PKA sites, the ATP/AMP signature motif, and the FACS signature motif in rat ACSL1.

Potential PKA sites: S74 (0.823) and S411 (0.986)

Putative AMP binding domain (273/274 – 292/293) (455/456 – 463/464)

Putative FA binding domain (534-551)
CHAPTER II

FUNCTIONS OF RAT ACYL-COA SYNTHETASES IN BACTERIA

The five rat ACSL isoforms display different substrate preferences, tissue expression, sensitivity to inhibitors and subcellular locations. We hypothesized that these isoforms were functionally specific, rather than being redundant. To test this hypothesis we transformed individual ACSL isoforms into \textit{E. coli} that contain mutated endogenous ACSL (FadD). Since FadD is the only ACSL in \textit{E. coli} and functions in vectorial acylation for exogenous fatty acid (Scheme 2.1), \textit{E. coli} with a mutation in FadD cannot grow on medium with fatty acid as the sole energy source. Transformation of rat ACSL isoforms into \textit{E. coli fadD} addressed the question of whether rat ACSL isoforms differed in their ability to replace the single FadD in fatty acid transport and utilization. An isoform that could replace FadD would enable the mutated \textit{E. coli} to transport exogenous fatty acids, to incorporate fatty acids into lipid metabolism, and to grow on medium with fatty acid as the only energy source.
Scheme 2.1. *E. coli* model to study the rat ACSL isoforms. When *E. coli* are grown in medium with exogenous fatty acids, LCFA is imported by outer membrane protein FadL and then esterified by inner membrane ACSL (FadD). This so called “vectorial acylation” associates LCFA uptake with activation. Fatty acyl-CoA binds to transcription factor FadR and releases its repression on the fatty acid degradative genes (*fad* genes). Expressed enzymes for LCFA degradation uses acyl-CoA to produce acetyl-CoA for β-oxidation to provide energy (37). When *fadD* is mutated, vectorial acylation is impaired and no acyl-CoA binds FadR, which results in inhibition of *fad* genes and fatty acid oxidation. This *fadD* mutant (*fadD*) thus cannot grow with LCFA as the sole energy source. Both wild type strain and mutated strain can grow with glucose as energy source because FadD is not essential under this condition, thus serving as a positive control. We use this mutated strain as the model to study the differences of rat ACSL isoforms in their ability to replace the single FadD in fatty acid transport and utilization.
2.1 Manuscript 1: Rat long-chain acyl-CoA synthetase 5, but not 1, 2, 3, or 4 complements *Escherichia coli* fadD

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‡ ACS2 was used instead of ACSL6 before revised nomenclature for ACSL family (50)

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§ Middle initial (O.) is added for all of Lei Li’s journal publications.

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Abstract. Long-chain fatty acids are converted to acyl-CoAs by acyl-CoA synthetase (fatty acid CoA ligase: AMP forming, E.C. 6.2.1.3; ACS). *Escherichia coli* has a single ACS, FadD, which is essential for growth when fatty acids are the sole carbon and energy source. Rodents have five ACS isoforms that differ in substrate specificity, tissue expression and subcellular localization, and are believed to channel fatty acids towards distinct metabolic pathways. We expressed rat ACS isoforms 1, 2, 3, 4 and 5 in an *E. coli* strain that lacked FadD. All rat ACS isoforms were expressed in *E. coli fadD* or *fadDfadR* and had ACS specific activities that were 1.6- to 20-fold higher than the wild-type control strain expressing FadD. In the *fadD* background, the rat ACS isoforms 1, 2, 3, 4 and 5 oxidized [14C]oleate at 5 to 25% of the wild-type levels, but only ACS5 restored growth on oleate as the sole carbon source. To ensure that enzymes of β-oxidation were not limiting, assays of ACS activity, β-oxidation, fatty acid transport, and phospholipid synthesis were also examined in a *fadD fadR* strain, thereby eliminating FadR repression of the transporter FadL and the enzymes of
β-oxidation. In this strain, fatty acid transport levels were low but detectable for ACS1, 2, 3, and 4 and were nearly 50% of wild-type levels for ACS5. Despite increases in β-oxidation, only ACS5 transformants were able to grow on oleate. These studies show that although ACS isoforms 1-4 variably supported moderate transport activity, β-oxidation, and phospholipid synthesis, and although their in vitro specific activities were greater than that of chromosomally encoded FadD, they were unable to substitute functionally for FadD regarding growth. Thus, membrane composition and protein-protein interactions may be critical in reconstituting bacterial ACS function.

Introduction

Acyl-CoA synthetase (fatty acid CoA ligase: AMP forming, E.C. 6.2.1.3; ACS)\(^1\) catalyzes the conversion of long chain free fatty acids into their CoA esters, and plays an important role in fatty acid metabolism in bacteria, yeast and mammalian cells (4,66). In yeast, the ACS isoforms Faap1 and 4 are required for the utilization of exogenous fatty acids for growth and transport (52), and in NIH 3T3 cells, overexpression of mouse ACS1 increases fatty acid uptake (16).

The \textit{E. coli} ACS enzyme FadD and the fatty acid transport protein FadL are essential components of a fatty acid uptake system (37). Because the outer membrane of \textit{E. coli} contains lipopolysaccharide which constitutes a barrier for hydrophobic molecules, fatty acids are not able to cross it by diffusion, but require the transport protein FadL. Protonated
fatty acids then cross the bacterial inner membrane in a process favored by the proton
gradient and are abstracted from the membrane and concomitantly trapped within the cell as
acyl-CoA esters by the membrane-associated ACS, FadD (37). FadL-mediated transport
and the subsequent FadD-mediated formation of acyl-CoA esters is defined as vectorial
acylation, a process that allows exogenous fatty acids to be used by bacteria as their energy
source as well as a substrate for phospholipid biosynthesis. Thus, when no other carbon
source is available, exogenous fatty acids are converted to acyl-CoA esters which bind to the
transcription factor FadR and derepress the expression of the fad genes that encode proteins
responsible for fatty acid transport (FadL), activation (FadD), and β-oxidation (FadA, FadB,
FadE and FadH) (37). When alternative sources of carbon are available, bacteria synthesize
fatty acids as acyl-ACPs which are used for phospholipid synthesis but are not substrates for
β-oxidation. Thus acyl-CoA and acyl-ACP constitute independent pools of fatty acids
destined for different fates.

Contrasting with the single ACS present in bacteria, eukaryotes express multiple ACS
isoforms that are not functionally equivalent (66). Six yeast isoforms (Faap 1 – 4, Fat1p
and Fat2p) have been cloned and characterized (82). Faap1 and Faap4 are essential for the
utilization of exogenous fatty acids, since faa1Δfaa4Δ strains cannot grow when endogenous
synthesis is inhibited by the fatty acid synthase inhibitor cerulenin unless supplemented with
14:0, 16:0, or 18:1 (52). Faap2 activates endogenous medium-chain fatty acids destined for
β-oxidation in peroxisomes (84), and Faap3 also uses endogenous fatty acids, but their
specific fate is uncertain (104). Fatlp has specificity for very long chain fatty acids (105) and is required for efficient fatty acid uptake (23).

In rats and humans, five long-chain ACS isoforms\(^2\) have been cloned (4,66). These isoforms have different substrate preferences, are expressed in different tissues and are present in different subcellular locations. The expression of each of the rat isoforms is likely to be regulated independently. For example, ACS1 mRNA in different tissues is variously upregulated by PPAR\(\alpha\) and PPAR\(\gamma\) (95,106) and by insulin (107), ACS2 in brain is upregulated by PPAR\(\beta\) (62), and ACS4 mRNA is induced by ACTH and by 20:4 and down regulated by dexamethasone in steroidogenic tissues (108). These and other data suggest that rat ACS isoforms, like those of yeast, are not redundant, but rather, that they have distinct functions, possibly directing fatty acids to different metabolic pathways. In human skin fibroblasts, triacsin C, which inhibits ACS1 and 4 but not ACS5 (21), blocks de novo glycerolipid synthesis but does not affect lysophospholipid reacylation (79), suggesting that different ACS isoforms generate independent acyl-CoA pools which have specific fates. In similar experiments with rat hepatocytes, thiazolidinediones, which are inhibitors of rat ACS4 but not ACS1 or 5 (21), markedly decrease the synthesis of TAG but have less effect on \(\beta\)-oxidation (81). Female mice heterozygous for ACS4 gene inactivation have increased production of eicosanoids in uterine tissue and reduced fertility, suggesting that other isoforms cannot compensate for even a partial lack of ACS4 (88). In humans, mutations in the FACL4 gene (ACS4 homolog) that inactivate the enzyme are linked to mental retardation.
(54,109), again suggesting that the unique function of this isoform in brain cannot be replaced.

Expression of rat ACS1 rescues yeast faa1Δ faa4Δ strains and allows growth on a fermentable carbon source when cerulenin and exogenous long-chain fatty acids are present (52), indicating that a mammalian ACS can functionally substitute in a lower eukaryote. Because the long-chain ACSs may have independent functions, we wondered whether they would vary in their ability to replace the single ACS in E. coli, FadD, in fatty acid vectorial transport and the activation of exogenously provided fatty acids. To answer this question, we transformed rat ACS1-5 into E. coli fadD and measured transport, β-oxidation, lipid synthesis, and growth in the presence of oleate as the only source of carbon and energy.

**Experimental Procedures**

**Construction of recombinant pFLAG-CTC-ACS plasmids.** Plasmids pFLAG-CTC-ACS1, -ACS4, and –ACS5 express the ACS isoforms as fusion proteins with a C-terminal FLAG epitope, under the control of a tac promoter (21). In order to obtain cDNA clones of ACS2 and ACS3, rat total brain RNA was isolated using Trizol reagent (Invitrogen) and cDNAs were synthesized using M-MLV reverse transcriptase (Invitrogen). Rat ACS3 (GenBank accession number D30666) (46) was amplified by PCR using the upper primer 5’-CTAAGCTTCACATGAATAACCAGTATCTTCAACAC-3’ and the lower primer 5’-AAGTCGACTTTTCTTCCGTACATCCGCTCAATG-3’. The product included
the open reading frame flanked by the recognition sequences for HindIII and SalI restriction enzymes. Following digestion with HindIII and SalI, the amplified ACS3 cDNA was ligated into the pFLAG-CTC vector to produce pFLAG-CTC-ACS3. Rat ACS2 (GenBank accession number D10041) (49) was amplified by PCR using the upper primer 5’-ATAAGCTTAAGATGCAGACCCAGGAGATCCTG-3’ and the lower primer 5’-GTACTCGAGACATGGAGATTGAGTACAGCTCTTCT3’, and ligated into pCR-BluntII-TOPO vector (Invitrogen) according to the manufacturer instructions. The ACS2 cDNA was excised with HindIII and XhoI, and then ligated into the pFLAG-CTC vector to generate pFLAG-CTC-ACS2. The sequences of ACS2 and 3 were verified by automated DNA sequencing (UNC-CH Automated DNA Sequencing Facility).

**Strains, transformation and culture conditions.** *E. coli* strain BL21-CodonPlus(DE3)-RIL was obtained from Stratagene and will be referred to as BL21(DE3)RIL or wildtype throughout this paper. A strain containing a fadD deletion was generated by P1 transduction of BL21-(DE3)RIL with fadD::kan as described previously (12) and is referred to as BL21(DE3)RILfadD. A spontaneous fadR mutant was obtained by plating BL21(DE3)RIL on minimal medium containing decanoate (110). This mutant, BL21(DE3)RILfadR constitutively expresses the fatty acid degradative enzymes (Fad) without oleate induction. It was transduced with a P1 bacteriophage grown on BL21(DE3)RILfadD to generate the strain BL21(DE3)RILfadR fadD. Bacteria were transformed by the Hanahan protocol (111); strains transformed with pFLAG-CTC vector
without an insert were used as control. For routine culture, liquid Terrific Broth (TB) media (Invitrogen) and Luria-Bertani agar (Fisher) plates were used; 50 µg/ml carbenicillin (Life Technologies) was added to maintain the plasmids. Growth in liquid cultures was monitored by measuring optical density at 600 nm (OD$_{600}$). Frozen stocks were prepared in LB media 7% DMSO and stored at -80°C.

Transport assay. BL21(DE3)RIL$fadR fadD$ transformed with pFLAG derivatives of rat ACS 1-5 was used to inoculate an overnight culture in TB. The next morning cells were subcultured at 1:100 dilution in the same medium. When growth reached mid-log phase, protein production was induced with 1 mM IPTG for 1 h. Cells were then harvested by centrifugation (3400 x g 15 min at room temperature), rinsed once with M9 medium (112) and 0.5% Brij 58, resuspended in one-half volume of the same media containing 100 µg/ml chloramphenicol, and starved for 15 minutes at 30°C. Fatty acid transport was measured by adding 100 µM [³H]potassium oleate (final concentration). At 0, 2, 4, and 6 min aliquots were pipetted onto filter GN-6, washed twice with M9 0.5% Brij 58, air dried and counted (113).

Induction of rat ACSs and fad genes for β-oxidation and lipid synthesis experiments. Bacteria were grown overnight in 3 ml of TB media at 37°C with shaking. The next morning the bacteria were harvested by centrifugation and resuspended in Medium E with 0.01 g/l thiamine (Med EB$_1$), 25 mM potassium acetate, 1 g/l casamino acids. The cell
density was adjusted to OD$_{600}$ $\approx$ 0.1 and the bacteria were grown in 250 ml Erlenmeyer flasks at 37°C with shaking at 240 rpm. When the cultures reached mid-log phase (OD$_{600}$ $\approx$ 0.5), the expression of the recombinant rat ACS isoforms was induced with 1 mM IPTG. At the same time, in the BL21(DE3)RIL and BL21(DE3)RILfadD strains the expression from the endogenous fad genes responsible for $\beta$-oxidation was induced with 5 mM potassium oleate in 0.5% Brij58 (114). No oleate was necessary for fadR and fadR fadD strains because these fadR mutants constitutively express the proteins of the fad regulon. The cultures were allowed to grow for one hour and aliquots were used to measure lipid incorporation. To measure $\beta$-oxidation, cells were harvested by centrifugation and washed twice with Med EB$_1$, 0.5% Brij58, 1 mM IPTG with no carbon source and resuspended in Med EB$_1$, 1 mM IPTG 0.1 g/l chloramphenicol, bringing the cell concentration to OD$_{600}$ $\approx$ 1. Protein content was determined in separate aliquots. The remaining cultures were further processed to measure ACS activity and to perform immunoblot analyses.

**$\beta$-oxidation assay.** To measure $\beta$-oxidation, 2 ml aliquots of the bacterial cultures described above were incubated for one hour at 37°C with shaking at 240 rpm with 500 $\mu$M [$^{14}$C]oleic acid in 0.5 % Brij58 in rubber-stoppered vials with center wells. Then, 0.5 ml 5 N H$_2$SO$_4$ was added to the medium to promote the evolution of [$^{14}$C]CO$_2$, which was trapped in center wells filled with 0.2 ml ethanolamine:ethanol 1:1 (v/v). After an additional hour of shaking, the contents of the center wells were transferred to vials and the [$^{14}$C]CO$_2$ produced was measured by scintillation counting (115). Experiments were performed in
triplicate and results are presented as mean ± S.E. of a representative experiment, which was repeated two to six times for BL21(DE3)RIL wildtype and fadD strains and twice with fadR and fadR fadD strains. Differences were analyzed by Student’s t test.

**Lipid synthesis.** Bacteria were grown to midlog phase and the expression of rat ACS isoforms was induced for 1 h as described above. Then 1 ml of culture was incubated with 20 µM of [1-14C]oleate (1 µCi) (final concentration) for 30 min at 37°C. Cultures were then chilled on ice and washed three times with PBS buffer containing 0.5% Brij58. Lipids were extracted (116), and [14C]oleate incorporation was measured by scintillation counting. Incorporation into phospholipid species was determined by thin layer chromatography in CHCl3/ethanol/H2O/triethylamine (30/35/7/35; v/v) using authentic standards (117).

**Preparation of bacterial lysate.** Bacterial cultures described above were harvested by centrifugation for 10 minutes at 5000 rpm in a Sorval SA-600 rotor at 4°C, and resuspended in 10 mM HEPES, pH 7.8, 0.5 mM EDTA buffer. The bacteria were lysed by sonication on ice, with six 10 seconds bursts and 10 second rest intervals using a heat system-ultrasonic cell disruptor sonicator (Heat systems-Ultrasonics) at setting 4. Lysate aliquots were stored at -80°C until used for enzyme assay.

**ACS activity assay.** ACS activity was measured using 17 to 80 µg of bacterial lysate. Assays contained 175 mM Tris-HCl pH 7.4, 8 mM MgCl2, 5 mM DTT, 10 mM ATP, 0.25
mM CoA and 50 \( \mu \text{M} [^{14}\text{C}]\text{palmitic acid} \) (New England Nuclear) in 0.5 mM Triton X-100, 0.01 mM EDTA in a total volume of 200 \( \mu \text{l} \). Assays were performed at 37°C for 5 min with shaking. The reaction was started by adding lysate protein and terminated by adding 1 ml Dole’s reagent (isopropanol:heptane:1 M \( \text{H}_2\text{SO}_4 \) 80:20:2). The unreacted free fatty acid was removed with two 2-ml heptane washes, and the labeled acyl-CoA produced by the reaction was measured by scintillation counting (118). The assays measured initial rates.

**Immunoblot analysis.** Proteins from the bacterial lysate (1 or 2 \( \mu \text{g} \)) were resolved on a 10 \% polyacrylamide gel containing 1\% SDS and transferred onto polyvinylidene difluoride membranes (BioRad). Immunoreactive bands were detected by incubating the membranes with anti-FLAG M2 monoclonal antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce) and SuperSignal West Pico Chemiluminescent Reagent (Pierce).

**Growth complementation of fadD deletion.** Bacteria were transformed with plasmids containing different isoforms of rat ACS or no insert. These were examined for their ability to grow on oleate as the sole energy and carbon source. Transformants were streaked on Med EB\(_1\) agar plates supplemented with 50 \( \mu \text{g/ml carbenicillin}, 5 \text{mM potassium oleate solubilized in 0.5 \% Brij 58 (final concentration)}, \) and different concentrations of IPTG (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 \( \text{mM} \)). Control plates were supplemented with 25 mM glucose and the appropriate concentration of IPTG. The plates were incubated at 37°C and growth was checked daily for seven days.
Other methods. Protein content was measured using bovine serum albumin (BSA) as the standard (119).

RESULTS

Rat ACS isoforms 1-5 are expressed and active in BL21(DE3)RILfadD. In order to determine whether rat ACS isoforms can functionally replace FadD, the sole ACS enzyme in bacteria, the coding region of fadD was deleted in BL21(DE3)RIL to generate strain BL21(DE3)RILfadD. The fadD strain had ACS activity levels indistinguishable from background and was unable to grow when oleate was the sole energy and carbon source (Fig. 2.2, Table 2.1). BL21(DE3)RILfadD was transformed with the expression vector pFLAG-CTC-ACS1, 2, 3, 4, or 5 or with the empty vector as a control. The rat ACS isoforms were each expressed as a fusion protein with a C-terminal FLAG epitope. The FLAG epitope does not interfere with ACS activity or kinetic properties (21).

All five ACS isoforms were expressed in BL21(DE3)RILfadD, with ACS1 and 4 exhibiting the greatest expression and ACS5 the least (Fig. 2.1A). All isoforms exhibited ACS activity (Fig. 2.2A). In each case, the specific activity was at least 1.6-fold higher (ACS5) and as much as 20-fold higher (ACS4) than the specific activity of the bacterial enzyme FadD in the wildtype control strain containing the empty vector. Further, the correlation was poor between expressed protein and enzyme activity. Although the expression of ACS4 protein was not substantially different from that of ACS1, ACS4 specific
Table 2.1. *Only rat ACS5 complements absence of FadD.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>EB₁ Media</th>
<th>25 mM Glucose</th>
<th>5 mM oleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)RIL</td>
<td>Empty</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Empty</td>
<td>+++</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>ACS1</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ACS2</td>
<td>+++</td>
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<td></td>
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<tr>
<td></td>
<td>ACS5</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

| fadD            | Empty  | +++       |+++          |
|                 | Empty  | +++       | -           |
|                 | ACS1   | +++       | -           |
|                 | ACS2   | +++       | -           |
|                 | ACS3   | +++       | -           |
|                 | ACS4   | +++       | -           |
|                 | ACS5   | +++       | ++           |

| fadR            | Empty  | +++       |+++          |
|                 | Empty  | +++       | -           |
|                 | ACS1   | +++       | -           |
|                 | ACS2   | +++       | -           |
|                 | ACS3   | +++       | -           |
|                 | ACS4   | +++       | -           |
|                 | ACS5   | +++       | ++           |

BL21(DE3)RIL and BL21(DE3)RILfadR containing the empty vector (EV) or BL21(DE3)RILfadD and fadR fadD containing vectors expressing ACS1, 2, 3, 4 or 5 were streaked on medium EB₁ agar plates supplemented with 5 mM oleate in 0.5 % Brij58 as the sole energy and carbon source. EB₁ agar plates containing 25 mM glucose were used as controls. The plates were incubated at 37°C and checked daily. This experiment was repeated 6 to 11 times with both carbon sources and IPTG concentrations of 0.005 to 0.5 mM. +++*, WT growth; ++, intermediate growth; -, no growth.
Figure 2.1. **Rat ACS isoforms 1-5 are expressed in E. coli fadD.** BL21(DE3)RILfadD (A) or BL21(DE3)RILfadR fadD (B) transformed with different ACS isoforms was grown as described under Experimental Procedures. Expression was induced for 1 h with 1 mM IPTG. Lysates were prepared and 1 µg (A) or 2 µg (B) of protein was resolved by 10 % SDS-PAGE and subjected to immunoblot analysis using M2 anti-FLAG antibody. Exposure time was 2 seconds except for ACS5 which was exposed for 20 sec (A) or 30 min (B). The experiment was repeated twice. Representative results are shown.

Figure 2.2. **Rat ACS isoforms 1-5 expressed in E. coli fad D are active.** BL21(DE3)RILfadD (A) or BL21(DE3)RILfadR fadD (B) transformed with the empty vector (EV) or in which rat ACS expression was induced for 1 h with 1 mM IPTG was used to prepare lysates as described in the Experimental Procedures. ACS specific activity was assayed with 14 to 70 µg protein. BL21(DE3)RIL (WT) (A) and BL21(DE3)RILfadR (B) strains transformed with the EV were used as controls. Data are presented as means ±SE (n ≥ 3).
activity was 8-fold higher than that of ACS1, perhaps because ACS4 is a peripheral membrane protein like FadD. In contrast, ACS1 and ACS5 (and probably ACS2 and 3) are integral membrane proteins whose activity might be negatively affected by the composition of the bacterial membrane, which differs considerably from the phospholipid composition of mammalian membranes (120).

**Rat ACS isoforms 1-5 activate exogenous fatty acids for β-oxidation.** When carbohydrate is not available, *E. coli* can import and activate exogenous fatty acids to acyl-CoA esters that are then oxidized to provide energy. BL21(DE3)RIL*fadD* expressing each of the rat ACS isoforms was studied to determine whether these rat ACS isoforms would be able to supply fatty acyl-CoA for β-oxidation in the absence of FadD (Fig. 2.3A). As expected, BL21(DE3)RIL*fadD* transformed with the empty vector was unable to oxidize exogenous oleate. However, transformation with rat ACS isoforms 1, 2, 3, 4, or 5 allowed some β-oxidation to occur, indicating that each of the rat ACS isoforms partially replaced FadD for this function. There was no correlation between in vitro specific activities of the ACS isoform and the rates of in vivo β-oxidation. Despite ACS activities that were 1.6-fold to 20-fold higher than that of the control BL21(DE3)RIL which contains active FadD (Fig. 2.2), the relative rates of β-oxidation ([14C]CO₂ produced/ACS activity) were 0.2 to 7.5% of those observed in the wild-type strain (Fig. 2.3B). Thus, although the ACS isoforms were able to catalyze the formation of acyl-CoAs in vitro, they either did not form acyl-CoAs in
Figure 2.3. Rat ACS isoforms 1-5 partially restore β-oxidation in E. coli fad D. BL21(DE3)RIL fadD (A, B) or BL21(DE3)RIL fadR fadD (C, D) were transformed with the empty vector (EV) or with the ACS isoforms. BL21(DE3)RIL WT (A, B) and BL21(DE3)RIL fadR (C, D) strains transformed with the EV were used as controls. ACS expression was induced for 1 h with 1 mM IPTG. A and B: β-oxidation was measured using 0.5 mM [14C]oleic acid for 1 h at 37°C as described in Experimental Procedures. Experiments were performed in triplicate. The mean ± S.E. of a representative experiment, repeated two to six times, is plotted. C and D: Relative β-oxidation was calculated ([14C]CO₂ produced/ACS activity) and normalized (WT and fadR controls set at 1).
vivo, the acyl-CoAs formed were not accessible to the β-oxidation enzymes, or the activities of the β-oxidation enzymes were limiting due to the presence of FadR.

β-oxidation by *E. coli* requires expression of the *fad* genes encoding proteins that mediate transport (FadL), activation (FadD), and β-oxidation of exogenous fatty acids (FadA, FadB, FadE, and FadH) (114,121). When carbohydrate is present, the transcription factor FadR represses the expression of these proteins. If long-chain fatty acids are provided as the sole carbon and energy source in the culture media, however, the resulting acyl-CoAs produced by the activity of FadD bind to FadR and regulate FadR activity (110) by promoting the dissociation of FadR from the promoter region of the genes of the *fad* regulon, thereby inducing their expression (122). If the rat ACSs were not as efficient as FadD in providing the long-chain acyl-CoA ligand for FadR, the *fad* genes might be expressed at low levels and limit β-oxidation. To rule out this possibility, we generated BL21(DE3)RIL*fadR* and *fadR fadD* strains. These strains have a mutation in the *fadR* gene that leads to constitutive expression of FadL and the β-oxidation enzymes, independent of the presence of acyl-CoA. Expression of the ACS isoforms and their activities in the *fadD fadR* strain were similar to that shown for BL21(DE3)RIL*fadD* (Figs. 2.1B, 2.2B). As expected, β-oxidation in BL21(DE3)RIL*fadR* was 3.7-fold higher than observed in the wildtype strain (Compare the first bars in Fig. 2.3A and 2.3C). However, the rescue of *fadR fadD* by the five rat ACS isoforms was still only partial (Fig. 2.3C); values for relative β-oxidation remained low for ACS 1-4 at 0.6 to 16% of the positive control BL21(DE3)RIL*fadR* (Fig 2.3D). In contrast,
relative β-oxidation for $fadDfadR$ bacteria expressing ACS5 was 55% of the $fadR$ control. The lower efficiency of rat ACS isoforms 1-4 was not due to low expression of FadL or limiting enzymes of β-oxidation. In both the $fadD$ (Fig. 2.3B) and $fadD fadR$ (Fig. 2.3D) strains, ACS5 had the highest rate of β-oxidation relative to expression of activity.

**Rat ACS isoforms 1-5 partially restore fatty acid transport in BL21(DE3)RIL$fadR fadD$.**

FadD and FadL comprise the *E. coli* fatty acid import system that pairs fatty acid transport and acylation (37). To determine whether rat ACS isoforms can replace FadD functionally in this import system, oleate transport was measured in BL21(DE3)RIL$fadR fadD$ expressing each of the five rat ACS isoforms. Transport was defined as $[^3H]$-oleate incorporation into cells in a traditional filtration assay (see Experimental Procedures). While transport cannot be distinguished from metabolic use in this assay, experiments were performed during a 4 minute period in which the data points are linear and dependent upon both the transporter FadL and an acyl-CoA synthetase, generally bacterial FadD. In the absence of FadD, $[^3H]$-oleate import into cells was minimal, reflecting the importance of ACS for this process (Fig. 2.4A). Expression of rat ACS isoforms increased fatty acid transport with respect to the negative control harboring the empty vector (Fig. 2.4A). Compared with the transport activity of wild type *E. coli* (1000 pmol/min/mg protein) (113), the relative transport ranged from 0.02% (ACS4) to 12% (ACS5) (Fig. 2.4B). Thus, relative to specific activity, the amount of transport was greatest for ACS5. Although ACS3 protein expression was also low (Fig. 2.1B), its ability to promote fatty acid transport was equivalent to that of ACS1 and
2; thus, toxicity from protein overexpression itself cannot explain these results.

**Rat ACS isoforms 1-5 activate fatty acids for lipid synthesis.** Vectorial acylation by FadL and FadD allows *E. coli* to use exogenous fatty acids for phospholipid synthesis (121). To study the ability of the rat ACS isoforms to substitute for FadD in this process, we incubated BL21(DE3)RIL*fadR* and BL21(DE3)RIL*fadD*fadR* with [14C]oleate and measured label incorporation into lipid. When FadD was absent, the incorporation of exogenous fatty acid into phospholipid was negligible (Fig. 2.5A). The residual incorporation probably reflects [14C]oleate incorporation into PE by the acyl-ACP synthetase/2-acyl-GPE acyltransferase system (123). Expression of the rat ACS isoforms significantly increased the incorporation of [14C]oleate into phospholipid compared to the vector control (Fig. 2.5A), reaching 10 to 41 % of the values for the control strain BL21(DE3)RIL*fadR* during the 30 min incubation. When expressed relative to activity, ACS5 substituted most effectively (85%) and ACS4 least (1%) (Fig. 2.5B). The percent of [14C]oleate incorporated into different phospholipid species was similar for each of the ACS isoforms (data not shown). Thus, all five rat ACS isoforms could partially replace FadD in providing acyl-CoA for phospholipid synthesis, although each was less efficient than the endogenous bacterial enzyme.

**Only ACS5 supports the growth of *E. coli* fadD mutants on fatty acid.** Growth of *E. coli* when long-chain fatty acids are its sole energy and carbon source requires FadD to activate fatty acids used for both β-oxidation and for the synthesis of membrane
Figure 2.4. Rat ACS isoforms 1-5 partially restore fatty acid transport in E. coli fadR fadD. A: BL21(DE3)RIL fadR fadD transformed with rat ACS isoforms 1-5 or empty vector (EV) were grown to mid-log phase in LB media. Expression of rat ACSs was induced with 1 mM IPTG for 1 h and fatty acid transport was measured by adding 100 µM [3H]oleate as described in Experimental Procedures. Results represent averages of two independent experiments, each performed in triplicate and plotted as the mean ± S.E. B: Relative transport was calculated ([3H]oleate transported/ACS activity) and normalized (fadR control set at 1).

Figure 2.5. Lipid synthesis is partially restored by rat ACS isoforms 1-5. A: BL21(DE3)RIL fadR was transformed with empty vector (EV). BL21(DE3)RIL fadR fadD was transformed with EV or with ACS isoforms. ACS expression was induced for 1 h with 1 mM IPTG. Incorporation of fatty acid into phospholipid was then measured with 20 µM [14C]oleic acid for 30 min at 37°C as described in Experimental Procedures. Experiments were performed in triplicate. The mean ± S.E. of a representative experiment, repeated two times, is plotted. B: Relative oleate incorporation into phospholipids was calculated ([14C]oleate incorporated/ACS activity) and normalized (fadR control set at 1).
phospholipids. To determine whether the low rates of transport, $\beta$-oxidation and phospholipid synthesis observed with the rat ACSs were sufficient for growth, bacteria were streaked on medium EB$_1$ agar plates supplemented with 5 mM oleate in 0.5 \% Brij58. Plates containing 25 mM glucose were used as controls. As expected, BL21(DE3)RILfadD transformed with the empty vector was unable to grow on oleate (Table 2.1). BL21(DE3)RILfadD transformed with rat ACS5 grew on oleate, although growth was slower than for the wildtype strain BL21(DE3)RIL. Bacteria lacking fadD but expressing ACS1, 2, 3, or 4, however, did not grow on EB$_1$ plus oleate. BL21(DE3)RILfadD transformed with either empty vector or the rat ACS isoforms grew in media that contained glucose, indicating that lack of growth on oleate was due to the unavailability of a usable carbon and energy source. These experiments were repeated using BL21(DE3)RILfadR and BL21(DE3)RILfadD fadR with comparable results (Table 2.1).

**Discussion**

It has become increasingly apparent that the presence of multiple homologs of an enzyme may permit them to perform non-overlapping functions and that the ability of a specific mammalian enzyme involved in lipid biosynthesis to complement a defective bacterial or yeast gene may provide critical clues about the function or active site of the mammalian ortholog. For example, complementation of the yeast $faa1\Delta faa4\Delta$ strain by rat ACS1 showed the ability of this ACS isoform to activate exogenously provided 14:0 and 16:0 for incorporation into neutral lipid and phospholipid (52). Further, the triacsin C
sensitivity of both Faa4p and ACS1 suggested functional analogy. Therefore, we attempted to determine whether any or all of the rat ACS isoforms could complement *E. coli* FadD. FadD contains the AMP/ATP consensus binding site and the putative fatty acid binding site (55) present in the luciferase/ACS superfamily and its amino acids show considerable similarity to those of the rat ACSs. Clustal W (http://www.ebi.ac.uk/clustalw/) analysis shows that the rat ACS isoforms most similar to FadD are 1, 2, and 5 with 143, 132, and 131 identical amino acids, respectively, and that ACS3 and 4 are least similar with only 115 and 119 identical amino acids, respectively. LALIGN analysis (124) showed no regions of similarity that were uniquely specific to ACS5 and FadD, nor did ACS3, which was functionally the least active, have unique differences with FadD.

Except for ACS4 (65), the rat ACSs are, or are predicted to be, integral membrane proteins that have active sites facing the cytosol (125). Like FadD, the ACSs use a broad range of fatty acid substrates from 10 to 22 carbons, but they are differentially inhibited by triacsin C and thiazolidinediones and are believed to link acyl-CoAs to different and distinct fates within mammalian cells (66). For example, rat liver ACS1 and 4 are present on endoplasmic reticulum and the related mitochondrial associated membrane (64), and in NIH 3T3 cells ACS1 is located at the plasma membrane (16). In addition, the fungal metabolite triacsin C inhibits recombinant ACS1 and 4 (21), and in human fibroblasts and HepG2 human hepatoma cells, triacsin inhibits triacylglycerol synthesis and de novo synthesis of phospholipids from glycerol-3-phosphate (79,126). Taken together, these data suggest that
ACS1 and 4 provide acyl-CoAs for glycerolipid biosynthesis. Conversely, ACS5 is present on mitochondrial membranes and is not inhibited by triacsin C (21,64), suggesting that ACS5 might play a role in β-oxidation. ACS2 and 3 have not been extensively studied.

Bacteria synthesize fatty acids as acyl-ACPs that are used primarily for the biogenesis of phospholipids, but cannot be used as substrates for β-oxidation. In contrast, exogenous fatty acids are converted by FadD to acyl-CoAs, which are primarily substrates for β-oxidation and, to a minor extent, can be used for phospholipid synthesis (2% activity of acyl-ACP pathway). Because mammalian ACS isoforms appear to be linked to specific pathways that use acyl-CoAs, we wondered whether they would vary in their ability to substitute for FadD in providing acyl-CoAs for β-oxidation and phospholipid synthesis.

We found that rat ACS5, but not ACS1, 2, 3, or 4, was able to complement E. coli fadD functionally to allow growth on oleate as the sole carbon source. Whereas all the rat ACS isoforms exhibited some ability to allow fatty acid transport, β-oxidation, and phospholipid synthesis, each of these actions was minimal for ACS1, 2, 3, or 4. It should be noted that fatty acid transport, phospholipid synthesis and β-oxidation were measured in assays of 2-6, 30, and 60 min, respectively. In contrast, complementation for growth requires sustained energy production and phospholipid synthesis from exogenous fatty acids sufficient for bacterial doubling over about 70-140 generations (24-48 h). The differences we observed are particularly striking when viewed relative to ACS activity. The most divergent pair,
ACS4 and ACS5 showed large differences in β-oxidation, oleate transport, and oleate incorporation into phospholipids relative to activity.

Our data are limited by differences in ACS protein expression and relative differences in enzyme activity. It is unclear why expression was excellent for ACS1, 2, 3, and 4 and poor for ACS5 in both bacterial strains, and why specific activity relative to expressed protein was excellent for ACS5, moderate for ACS4, and poor for ACS1, 2, and 3.

Why then were four of the rat ACS isoforms functionally ineffective in *E. coli*? It may be that FadD normally interacts with other proteins. These could be related to fatty acid transport across the bacterial membrane or in pathways of β-oxidation or glycerolipid synthesis. Such protein-protein interactions have not been reported in *E. coli* lipid metabolic pathways, although it has been proposed that mammalian cells channel acyl-CoAs towards distinct fates (66). Although FadD could interact directly with bacterial enzymes of β-oxidation, such interactions would not be possible for the mammalian ACSs, whose active sites face the cytosol (125) rather than the site of fatty acid oxidation within peroxisomes or the mitochondrial matrix.

On the other hand, FadD is thought to “abstract” fatty acids from the inner membrane after their transport through the cell wall by FadL. Unlike most of the rat ACSs, FadD is a soluble protein that is activated upon movement to the bacterial membrane (10). The
mammalian ACSs, which would be present in the bacterial membrane (21), should have been able to function similarly in a transport assay. Except for ACS4, the ACSs are integral membrane proteins, and ACS1, 2, and 3 would interact in E. coli in a phospholipid milieu very different from the one they would encounter in a mammalian cell. ACS5, however, is positioned on the outer mitochondrial membrane which, like bacterial membranes, contains cardiolipin (127). No studies have been performed on the possible cardiolipin dependence of ACS5. If, however, the E. coli membrane composition altered the transmembrane or activating domains of the ACSs such that catalysis was poor, a false estimate of activity may have been obtained during an in vitro measurement in the presence of detergent. Alternatively, the mammalian ACSs might require post-translational modifications that are critical for their in vivo function.

Another possibility is that over-expression of the rat ACSs was toxic to the bacteria and that ACS5 supported growth only because less ACS protein was expressed. Certainly the rates of growth on oleate were markedly decreased in the bacteria expressing the rat enzymes. However, if the amount of protein expressed was the problem, growth should also have been poor on minimal glucose plates containing the inducer IPTG. The high expression of exogenous proteins in bacteria growing in a minimal media might also have diverted limited substrates like amino acids and ATP, thereby compromising the synthesis of other proteins that decrease the growth rate, but growth remained poor, even when low IPTG concentrations were used to decrease the amount of protein expressed. Further, when we repeated the
studies of the ACSs in a FadR mutant to ensure that FadL and enzymes of β-oxidation were not limiting and that ATP and amino acids were not being diverted, growth did not improve despite the expected 3.7-fold increase in β-oxidation.

In complementation experiments with both the BL21(DE3)RIL\textit{fadD} and \textit{fadD fadR} strains, ACS5 consistently supported growth in oleate, whereas ACS isoforms 1-4 were unable to substitute for a lack of FadD. These studies show that although ACS isoforms 1-4 variably exhibited moderate transport activity, β-oxidation, and phospholipid synthesis, and although their in vivo specific activities were greater than that of FadD, they were unable to substitute functionally for FadD regarding growth. ACS5 consistently showed the highest relative rates of β-oxidation, fatty acid transport, and oleate incorporation into phospholipids. Thus, the membrane composition and protein-protein interactions may be critical in reconstituting bacterial ACS function.
CHAPTER III
THE ROLE OF RAT ACYL-COA SYNTHETASE 1 IN FATTY ACID CHANNELING IN RAT PRIMARY HEPATOCYTES

The work presented in this chapter focuses on the role of rat ACSL1 in fatty acid channeling in hepatocytes. Although studies suggest that ACSL1 is important for TAG synthesis in adipocytes and fibroblasts, conflicting data exist concerning the function of ACSL1 in liver. Upregulation of ACSL1 in liver is shown under both lipogenic and oxidative conditions. All the previous studies focused on the subcellular locations, inhibitor effects, expression of mRNA or protein of ACSL1, but no study has been conducted on the role of ACSL1 by directly measuring metabolism change imposed by ACSL1. In the current work, we overexpressed rat ACSL1 in rat primary hepatocytes and examined its effects on fatty acid channeling (Scheme 3.1). Consistent with our hypothesis that ACSL provides fatty acyl-CoAs to specific downstream pathways, our data showed that adenovirus-mediated overexpression of rat ACSL1 channeled oleate toward some specific pathways and away from others. In contrast to overexpression studies of ACSL1 in other cell types, overexpressed ACSL1 in hepatocytes increased oleate incorporation into DAG and PL, but not TAG, suggesting that the function of ACSL1 might be tissue-specific.
Scheme 3.1.  Experiment outline of rat ACSL1 overexpression on lipid metabolism. The effects of overexpressed ACSL1 is examined on A) 3 h substrates incorporation and on B) prelabeled substrates recycling by pulse-chase experiments. Rat primary hepatocytes were infected with adenovirus carrying either ACSL1 or GFP (control) and labeled with \([^{14}C]\)oleate, \([^{3}H]\)glycerol, or \([^{14}C]\)acetate for 3 h. Lipids were extracted from cell and medium samples, and oxidation products were collected. Labeled lipids were quantified after separating by thin layer chromatography. For recycling study in pulse-chase experiment, radio label was removed after 3 h labeling, turnover of cellular lipids was examined by measuring the remaining label in different lipids species after a 14 h chase.
3.1 Manuscript 1: Overexpression of rat long chain acyl-CoA synthetase 1 alters fatty acid metabolism in rat primary Hepatocytes

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**Abstract.** Long chain acyl-CoA synthetases (ACSL) activate fatty acids (FA) and provide substrates for both anabolic and catabolic pathways. We have hypothesized that each of the five ACSL isoforms partitions fatty acid (FA) towards specific downstream pathways. ACSL1 mRNA is increased in cells under both lipogenic and oxidative conditions. To elucidate the role of ACSL1 in hepatic lipid metabolism, we overexpressed an ACSL1 adenovirus construct (Ad-ACSL1) in rat primary hepatocytes. Ad-ACSL1, located on the endoplasmic reticulum but not on mitochondria or plasma membrane, increased ACS specific activity 3.7-fold 24 h after infection. With 100 or 750 µM [1-14C]oleate, Ad-ACSL1 increased oleate incorporation into diacylglycerol and phospholipids, particularly phosphatidylethanolamine and phosphatidylinositol, and decreased incorporation into cholesterol esters and secreted triacylglycerol. Ad-ACSL1 did not alter oleate incorporation into triacylglycerol, β-oxidation products, or the total amount of FA metabolized. To examine the effects of Ad-ACSL1 on lipid turnover, we performed pulse-chase experiments. In Ad-ACSL1 cells, more labeled triacylglycerol and phospholipid, but less labeled diacylglycerol remained, suggesting that ACSL1 increased reacylation of hydrolyzed oleate derived from triacylglycerol and diacylglycerol. In addition, less hydrolyzed oleate was
used for cholesterol ester synthesis and β-oxidation. The increase in [1,2,3-\(^3\)H]glycerol incorporation into diacylglycerol and phospholipid was similar to the increase with \([^{14}\text{C}]\text{oleate}\) labeling. Labeling Ad-ACSL1 cells with \([^{14}\text{C}]\text{acetate}\) increased triacylglycerol synthesis, but did not channel endogenous FA away from cholesterol ester synthesis. Thus, consistent with the hypothesis that individual ACSLs partition FA, Ad-ACSL1 increased FA reacylation and channeled FA towards diacylglycerol and phospholipid synthesis and away from cholesterol ester synthesis.

INTRODUCTION

Long-chain acyl-CoA synthetases (ACSL) catalyze the first step in FA metabolism by converting long-chain FA into acyl-CoA thioesters. Acyl-CoAs enter both anabolic and catabolic pathways (4), and the disturbance of these pathways is linked to disorders such as hepatic steatosis, hyperlipidemia, and insulin resistance. Five ACSL isoforms, each the product of a separate gene, have been cloned and characterized in mammals (44,50). Even though individual ACSL isoforms have different substrate preferences, enzyme kinetics, and cellular and subcellular locations, and are regulated uniquely (21,51,66), the significance of this diversity is unknown. We hypothesized that instead of being redundant, individual ACSL isoforms might channel FA into distinct metabolic pathways.

Evidence for the importance of ACSL in FA channeling comes from studies with triacsin C, an inhibitor of recombinant ACSL1, 3 and 4, but not ACSL5 or 6 (21,51). For example,
in hepatocytes in which ACSL1, 3, 4 and 5 are abundant, triacsin C inhibits TAG synthesis 70% but inhibits oleate incorporation into phospholipids and β-oxidation only 34% (80). More direct evidence comes from studies that overexpress individual ACSL isoforms. In ACSL1 heart-specific transgenic mice, TAG and PL accumulate in heart muscle in the absence of changes in CE or β-oxidation (86), and when ACSL5 is overexpressed in rat hepatoma McArdle-RH777 cells, it partitions exogenously derived FA towards TAG synthesis and storage, but not towards PL or CE synthesis (22). However, the exact function of other ACSL isoforms in liver remains largely unexplored, in part because we lack inhibitors that can exclusively inhibit one isoform without affecting others.

Although studies suggest that ACSL1 is important for TAG synthesis in adipocytes and fibroblasts (66,128), conflicting data exist concerning the function of ACSL1 in liver. Supporting a role for ACSL1 in TAG synthesis is its location in the ER and mitochondria-associated membrane, which are sites of TAG synthesis, and its absence from mitochondria, the major site of FA β-oxidation (64). Further, ACSL1 mRNA is induced when previously fasted rats are refed with a high fat or high sucrose diet that favors lipogenesis (45,129), and hepatic ACS specific activity and ACSL1 mRNA is enhanced in obese and hypertriglyceridemic rats that have fatty livers (92,93). On the other hand, in support of a role for ACSL1 in providing FA for β-oxidation are data showing that PPARα agonists, which upregulate genes for both FA β-oxidation and de novo FA synthesis (130), increase total ACS activity in rat liver (97) and ACSL1 mRNA expression in rat liver (97)
and in the rat liver-derived cell line AML-12 (95). This upregulated transcription is mediated by a PPAR-responsive element in the promoter of the Acsl1 gene (99).

To elucidate the specific role of ACSL1 in hepatic FA metabolism, we overexpressed rat ACSL1 in rat primary hepatocytes. We hypothesized that ACSL1 would channel FA towards some specific lipid metabolic pathways and away from others.

**EXPERIMENTAL PROCEDURES**

**Materials.** DNA restriction endonucleases and ligase for recombinant adenovirus construction were from New England Biolabs. HEK-293 and CHO cells were from the American Type Culture Collection. MEM, nonessential amino acids (NEAA), FBS and tissue culture dishes were from GIBCO-BRL Life Technologies. Rat-tail collagen I was from Collaborative Biomedical Products. Silica gel G plates were from Whatman (cat. 4865-821). [1-\(^{14}\)C]acetate, [1,2,3-\(^{3}\)H]glycerol and [1-\(^{14}\)C]oleate were obtained from PerkinElmer Life Sciences and [\(^{14}\)C]palmitate was from New England Nuclear. Lipid standards were from Sigma and Avanti Polar Lipids. Polyacrylamide stock was from National Diagnostics. Lab-Tek™ II Chamber Slides™ were from NUNC. RNeasy kit was from Qiagen. Chemicals were from Sigma-Aldrich unless otherwise indicated.

**Construction of pACCMV-ACSL1FLAG adenovirus.** A full-length rat ACSL1 cDNA with a C-terminal FLAG epitope (DYKDDDDK) was subcloned from a previously
constructed pFLAG-CTC plasmid (21) into a shuttle vector, pACCMVpLpA at the Bam HI and Sal I sites. Expression of the inserted ACSL1FLAG cDNA is driven by the cytomegalovirus promoter. The inserted ACSL1FLAG in the pACCMV-ACSL1FLAG construct was verified by restriction enzyme analysis and confirmed by DNA sequencing at the UNC DNA sequencing facility. Expression and activity of the pACCMV-ACSL1FLAG construct were confirmed by transient transfection into CHO cells for 24 h, followed by ACS activity assay and anti-FLAG Western blot. The pACCMV-ACSL1FLAG construct was cotransfected with an adenoviral DNA, pJM17, into HEK293 cells for homologous recombination to form recombinant adenovirus carrying ACSL1FLAG cDNA (Ad-ACSL1) (131). After plaque purification, virions were further purified and amplified by the UNC Vector Core Facility. A virus containing a green fluorescent protein (GFP) gene under control of the cytomegalovirus promoter (Ad-GFP) was used for control infections (22).

**Hepatocyte isolation and adenovirus infection.** Animal protocols were approved by the UNC Institutional Animal Care and Use Committee. Male Wistar rats (250-300 g) were housed in a 12:12-h light-dark cycle and were allowed free access to food (Prolab® Rat/Mouse/Hamster 3000 diet, Labdiet) before hepatocyte isolation. Primary hepatocytes were isolated by collagenase perfusion by the UNC Cellular Metabolism and Transport Core. Cell viability, determined by trypan blue exclusion, exceeded 90%. Hepatocytes were seeded at a density of $1.5 \times 10^6$ cells per 60 mm or $4.5 \times 10^6$ cells per 100 mm collagen-coated dish in MEM supplemented with 10% FBS (v/v), 50 U/ml penicillin, and 50
mg/ml streptomycin (132). After cells attached (4-5 h), recombinant adenoviruses (Ad-GFP or Ad-ACSL1) were added for 2 h at 37°C in serum-free MEM. Infection medium was removed and replaced by MEM containing 10% FBS, 10 nM dexamethasone, and 0.1 mM NEAA (MEM-DA).

For dose-dependent expression, hepatocytes were infected with Ad-ACSL1 at multiplicities of infection (MOIs) of 5, 10, 20 or 50 for 2 h. Uninfected cells or Ad-GFP infected cells (20 MOI) served as controls. After 18 h, cells were washed with cold PBS and homogenates were collected as described below. For the time-course, hepatocytes were infected with Ad-GFP or Ad-ACSL1 at an MOI of 20. Homogenates were collected after 12, 18, 24, 26, or 36 h of incubation for Ad-ACSL1 infected cells and after 18 h of incubation for Ad-GFP infected cells.

**Cell labeling and lipid extraction and analysis.** Twenty-one h after adenoviral infection (Ad-GFP or Ad-ACSL1) at 20 MOI, hepatocytes were labeled with MEM containing 1.0 μCi of [1-14C]oleate bound to FA-free BSA in a 3:1 molar ratio for 3 h (22). The radiolabeling medium, which included 1 mM carnitine, contained a final concentration of 100 μM or 750 μM oleate (132). In some experiments cells were incubated with 250 μM [1,2,3-3H]glycerol (1.1 μCi) or 2.5 mM [1-14C]acetate (1.0 μCi). The medium was collected for acid-soluble metabolites (ASM) measurement or extracted to measure radiolabel incorporation into secreted lipids (22,132). Hepatocytes were washed twice with
1% BSA in PBS at 37°C and cellular lipids were extracted (116). For pulse-chase experiments, hepatocytes were infected with adenovirus for 21 h (as above) and incubated with 750 µM [1-14C]oleate in the presence of 250 µM unlabeled glycerol, or with 250 µM [1,2,3-3H]glycerol in the presence of 750 µM unlabeled oleate. After a 3 h incubation, the cells were either collected (pulse) as described above, or washed twice with 1% BSA in PBS and then incubated for an additional 14 h in MEM-DA without added oleate or glycerol (chase). The medium and cells were collected and lipids were extracted as described.

Aliquots of the lipid extracts from the cells and media were separated by thin layer chromatography (TLC) on 0.25-mm silica gel G plates in either hexane:ethyl ether:acetic acid (80:20:1; v/v) for neutral lipids (22) or in chloroform:methanol:acetic acid:water (50:37.5:3.5:2; v/v) for PL (133), together with authentic lipid standards in parallel. The 14C- or 3H-labeled lipids were detected and quantified with a Bioscan 200 Image System.

**Cell homogenate preparations for ACS activity and ACSL protein assays.** Hepatocytes infected with Ad-ACSL1 or Ad-GFP were washed twice with cold PBS and collected in cold Medium A (10 mM Tris pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol and Protease Inhibitor Cocktail [Sigma]) and homogenized on ice with 10 up-and-down strokes with a Teflon-glass motor-driven homogenizer. Homogenate aliquots were stored at –80°C until use. Protein concentrations were determined by the BCA method (Pierce). ACS specific activity was determined by measuring the production of [14C]acyl-CoA in the
presence of 175 mM Tris-HCl, pH 7.4, 8 mM MgCl₂, 5 mM dithiothreitol, 10 mM ATP, 0.25 mM CoA, and 50 µM [¹⁴C]palmitic acid in 0.5 mM Triton X-100, 0.01 mM EDTA. The assays were performed in a total volume of 200 µl at 37 °C for 5 min. The reaction was started by adding 0.5 to 1.5 µg of homogenate protein, terminated with 1 ml Dole’s reagent (isopropanol, heptane, 1M H₂SO₄; 80:20:2; v/v), and extracted (134). Enzyme assays measured initial rates.

**Western Blot analysis.** Homogenates from the dose-dependent infection and time-course incubations (10 µg) were separated by electrophoresis on a 10% polyacrylamide gel with 0.1% SDS, and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Immunoreactive bands were detected by incubating the membranes with anti-FLAG M2 monoclonal antibody (Sigma), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, and SuperSignal West Pico Chemiluminescent Reagent (Pierce) (135).

**Quantitative Real-time-PCR.** Hepatocytes were plated at 4.5 x 10⁶ cells per 100 mm dish and infected with Ad-GFP or Ad-ACSL1 at an MOI of 20 for 2 h as described above. Thirty h after infection, RNA was isolated (RNeasy, Qiagen) and stored at -80°C until use. Samples were analyzed on an ABI Prism 7700 sequence detection system (Applied Biosystems). Primers and corresponding FAM probes are listed in Table 3.1 except for CPT1 (Applied Biosystems, Rn00580702_m1). Data were analyzed using the relative standard curve method (22).
**Immunocytochemistry.** Primary hepatocytes (100,000 cells/well) were plated on 8-well chamber slides (Lab-Tek) coated with collagen I (Sigma) and infected with Ad-ACSL1 for 18 h as described above. After washing three times with PBS at room temperature cells were fixed with 4 % formaldehyde and permeabilized with 0.1% Triton-X100. After blocking with 10% FBS in PBS cells were labeled with anti-FLAG M2 monoclonal antibody [1:400, Sigma] and individual organelle markers (anti-calnexin polyclonal antibody [1:100, Abcam], anti-VDAC polyclonal antibody [1:100, ABR], rhodamine phalloidin [1:100, Molecular Probes]) in blocking buffer for 1 h at room temperature. Secondary labeling was performed with Alexa Fluor® 488 goat anti-mouse IgG (1:1000) or Alexa Fluor® 568 goat anti-rabbit IgG (1:1000, Molecular Probes). Multichannel confocal microscopy was performed sequentially with a Zeiss 510 Meta Inverted Laser Scanning Confocal Microscope. LSM 510 Meta software was used for acquisition and image analysis.

**Statistical analysis.** Data from each group were expressed as means ± SE. Data were analyzed by Student’s t-test, and significance was declared at $p < 0.05$. 
RESULTS

Adenoviral overexpression of ACSL1 increased ACS activity in rat primary hepatocytes.

We infected rat primary hepatocytes with Ad-GFP or an adenovirus containing rat ACSL1 with a FLAG epitope at the C terminus (Ad-ACSL1) and measured the ACS specific activity with palmitate at different virus doses (Figure 3.1A) and incubation times (Figure 3.1B).

After an 18 h infection with Ad-GFP, ACS specific activity did not change (167.8 ± 4.9 vs. 155.0 ± 2.6 nmol/min/mg protein in uninfected cells). In contrast, Ad-ACSL1 (18 h) increased ACS specific activity 86% to 236% at 5 to 50 MOI, with a linear increase through 20 MOI. Ad-ACSL1 at 20 MOI increased ACS specific activity linearly for 36 h. Western blotting with anti-FLAG primary antibody showed a band of about 75 kDa, confirming that the increase in ACS specific activity was due to the overexpression of ACSL1. Consistent with the increase in activity, the density of the immunoreactive band increased with the time of incubation and the adenovirus dose. For labeling experiments, we chose 20 MOI and 24 h when ACS specific activity was increased 3.7-fold. ACSL4 or ACSL5 are the two other major ACSLs expressed in rat liver. Overexpressed ACSL1 increased ACSL5 mRNA expression 2-fold but ACSL4 mRNA expression was not altered (Table 3.2).

Overexpressed ACSL1 colocalized with ER but not mitochondria or plasma membrane.

Previous studies that used subcellular fractionation showed that endogenous ACSL1 in rat liver is located in ER, but not mitochondria (64,80). To characterize the intracellular location of overexpressed ACSL1, we used confocal microscopy to examine its distribution.
Table 3.1.  **Primer and probe sequences used for gene expression by qRT-PCR for rat primary hepatocytes.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers &amp; Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACSL4</strong></td>
<td><strong>Forward:</strong> TATGGGCTGACAGAATCATG  <strong>Reverse:</strong> CAACTCTTCCAGTAGTGTA  <strong>Probe:</strong> TAACTTCAGTAACTGTTCCAGCCC</td>
</tr>
<tr>
<td><strong>ACSL5</strong></td>
<td><strong>Forward:</strong> GGCACACAGAATGCACAG  <strong>Reverse:</strong> GGAGTCCCAACATGACCTG  <strong>Probe:</strong> TGTCCAGTCCCCAGGTGATGTAAT</td>
</tr>
<tr>
<td><strong>DGAT2</strong></td>
<td><strong>Forward:</strong> GGAACCGCAAAGGCTTTGTA  <strong>Reverse:</strong> CTCAAAGGAATAGTGGA  <strong>Probe:</strong> AGCTGGCCCTGCGCCATGG</td>
</tr>
<tr>
<td><strong>ACAT2</strong></td>
<td><strong>Forward:</strong> TATACTGCCAGGAGTGGTAC  <strong>Reverse:</strong> AGGTGTCACCAGCTCCAAA  <strong>Probe:</strong> CTGTCCCTTGCCCCAGCCGA</td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td><strong>Forward:</strong> TGCCTGACGGTCAGGTCA  <strong>Reverse:</strong> CAGGAAGGAAGGCTGGAAG  <strong>Probe:</strong> CACTATCGGCAATGAGCGGTTCG</td>
</tr>
</tbody>
</table>

Table 3.2.  **Comparison of liver gene expression levels between Ad-GFP and Ad-ACSL1 infected rat primary hepatocytes.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Ad-GFP</th>
<th>Ad-ACSL1</th>
<th>T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACSL4</strong></td>
<td>0.87 ± 0.30</td>
<td>1.56 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ACSL5</strong></td>
<td>0.66 ± 0.31</td>
<td>1.39 ± 0.66</td>
<td>*</td>
</tr>
<tr>
<td><strong>DGAT2</strong></td>
<td>0.86 ± 0.20</td>
<td>1.23 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ACAT2</strong></td>
<td>0.66 ± 0.20</td>
<td>1.02 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CPT1</strong></td>
<td>0.54 ± 0.06</td>
<td>0.51 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abundance of mRNA was quantified using qRT-PCR and normalized to β-actin. Data represent means ± SE (n = 6). *p < 0.05 by paired Student’s t test. NS, non significant, p > 0.05.
Figure 3.1. *Acyl-CoA synthetase (ACS) activity increased in rat hepatocytes overexpressing ACSL1*. Rat primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were uninfected (control) or infected with adenoviruses carrying either GFP (Ad-GFP) or rat ACSL1 (Ad-ACSL1). A) Cells were infected with Ad-ACSL1 at different MOI as indicated. After an 18 h incubation, cells were scraped and homogenized. B) Cells were infected with Ad-GFP or Ad-ACSL1 (MOI = 20) and homogenates were collected at the indicated times. Total ACS activity was measured and Western blot with anti-FLAG monoclonal antibody was performed as described under “Experimental procedures”. Data are reported as means ± SE from triplicate dishes. All Ad-ACSL1 versus Ad-GFP, p < 0.001.
ACSL1-FLAG labeling was confined to intracellular membranes that partially colocalized with the ER marker calnexin (Figure 3.2A), but did not colocalize with the mitochondrial marker VDAC (Figure 3.2B), or sub-plasma membrane actin filaments and stress fibers (Figure 3.2C), indicating the absence of mitochondrial and plasma membrane association in primary hepatocytes. The location of ACSL1-FLAG did not change with prior incubation with 750 µM oleate or 10 µM forskolin, and ACSL1-FLAG labeling was well colocalized with large regions of fluorescent-Concanavalin A, an ER-Golgi stain (data not shown). These data define a fixed location for ACSL1-FLAG on ER membranes. Thus, the subcellular location of overexpressed ACSL1-FLAG in hepatocytes was similar to that of endogenous ACSL1 in liver (64).

**Ad-ACSL1 increased [1-14C]oleate incorporation into DAG but not TAG, and decreased [1-14C]oleate incorporation into CE.** To determine the effects of overexpressed ACSL1 on lipid metabolism, we incubated the adenovirus infected hepatocytes with 100 µM or 750 µM [1-14C]oleate, representing physiological concentrations of exogenous FA under fed or fasting conditions, respectively. At 100 µM oleate, incorporation of [1-14C]oleate into [14C]DAG doubled with Ad-ACSL1 (Figure 3.3A). Increasing exogenous oleate to 750 µM resulted in a 1.7-fold increase of [14C]DAG in cells infected with Ad-GFP, and this was doubled again by the presence of Ad-ACSL1. Surprisingly however, despite the Ad-ACSL1-mediated increase in [14C]oleate incorporation into DAG, the incorporation of oleate into TAG remained unchanged at both oleate concentrations (Figure 3.3B). The
Figure 3.2.  *Ad-ACSL1 colocalized with endoplasmic reticulum (ER).* Primary hepatocytes were plated on 8-well chamber slides (100,000 cells/well) and infected with Ad-ACSL1 at 10 MOI for 18 h. Cells were fixed, permeabilized, and doubly labeled with primary antibodies against FLAG (green) and one of the organelle markers (red). Appropriate species Alexa-coupled secondary antibodies were used for detection. A) Anti-calnexin (ER marker); B) Anti-VDAC (mitochondria marker); C) Rhodamine phalloidin (actin dye). Yellow dots in merged A) indicate partial colocalization of ACSL1FLAG with ER. Cells stained only with red were uninfected cells. Cells were examined by confocal microscopy at 63x magnification. Scale bar, 10 µm. Images are from a representative experiment that was repeated three times.
ACSL1 overexpression increased [1-14C]oleate incorporation into cellular DAG but not TAG. Primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) at 20 MOI. After a 21 h infection, cells were incubated with 100 µM or 750 µM [1-14C]oleate for 3 h and harvested. Cellular lipids were extracted and [1-14C]oleate incorporation into neutral lipid species was determined by TLC as described under “Experimental procedures”. [1-14C]oleate incorporation into cellular A) DAG; B) TAG; C) CE; and D) unesterified fatty acid (FA). Data are reported as means ± SE from a representative experiment performed in triplicate dishes that was repeated four times. Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01.
addition of 750 µM oleate increased $[^{14}\text{C}]$TAG 5-fold in both Ad-GFP and Ad-ACSL1 infected cells. Cellular TAG mass also remained unchanged by the presence of excess ACSL1 (data not shown). Decreasing the adenovirus dose to 10 MOI did not change the incorporation pattern (data not shown), thereby excluding the possibility that cell toxicity related to excess Ad-ACSL1 had inhibited TAG synthesis. Further, the mRNA level of DGAT2, the major enzyme that converts DAG to TAG in liver (136), was unchanged in Ad-ACSL1 infected cells (Table 3.2).

Acyl-CoAs are also substrates for cholesterol esterification. Compared to Ad-GFP control hepatocytes, the incorporation of $[^1\text{H}]$oleate into CE was 70% lower in Ad-ACSL1 cells at both 100 µM and 750 µM (Figure 3.3C), suggesting that ACSL1 channels acyl-CoAs away from cholesterol esterification. An alternate interpretation, is that ACAT2, the major enzyme for CE synthesis in rodent liver (137) was downregulated. This seemed less likely because the abundance of ACAT2 mRNA was unchanged (Table 2). Finally, despite a likely increase in the rate of FA conversion to acyl-CoA, the amount of unesterified $[^{14}\text{C}]$oleate present in the cells did not decrease (Figure 3.3D).

**ACSL1 overexpression increased $[^1\text{H}]$oleate incorporation into specific PL.** In addition to its effects on neutral lipids, Ad-ACSL1 increased $[^1\text{H}]$oleate incorporation into total cellular PL 70% and 21% with 100 µM and 750 µM oleate, respectively (Figure 3.4A). At 100 µM oleate, Ad-ACSL1 increased oleate incorporation into (phosphatidylethanolamine)
PE 164% (Figure 3.4B), phosphatidylinositol (PI) 104% (Figure 3.4C), and phosphatidylcholine (PC) 54% (Figure 3.4D). At 750 µM oleate, Ad-ACSL1 overexpression increased labeled PE 99% and labeled PI 56%, with no change in labeled PC. Thus, it appears that overexpression of ACSL1 enhanced oleate incorporation predominantly into PE and PI, phospholipids that originate from DAG and phosphatidic acid, respectively.

**ACSL1 overexpression did not affect the use of [1-14C]oleate for β-oxidation.** To determine whether overexpressed ACSL1 provided acyl-CoAs for FA oxidation, we measured labeled ASM in the medium as an indicator of FA β-oxidation (132). ASM is considered a more accurate measure of β-oxidation than is CO₂ production (138). The incorporation of [1-14C]oleate into ASM was equal in Ad-ACSL1 infected cells and Ad-GFP control cells at both 100 µM and 750 µM oleate (Figure 3.5A), and there was no difference in the mRNA abundance of CPT1, the rate-limiting enzyme for β-oxidation (Table 3.2). Thus, overexpression of ACSL1 did not enhance FA degradation.

**Ad-ACSL1 decreased [14C]TAG secreted into the medium.** Despite similar incorporation of oleate into cellular TAG in Ad-ACSL1 and Ad-GFP infected hepatocytes, Ad-ACSL1 overexpression decreased the amount of labeled TAG secreted by about 53% (Figure 3.5B). In contrast, the incorporation of [14C]oleate into secreted DAG and PL remained unchanged (data not shown). These data suggested that ACSL1 activation of FA resulted in the synthesis of TAG that was not available for VLDL biogenesis.
Figure 3.4. *ACSL1* overexpression increased [1-14C]oleate incorporation into cellular PL. Hepatocytes were infected with either Ad-GFP (open bar) or ACSL1 (filled bar) for 21 h, and incubated with 100 µM or 750 µM [1-14C]oleate for 3 h. Cellular lipids were extracted and [1-14C]oleate incorporation into different PL species was determined by TLC. [1-14C]oleate incorporation into cellular A) PL; B) PE; C) PI; and D) PC. Data are reported as means ± SE from a representative experiment performed in triplicate dishes that was repeated four times. Ad-ACSL1 versus Ad-GFP, **p < 0.01, ***p < 0.001.
Figure 3.5.  

**ACSL1 overexpression decreased [1-14C]oleate metabolism to medium TAG but not to β-oxidation.**  Hepatocytes were seeded and infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) as described under “Experimental procedures”. Cells were incubated with 100 µM or 750 µM [1-14C]oleate for 3 h and the cells and medium were collected and extracted. Total FA metabolized includes cell and medium lipids and ASM. [1-14C]oleate incorporation into A) medium ASM; B) medium TAG; C) total [1-14C]oleic acid metabolized. Data are shown as means ± SE from a representative experiment performed in triplicate dishes and that was repeated four times.  Ad-ACSL1 versus Ad-GFP, ** p < 0.01, *** p < 0.001.
**Overexpression of ACSL1 did not increase the total amount of FA metabolized.** When ACSL5 is overexpressed in McArdle-RH7777 rat hepatoma cells (22), or when ACSL1 is overexpressed in fibroblasts, the uptake of exogenous FA is enhanced (16), suggesting that FA metabolism facilitates the ingress of FA. To determine whether ACSL1 similarly enhances FA uptake by hepatocytes, we calculated the total amount of [$1^{-14}$C] incorporated into cell and medium lipids and into medium ASM. Ad-ACSL1 did not significantly increase the total amount of FA metabolized at either 100 µM oleate ($p=0.057$) or 750 µM oleate ($p=0.093$) (Figure 3.5C). Thus, in hepatocytes, unlike other cells, exogenous FA uptake was not enhanced by excess ACSL1 activity, perhaps because ACSL1 had a different subcellular location or because there were tissue-specific differences in interacting proteins (16,20).

**ACSL1 increased oleate recycling to TAG and PL during the chase.** Cellular complex lipids undergo dynamic changes via hydrolysis, re-modeling, and re-esterification (139,140). Thus, we wondered whether the absence of enhanced [$^{14}$C]oleate incorporation into TAG despite a doubling of [$^{14}$C]DAG was due to increased TAG hydrolysis or to diminished TAG reacylation. To examine these possibilities, we labeled cells with 750 µM [$1^{-14}$C]oleate for 3 h and followed the fate of labeled complex lipids for 14 h afterwards.

In Ad-GFP infected hepatocytes, 33% of the labeled cellular TAG was lost during the 14 h chase (Figure 3.6A). Overexpression of ACSL1 attenuated the loss of label from TAG,
resulting in only an 18% decrease, and also helped to retain label present in PL. In contrast, the amount of labeled PL decreased 19% in the Ad-GFP infected cells. The diminished changes in the amount of [14C]-labeled TAG and lack of change in the amount of [14C]-labeled PL suggested that Ad-ACSL1 enhanced FA reesterification of TAG and PL. Since the amount of labeled DAG decreased 27% in Ad-GFP controls during the chase, whereas it decreased 59% in the cells that overexpressed ACSL1 (Figure 3.6A), it appeared that ACSL1 maintained label in TAG and PL partly by reesterifying FA that had been released from DAG.

**ACSL1 decreased the recycling of oleate into CE and β-oxidation metabolites.** In both the Ad-GFP and Ad-ACSL1 cells during the 14 h chase, the amount of [14C]-label in CE increased markedly compared to the label present at 3 h (Figure 3.6B). However, 64% less label was recycled into CE in Ad-ACSL1 cells. This decrease in [14C]CE was similar to the reduced labeling (70%) observed during the initial 3 h incubation, and was consistent with the interpretation that ACSL1 directs acyl-CoAs away from CE synthesis. In contrast, however, to the lack of effect of Ad-ACSL1 on [14C]ASM observed during the 3 h pulse (Figure 5A), Ad-ACSL1 infected cells incorporated 42% less label into ASM during the chase. These data suggested that the 14C-label in TAG and PL was retained at the expense of FA oxidation (Figure 3.6C).
Figure 3.6. **Overexpression of ACSL1 altered [14C]oleate recycling in hepatocytes.** Hepatocytes were plated and infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) for 21 h. Cells were labeled with 750 µM [1-14C]oleate for 3 h, and then were either collected for lipid extraction (pulse), or were washed and incubated with new media containing no FA for 14 h (chase), as described under “Experimental procedures”. The remaining [14C]-label was analyzed in cellular lipid extracts or medium ASM in chased cells, and compared with pulsed cells. **A)** Remaining label in cellular lipids as a percentage of the label present at 3 h pulse, label in TAG decreased from 179,114 ± 4,242 DPM to 120,231 ± 5,106 DPM/1.5 x 10^6 Ad-GFP cells and from 167,514 ± 4,843 DPM to 138,119 ± 2,032 DPM/1.5 x 10^6 Ad-ACSL1 cells, label in DAG decreased from 11,732 ± 588 DPM to 8,577 ± 242 DPM/1.5 x 10^6 Ad-GFP cells and from 21,740 ± 488 DPM to 8,912 ± 374 /1.5 x 10^6 Ad-ACSL1 cells; **B)** [14C]oleate incorporation into cellular CE during the 3 h pulse and 14 h chase; **C)** [14C]oleate incorporation into ASM during the 14 h chase. Data are shown as means ± SE from a representative experiment performed in triplicate dishes and that was repeated three times. # Significantly different from pulsed cells, p < 0.01; Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01, *** p < 0.001.
Overexpressed ACSL1 increased [$^{14}$C]oleate incorporation into DAG and PL via both de novo and reacylation pathways. To determine whether overexpressed ACSL1 affected de novo glycerolipid synthesis, we incubated hepatocytes with 250 µM [1,2,3-$^3$H]glycerol in the presence of 750 µM unlabeled oleate for either 3 h or for 3 h followed by a 14 h chase in the absence of labeled glycerol. Similar to the pattern of oleate incorporation during a 3 h incubation, overexpressed ACSL1 increased [$^3$H]glycerol incorporation into DAG and PL 79% and 14%, respectively (Figure 3.7A), suggesting that ACSL1 increased de novo synthesis of DAG and PL. Since ACSL1 overexpression increased incorporation into DAG and PL from oleate more than from glycerol, it is likely that reacylation contributed to the increase in labeled DAG and PL. Similar to the [$^{14}$C]oleate study, [$^3$H]TAG remained unchanged by Ad-ACSL1, again suggesting that ACSL1 overexpression did not increase de novo TAG synthesis despite the increase in [$^3$H]DAG. During the chase, again similar to the effects of ACSL1 on [$^{14}$C]oleate recycling, more [$^3$H]TAG and [$^3$H]PL and less [$^3$H]DAG remained in the Ad-ACSL1 infected cells (Figure 3.7B), confirming that ACSL1 altered lipid recycling in the hepatocytes by retaining labeled TAG and PL and by metabolizing DAG.

ACSL1 overexpression increased the incorporation of FA derived from de novo synthesis into TAG, DAG and PL. Liver metabolizes both FA imported from the blood and FA synthesized de novo. It has been suggested that FA derived from different sources might have distinct fates (141). We reported that ACSL5 exclusively activates exogenous FA but not FA synthesized endogenously (22). To examine the selectivity of ACSL1, we
labeled the adenovirus infected hepatocytes with 2.5 mM [1-\textsuperscript{14}C]acetate. ACSL1 increased [1-\textsuperscript{14}C]acetate incorporation into DAG and PL 83%, and 61%, respectively (Figure 3.7C), similar to the effect observed with \textsuperscript{14}C]oleate incorporation (Figure 3). In contrast, however, to the \textsuperscript{14}C]oleate study, overexpressed ACSL1 enhanced [1-\textsuperscript{14}C]acetate incorporation into TAG 13%, and did not significantly decrease \textsuperscript{14}C]acetate incorporation into CE. Lack of effect on \textsuperscript{14}C]CE may reflect the fact that most of the \textsuperscript{14}C]-label in CE was probably derived from \textit{de novo} synthesized cholesterol rather than from FA synthesis. Thus, it appeared that ACSL1 can activate FA derived from both exogenous and \textit{de novo} sources and commit them to the synthesis of DAG and PL.
Figure 3.7. ACSL1 overexpression increased [1,2,3-3H]glycerol incorporation into DAG and PL, and activates FA derived from de novo synthesis. Primary hepatocytes (1.5 x 10^6 cells/60 mm dish) were infected with Ad-GFP (open bar) or Ad-ACSL1 (filled bar) at 20 MOI for 21 h. Cells were labeled with 250 µM [1,2,3-3H]glycerol in the presence of 750 µM unlabeled oleate (A, B) or 2.5 mM [1-14C]acetate (C). Cells were either collected for lipid extraction after 3 h labeling (pulse) (A, C), or were washed and incubated with new media containing no glycerol nor FA for 14 h (chase) (B), as described under “Experimental procedures”. Remaining [14C] label was analyzed in cellular lipid extracts in chased cells, and compared with pulsed cells. Data are shown as means ± SE from 6 dishes. # Significantly different from pulsed cells, p < 0.05; Ad-ACSL1 versus Ad-GFP, * p < 0.05, ** p < 0.01, *** p < 0.001.
DISCUSSION

The major findings of this study are that overexpression of ACSL1 altered FA incorporation into specific pathways in rat primary hepatocytes, and that these pathways differed from those previously observed after overexpression of ACSL1 in other types of cells. Previous studies in NIH3T3 fibroblasts and PC12 neuronal cells strongly suggest that overexpressed ACSL1 provides acyl-CoAs that are incorporated primarily into TAG (63,128), and in vivo studies of ACSL1 heart-specific transgenic mice show a marked increase in heart TAG mass (86). In addition, during the differentiation of 3T3-L1 preadipocytes into adipocytes, ACSL1 mRNA increases 160-fold whereas other ACSL isoforms remain unchanged, concomitant with a 100-fold increase in microsomal ACS specific activity and TAG accumulation (63,90). ACSL1 mRNA is upregulated in liver by PPARα (97,99) and in adipose tissue by PPARγ (142), suggesting that the function of ACSL1 might differ in these tissues.

In contrast to other overexpression studies of ACSL1 (128) and ACSL5 (22), overexpressed ACSL1 in hepatocytes did not increase TAG mass or [14C]oleate incorporation into TAG despite a doubling in [14C]oleate incorporation into DAG. Because less [14C]TAG was hydrolyzed in the Ad-ACSL1 infected cells during the 14 h chase, it appeared that ACSL1 overexpression either diminished the rate of lipid hydrolysis or increased recycling of hydrolyzed [14C]oleate back to TAG. We favor the latter explanation because more [14C]DAG was lost from the Ad-ACSL1 infected cells. Thus, an increase in glycerolipid
turnover cannot explain the inconsistency between the increased \[^{14}\text{C}]\text{DAG}\) without a concomitant increase in \[^{14}\text{C}]\text{TAG}\). Incubating cells with \[^{3}\text{H}]\text{glycerol}\) confirmed the pattern seen with \[^{14}\text{C}]\text{oleate}\) incorporation. In addition, consistent with the pattern of oleate recycling during the chase, the \[^{3}\text{H}]\) label increased in \text{TAG}\) and \text{PL}\), and decreased in \text{DAG}\), again supporting the hypothesis that ACSL1 increases FA reacylation.

In addition to the increase in \[^{14}\text{C}]\text{DAG}\), overexpressing ACSL1 increased \[^{14}\text{C}]\text{oleate}\) incorporation into \text{PL}\), particularly \text{PE}\) and \text{PI}\). Thus, it appears that, instead of being channeled towards \text{TAG}\) synthesis, acyl-CoAs were used primarily to synthesize \text{PL}\). This is consistent with the previous finding that choline- and ethanolamine- glycerolphospholipid masses increased 50\% and 15\%, respectively in heart-specific ACSL1 transgenic mice (86). Because less \[^{14}\text{C}]\text{PL}\) and more \[^{14}\text{C}]\text{DAG}\) was hydrolyzed in Ad-ACSL1 cells during the chase, ACSL1 may have increased FA incorporation into \text{PL}\) by enhancing FA recycling or by increasing the use of labeled \text{DAG}\). These data are strikingly different from a study of overexpressed ACSL5 in McArdle-RH7777 rat hepatoma cells in which no increase was observed in either oleate or glycerol incorporation into cellular \text{PL}\) despite an increase in incorporation into \text{DAG}\) (22), again suggesting that ACSL1 and ACSL5 commit FA to different metabolic fates.

The incorporation of \[^{14}\text{C}]\text{oleate}\) into \text{CE}\) was markedly lower in cells that overexpressed ACSL1, despite unchanged ACAT2 mRNA, and increasing the exogenous oleate
concentration to 750 µM did not change this result, suggesting that acyl-CoA was not limiting. Further, even though both Ad-GFP and Ad-ACSL1 cells were able to use [14C]oleate hydrolyzed from labeled glycerolipids to esterify cholesterol during the chase, the Ad-ACSL1 cells continued to use less for CE labeling (Figure 6B). Thus, we conclude that overexpressed ACSL1 diverted oleate away from cholesterol esterification. This diversion by ACSL1 contrasts with studies of ACSL5 overexpression in rat hepatoma cells which did not decrease oleate incorporation into CE (22).

Overexpressed ACSL1 did not alter the amount of FA oxidized during the 3 h oleate incubation, but during the 14 h chase 42% less [14C]oleate was released from complex lipids for oxidization. Thus, even though hepatic ACSL1 is a target of PPARα (95,97,99), our data did not indicate that ACSL1 channels FA into the pathway of β-oxidation. Although PPARα agonists upregulate genes involved in FA oxidation like CPT1 (143), they also upregulate DGAT activity (144) and genes involved in de novo FA and TAG synthesis, including acetyl-CoA carboxylase and stearoyl-CoA desaturase-1 via PPARα-mediated increases in the amount of nuclear sterol regulatory element binding protein-1c (130). Thus, PPARα regulation of ACSL1 may enhance lipogenesis as well as increase β-oxidation.

Despite unchanged [14C]oleate incorporation into cellular TAG, Ad-ACSL1 cells secreted less [14C]TAG into the medium, perhaps due to insufficient CE for very low-density lipoprotein (VLDL) synthesis. Although the role of CE in the assembly and secretion of
VLDL is controversial (145,146), CE availability appears to be important because ACAT inhibitors reduce apoB100 secretion in primary rat hepatocytes and HepG2 cells (147) and overexpression of ACAT1 and ACAT2 stimulates apoB-containing lipoproteins in McArdle-RH7777 cells (146). Additionally, because in rat hepatocytes cytosolic TAG is not incorporated en bloc into the ER for VLDL biogenesis and secretion (148,149), the amount of cell TAG might not correlate directly with TAG secreted in VLDL. Finally, secretion might be affected by ACSL1-mediated changes in the cellular content of FA and acyl-CoA which are ligands for nuclear transcription factors like the PPARs (38) and HNF-4α (150,151).

Overexpression of several ACSL isoforms increase the uptake of exogenous FA (16,22,63). Uptake probably occurs because vectorial acylation and enhanced FA metabolism diminish the rate of efflux of unesterified FA from the cell (152). For example, in 3T3-L1 adipocytes, in addition to its location in the ER, ACSL1 is found on glucose transporter 4 vesicles (153) and on the plasma membrane where it interacts with FATP1 to enhance the uptake and metabolism of FA to TAG (20). Most studies have measured the initial rate of FA import within 1-2 min, a time frame that may not represent the physiological uptake of FA that is driven by transporters and metabolic demand (152). We measured the total FA metabolized in cell and medium lipids and in β-oxidation products during a period of time that takes metabolic demands into account (22,152). In our study, ACSL1 did not significantly increase the total amount of FA metabolized by hepatocytes.
Since FATP1 is not present in hepatocytes (154), and since we showed that hepatocyte ACSL1 is not present on the plasma membrane, lack of enhanced FA uptake and metabolism in hepatocytes may reflect the different intracellular location of ACSL1, its interaction with a different FATP isoform, or its association with different downstream enzymes that use acyl-CoA.

To determine the selectivity of ACSL1 for endogenous versus exogenous FA, we incubated hepatocytes with [14C]acetate, which is used for de novo FA synthesis. In contrast to incubations with [14C]oleate, Ad-ACSL1 increased label incorporation into TAG, as well as DAG and PL, showing enhanced use of de novo synthesized FA for TAG synthesis. It has been suggested that TAG and CE synthesis in hepatocytes requires some FA derived from de novo synthesis (144,155). Our data suggest that overexpression of ACSL1 channeled exogenous FA into DAG and PL, but not into TAG, in part due to insufficient endogenous FA. However, during the 14 h chase, the required pool of endogenous FA may no longer have been limiting because of the hydrolysis of TAG and PL, so that overexpressed ACSL1 could increase [14C]oleate incorporation into TAG. Differing from the decrease in exogenous oleate used for CE synthesis, ACSL1 overexpression did not diminish [14C]acetate incorporation into CE, suggesting that [14C]-label in CE was derived primarily from cholesterol rather than FA.

In summary, consistent with our hypothesis that ACSL1 channels FA towards specific
pathways, adenovirus-mediated overexpression of rat ACSL1 in rat primary hepatocytes channeled $[^{14}\text{C}]$oleate towards DAG, PE, PI and PC synthesis and away from cholesterol esterification. Overexpressed ACSL1 also increased the reacylation of hydrolyzed oleate to TAG and PL, but diminished the amount of hydrolyzed oleate used for $\beta$-oxidation. In contrast to its role in adipocytes (15), fibroblasts (16) and heart muscle (86), overexpression of ACSL1 in hepatocytes did not increase incorporation of $[^{14}\text{C}]$oleate into TAG or increase the total amount of FA metabolized. It seems likely that ACSL1 channels FA differently in different tissues, perhaps depending on the subcellular location of ACSL1 or the presence of interacting proteins specific to each cell type. In addition, overexpressed ACSL1 activated both exogenous FA and FA derived from de novo synthesis, but channeled the resulting acyl-CoA products into different pathways. Our study suggests that ACSL1 in hepatocytes plays an important role in directing FA into pathways of phospholipid synthesis, and away from cholesterol esterification and $\beta$-oxidation.
CHAPTER IV

PHOSPHORYLATION REGULATION OF ACYL-COA SYNTHETASE 1 AND 5

Many key enzymes in lipid metabolism are under acute regulation by lipogenic and lipolytic hormones. In view of the important role of ACSL in lipid metabolism, it is very possible that individual ACSL isoforms are under tight regulations. Although lipogenic and lipolytic hormones caused the microsomal ACS activity to change rapidly in adipocytes, no data existed about acute regulation of individual ACSL isoforms. We therefore initiated studies in primary hepatocytes and a cultured adipocyte cell line aimed at examining the potential acute regulation of ACSL1 and ACSL5 by phosphorylation. Although we found phosphorylated forms of ACSL1 and ACSL5, the mechanism and physiological significance were not solved in the data from these studies. However, the data presented in this chapter provide evidence that phosphorylation might be an important regulation on ACSL isoforms, and this regulation might be tissue and isoform specific.
4.1 Hormone effects on acyl-CoA synthetase 1 and 5 - phosphorylation regulation

INTRODUCTION

Long-chain acyl-CoA synthetase (ACSL) catalyzes the first step of fatty acid activation in lipid metabolism (66). Since the identification of the first isoform in rat (ACSL1), our knowledge on ACSL has been expanded, mainly on enzyme kinetics, tissue and subcellular locations, functions in fatty acid uptake and channeling, and changes under physiological and pathological conditions (21,22,51,64,65,80). However, acute regulation of ACSL has been ignored. Whether there is acute regulation of ACSL isoforms or what the role of acute regulation in lipid metabolism is remains unknown.

Studies of rat primary adipocytes have shown that microsomal ACS activity is increased rapidly by insulin and decreased by lipolytic hormones such as norepinephrine and glucagon (101,102). Since the dose-response curve for inactivation of ACS by lipolytic hormones issimilar to the curve for stimulation of lipolysis, it is possible that acute regulation of ACS plays a role in the lipid mobilization of adipocytes (102). In fact, ACSL isoforms have been identified in association with lipid droplets in 3T3-L1 adipocytes, where the adipocyte-specific protein perilipin A and stimulated HSL are located (89,156). It is believed that perilipin A coats lipid droplets in adipocytes, and when unphosphorylated, suppresses basic lipolysis, presumably by blocking the access of lipases to the TAG core (157,158). Supporting evidence includes the decreased TAG hydrolysis by ectopically expressed perilipin A in 3T3-L1 preadipocytes and Chinese hamster ovary fibroblasts that
normally lack perilipin (157,159), and increased basal lipolysis in perilipin A knockout mice (160). During hormone-stimulated lipolysis, activated PKA phosphorylates both perilipin A and HSL, and the phosphorylated perilipin A induces the translocation of HSL from the cytosol to the lipid droplets, a critical step in the stimulated lipolysis (156,161). Mutating the PKA sites in both perilipin A and HSL eliminates HSL translocation to the lipid droplet upon PKA activation (159,162). Thus PKA mediated phosphorylation of both perilipin A and HSL is critical for stimulated lipolysis in adipocytes (163).

Phosphorylation is one of the most important and abundant reversible post-translational modifications. More than 30% of proteins are modified by the covalent attachment of phosphate, which is critical to protein function and activity (164). Since in adipocyte microsomes, ACSL1 is the major ACSL isoform, we hypothesized that ACSL1 in adipocytes is acutely regulated by phosphorylation and dephosphorylation. The rationale for this hypothesis is based on rapid changes of microsomal ACS activity in rat adipocytes by insulin, norepinephrine and glucagon (101,102). In adipocytes, insulin, norepinephrine and glucagon decrease and increase cellular cAMP levels, respectively, thus inhibiting or activating PKA. In fact, two potential phosphorylation sites for PKA (Ser 74 and Ser 411) have been predicted on ACSL1 (Scheme 1.4). Moreover, using a peptide antibody raised against ACSL1 on a Western blot, we observed a 2nd band above the major ACSL1 band in 3T3-L1 adipocytes (data not shown).
In liver, ACSL5 is present in mitochondria (64), where mitochondrial GPAT1 (mtGPAT1) for TAG synthesis (132,165) and CPT1 for fatty acid β-oxidation are located (4). It has been proposed that CPT1 and mtGPAT1 compete for acyl-CoA substrates for these opposing pathways (4). Supporting this notion, a 13-fold increase in GPAT activity by overexpression of mtGPAT1 decreases β-oxidation products ~ 60% in hepatocytes from fed rats, but increases TAG synthesis 2.4-fold (132). Moreover, the competition between CPT1 and mtGPAT1 is under reciprocal regulation by AMP-activated protein kinase (AMPK) (166). AMPK phosphorylates and inactivates mtGPAT1, thus reducing TAG synthesis (166). Conversely, AMPK phosphorylates and inactivates acetyl-CoA carboxylase, which results in the decrease of malonyl-CoA and the release of its inhibition of CPT1, thus increasing fatty acid β-oxidation (4,166). Since ACSL5 provides acyl-CoA for TAG synthesis (22), it may play a role in this competition between mtGPAT1 and CPT1 by channeling acyl-CoA to mtGPAT1. Because both CPT1 (167) and mtGPAT1 are regulated by phosphorylation, we wondered whether ACSL5 was also under acute regulation by phosphorylation.

This study is the first one that has reported phosphorylation of any ACSL isoforms. We found that in rat primary hepatocytes ACSL5 was phosphorylated on threonine 192, a non-classical phosphorylation site. Using an anti-phosphoserine antibody we also detected phosphorylated ACSL1 in 3T3-L1 adipocytes. However, we were not able to find any activity change of physiological significance. These data support our hypothesis that ACSL is under phosphorylation regulation. Further studies are needed to investigate the
mechanism of phosphorylation of ACSL isoforms and the significance of this acute regulation in lipid metabolism.

METHODS

Materials. Minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), fetal bovine serum (FBS) were from Gibco-BRL Life Technologies. Rat-tail collagen I was from Collaborative Biomedical Products. Dexamethasone and hormones were purchased from Sigma. Proteinase inhibitor cocktail and phosphatase cocktail I and II were from Sigma. Anti-FLAG M2 agarose beads and antibody were from Sigma. Anti-phosphoserine antibody was from Abcam. Tris-HCl pre-cast 10% gel was from Bio-Rad (161-1458). Triacsin C was from Biomol.

Cell culture and adenovirus infection. Animal protocols were approved by the UNC Institutional Animal Care and Use Committee. Rat primary hepatocytes were isolated and cultured as described previously in Chapter III. Cells (4.5 x 10^6 cell/100mm plate) were infected with recombinant adenoviruses carrying either GFP (Ad-GFP) or rat ACSL5 (Ad-ACSL5, with a C-terminal FLAG tag) at MOI 10 (22). Media were changed after 2 h and cells were incubated for 22 h before hormone treatment.

3T3-L1 cells that express a modified coxsackie and adenovirus receptor (CARΔ1) were
a generous gift from Dr. David J. Orlicky from the University of Colorado (168). 3T3-L1 CARΔ1 preadipocytes were grown in DMEM + 10% FBS to 60% ~ 70% confluent and then infected by Ad-ACSL1 (MOI 10) for 12~14 h. In some experiments, 3T3-L1 CARΔ1 preadipocytes were grown to confluence and differentiated 2 days later in DMEM + 10% FBS supplemented with 1 μg/ml insulin, 250 nM dexamethasone, and 500 μM isobutylmethylxanthine (169). Media were changed to DMEM + 10% FBS after 2 days. Six days after adding differentiation media, cells were infected with Ad-ACSL1 in DMEM + 2% FBS + P/S overnight at MOI 400 (after dose-despondence check on transfection efficiency) for 40 h. Cells were then subject to hormone treatment.

**Hormone treatment and sample collection.** Adenovirus infected rat primary hepatocytes or 3T3-L1 CARΔ1 preadipocytes were stimulated with individual hormones at such conditions unless otherwise indicated: 100 nM insulin x 20min, 100 nM Glucagon x 10min, 0.5 mM AICAR x 30min, 1 μM epinephrine x 30min, 1 mM or 2 mM 8Br-cAMP x 50 min, or 2 mM dibutyryl-cAMP x 60 min. For specific activity check, cells were collected in cold Medium I + DTT (250 mM sucrose, 10 mM Tris, pH 7.4, 1mM EDTA, 1 mM DTT) in the presence of proteinase inhibitor and phosphatase inhibitor cocktails. Homogenates were collected on ice with 10 up-and-down strokes and aliquots were stored at -80°C. For immunoprecipitation and Western blot, cells were washed with cold PBS and incubated with 1 ml/plate cold RIPAI buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, supplemented with proteinase and
phosphatase inhibitor cocktails). Lysates were collected after 30 min rotate on ice and centrifuged at 14,000 x g for 10 min at 4°C to get rid of debris. The supernatant were transferred and stored at -20°C. Protein concentrations were determined by the BCA method (Pierce).

**Immunoprecipitation & Western blot.** All steps of immunoprecipitation were conducted at 4°C. Lysates were diluted in RIPAI buffer (300 µg in 1 ml unless otherwise indicated) and incubated with anti-FLAG agarose beads (20 µl/1 ml) on a nutator overnight. The agarose beads were centrifuged at 1,000 g for 5 min and the supernatant was collected to check how much protein was not bound in immunoprecipitation (Figure 4.3 B). The beads were then washed 3 times in 1 ml cold RIPAI buffer, followed by resuspension in SDS-PAGE sample buffer. Immunocomplex was eluted by boiling the beads and then subject to SDS-PAGE. For Western blot, proteins separated by SDS-PAGE were transferred to PVDF or nitrocellulose membrane, which were then incubated with either anti-FLAG M2 monoclonal antibody (1/4,000) in 5% milk PBST, or rabbit anti-phosphoserine polyclonal antibody (1/1,500) in 3% BSA TTBS. Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Reagent after the membranes were incubated with corresponding secondary antibodies.

**Preparation for mass spectrometry.** Lysates collected from Ad-GFP or Ad-ACSL5 infected, hormone stimulated primary hepatocytes were subject to immunoprecipitation with
anti-FLAG M2 agarose (300 µg protein with 20 µl beads in 1 ml RIPAI). The eluted immunocomplex was loaded on 10% Tris-HCl pre-cast gel (Bio-Rad 161-1458) for SDS-PAGE. The gel was then fixed in 25% isopropanol/10% acetic acid/65% Milli-Q* water for 25 min and stained in 0.01% BioRad R-250 Coomassie/10% acetic acid overnight on an orbital shaker. Protein bands were in-gel tryptic digested and the peptides were extracted for mass spectrometry analysis in the UNC Proteomics Core Facility.

**Mouse mitochondria isolation and PPase treatment.** Liver mitochondria were isolated from mouse by differential centrifugation (170). Briefly, livers were removed and minced in Medium I + DTT + proteinase inhibitor (250 mM sucrose, 10 mM Tris, pH 7.4, 1mM EDTA, 1 mM DTT), in the absence or presence of phosphatase inhibitor cocktails I and II. Tissue was then homogenized on ice and centrifuged at 600 x g for 5 min to remove large debris and nuclei. The supernatant was centrifuged at 10,300 x g for 10 min to obtain mitochondrial pellet, which was then homogenized in Medium I + DTT + proteinase inhibitor cocktail, in the absence or presence of phosphatase inhibitor cocktails I and II, according to first homogenation step. Aliquots were stored at -80°C.

**ACS activity assay.** ACS activity was determined in adenovirus infected and hormone stimulated hepatocytes or 3T3-L1 cells, and mouse liver mitochondria fractions. The activity assay measured the production of labeled acyl-CoA as described in Chapter II and III. In some assays, ACS activity was determined in the presence of triacsin C dissolved in


RESULTS AND DISCUSSION

**Acute regulation of hormones on ACS activity in hepatocytes.** Because liver contains several ACSL isoforms and very-long-chain ACSs whose substrates overlap with those of ACSL, effects of hormones on liver ACS activity can be complicated and not representative of individual ACSL. To increase the amount of ACSL5 activity, we overexpressed ACSL5 in rat primary hepatocytes by adenovirus (Ad-ACSL5). In a previous study with rat hepatoma McArdle-RH7777 cells, Ad-ACSL5 increased total ACS activity and triacsin C-resistant activity 2-fold and 17-fold, respectively (22). Extending the method to the current study, primary hepatocytes were infected with Ad-ACSL5, and then incubated with insulin, glucagon and AICAR (agonist of AMPK) for 10 – 30 min. Surprisingly, Ad-ACSL5 increased total ACS activity only 12.9%, probably because of the high endogenous ACS activity in hepatocytes. However, ACS activity was decreased 12% by insulin but increased 5% by glucagon (**Figure 4.1 A**) (This experiment was performed only 2 times so that conclusion cannot be firmly drawn.). Since ACSL5 and ACSL6 are the only isoforms resistant to triacsin C inhibition (21,51) and ACSL6 is not present in liver (61), triacsin C will differentiate ACSL5 activity from other isoforms. Triacsin C inhibited ACS activity to only 8 - 10% in Ad-GFP infected cells and to 9% - 11% in Ad-ACSL5 infected cells (**Figure 4.1 B**), which is assumed to be mainly ACSL5 activity. Similarly, triacsin C resistant ACS activity was decreased 19% by insulin and increased 14% by glucagon,
Figure 4.1. **Effects of hormones on ACS activity in Ad-ACSL5 infected hepatocytes.** Rat primary hepatocytes (4.5 x 10^6 cell/10 cm plate) were infected with Ad-GFP or Ad-ACSL5 (MOI 10) for 24 h. Cells were treated with hormones (100 nM insulin x 20 min, 100 nM glucagon x 10 min, 0.5 mM AICAR x 30 min; NT, no hormone treatment). Homogenates were collected in MedI + DTT (10 mM Tris pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol) supplemented with protease inhibitor and phosphatase inhibitor. ACS specific activity was measured in the absence A) and in the presence B) of triacsin C 10 µM. Endogenous ACS specific activity in Ad-GFP infected cells was 138 nmol/min/mg protein. ACS activity was measured with 0.5, 1.0, 1.5 µg protein. Triacsin C does not inhibit ACSL5 and ACSL6 (21,51) and ACSL6 is not present in liver (61), so the triacsin C resistant activity is assumed to be ACSL5 activity. The experiment was repeated once.
indicating that ACSL5 might be acutely regulated by these hormones.

If the observed ACS activity increase by glucagon and decrease by insulin were mediated by phosphorylation and dephosphorylation of ACSL5, respectively, protection of the phosphorylated state should then increase ACSL5 activity. Because ACS5 is located in mitochondria (64), to answer this question, we collected mitochondria from mouse liver in the absence and presence of phosphatase inhibitors. Since in addition to ACSL5, mitochondria contain an undescribed triacsin-sensitive ACSL (64), triacsin C at different concentrations was used to differentiate ACSL5 activity from triacsin C-sensitive ACSL in mitochondria (Figure 4.2). At 8 µM, triacsin C inhibited mitochondrial ACS activity to 53% - 56%, supporting the notion that at least another triacsin C-sensitive ACSL isoform existed in liver mitochondria. At all tested concentrations of triacsin C, ACS activity was 5 – 10% higher in mitochondria collected in the presence of phosphatase inhibitors compared to mitochondria collected in the absence of phosphatase inhibitors. This increase was more obvious at 8 µM triacsin C (10%), when ACS activity was mainly comprised of ACSL5, suggesting that phosphorylation might increase ACSL5 activity (Figure 4.2 B).

**Identification of phosphorylated ACSL5.** Following the above observation, we asked the question whether ACSL5 was phosphorylated and if so, by what kinase(s). A way of testing this would be mass spectrometry analysis of relatively pure protein, which would identify
Figure 4.2.  Effects of phosphatase inhibitor treatment on ACS activity in mouse liver mitochondria.  A) Mitochondria from mouse liver were collected in MedI + DTT (10 mM Tris pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitor cocktail), in the absence or presence of phosphatase inhibitor cocktail I and II (Sigma).  ACS specific activity was measured without triacsin C or with triacsin C at indicated concentrations.  Endogenous mitochondrial ACS specific activity without phosphatase inhibitors or triacsin treatments was 116.2 nmol/min/mg.  ACS activity was measured with 0.5, 1.0, 1.5 µg protein.  B) Data were presented as percentage change of ACS activity by phosphatase inhibitors treatment at indicated triacsin C concentrations.  The experiment was repeated once.
which amino acid(s) is phosphorylated and imply a potential kinase based on comparison with consensus sites (171). To get relatively pure ACSL5, we first tried to immunoprecipitate the endogenous ACSL5 from rat primary hepatocytes using a peptide antibody raised against ACSL5. This method did not work, possibly because peptide epitope was unexposed during the immunoprecipitation. Then we infected hepatocytes with Ad-ACSL5, which carries an ACSL5 cDNA with a C-terminal FLAG tag, and immunoprecipitated ACSL5FLAG using an anti-FLAG agarose. We were able to immunoprecipitate the overexpressed ACSL5FLAG (Figure 4.3 A). Western blotting with anti-FLAG primary antibody confirmed the pulled down protein as ACSL5FLAG. A second band of larger size was present above the major band (Figure 4.3 B).

Following immunoprecipitation and SDS-PAGE, an in gel digestion was used to extract peptides for MALDI-TOF mass spectrometry. Digestion in solution could not be used because detergents used to increase membrane protein solubility causes significant difficulties in mass spectrometry (172). The coverage of peptide extracted was 32% - 36%. We detected phosphorylation of threonine 192 from Ad-ACSL5 infected hepatocytes that had not been treated or that had been treated with AICAR. No phosphorylated ACSL5 was found in hepatocytes that had been treated with epinephrine. These data suggest that ACSL5 might be phosphorylated at threonine 192 under basal conditions, and that the phosphate might be removed with epinephrine, but not AICAR stimulation. Sequence
Figure 4.3.  **Immunoprecipitation of ACSL5FLAG**  

A) Ad-GFP or Ad-ACSL5 infected rat primary hepatocytes were treated with AICAR or epinephrine and collected in RIPA buffer with phosphatase inhibitors. Lysates were subjected to immunoprecipitation with anti-FLAG M2 agarose. The pull-down was loaded on 10% precast gel and subject to SDS-PAGE. Coomassie-stained protein bands at the right size (arrows) were excised from the gel and subjected to tryptic digestion. The tryptic peptides were extracted and analyzed by Mass Spectrometry in the UNC Proteomics Core Facility.  

B) Lysates from Ad-ACSL5 infected hepatocytes (30, 50, 150 µg) were subject to Western blot directly, or after immunoprecipitation. Anti-FLAG primary antibody confirmed ACSL5FLAG at right size and detected a 2nd band. WB: western blot directly; S: supernatant from immunoprecipitation; IP: pull-down from immunoprecipitation.  

C) Ad-ACSL5 infected hepatocytes were treated with hormones (insulin, 100nM x 20 min, 1 µM epinephrine x 30min, AICAR 0.5 mM x 30 min, glucagons 100 nM x 10 min). Lysates were collected for immunoprecipitation by anti-FLAG agarose, followed by Western blot with anti-phosphoserine antibody, which was then reprobed with anti-FLAG antibody. Ad-GFP infected hepatocytes served as control.
comparison of this site in ACSL5 from human, rat and mouse (ClustalW alignment http://www.ebi.ac.uk/clustalw/) showed that this threonine was located in a highly conserved region (Scheme 4.1) different from the ATP/AMP or the FACS signature motifs (Scheme 1.2). In this region, threonine 192 was one of the few amino acids that were conserved in rodent ACSL5, but not in human ACSL5. Sequence alignment also showed that among the subfamily of rat ACSL1, ACSL5 and two variants of ACSL6, threonine 192 only existed in ACSL5. Thus, it is likely that phosphorylation on this threonine is conserved in rodents, and is isoform specific. This could be a mechanism by which rodent ACSL5 and its associated pathways are specifically regulated.

To our surprise, however, this threonine site is not predicted by the Netphos program (http://www.cbs.dtu.dk/services/NetPhos/) as a consensus site for any common protein kinase, such as PKA, protein kinase C (PKC), or AMPK. It might be a site of casein kinase-II (TXXXE compared to the consensus sequence of [S/T]-(X)2-[D/E]) (Nicesite View http://ca.expasy.org/cgi-bin/nicesite.pl?PS00006). However, because the function of casein kinase-II is unclear, and phosphorylation on threonine 192 was found in the cells before and after exposure to AICAR, but not to epinephrine, our data did not suggest any physiological significance of this phosphorylation. Minimotif Miner (MnM) analysis (http://sms.engr.uconn.edu/servlet/SMSSearchServlet) showed that the region containing threonine 192 could be a FHA (forkhead associated protein) binding motif, which requires phosphorylation of threonine 192, suggesting that ACSL5 might be bound by some signal
Scheme 4.1. Identification of threonine phosphorylation on rat ACSL5. Immunoprecipitated ACSL5FLAG bands from Figure 4.3A (arrows) were sent for mass spectrometry. MALDI-TOF identified that bands as rat ACSL5 and detected phosphorylation on threonine 192 from cells that had not been treated or that had been treated with AICAR. Sequence comparison (ClustalW, http://www.ebi.ac.uk/clustalw/) showed that threonine 192 was conserved in rodent ACSL5, but not in human ACSL5. Among the subfamily of rat ACSL1, 5, and 6, threonine 192 was only present in rat ACSL5. Highlighted T is the threonine 192 found to be phosphorylated in rat ACSL5.
proteins and involved in signaling (173).

Since the in gel digestion extracted only 32% - 36% of the protein and did not include the potential PKA site or AMPK sites on ACSL5, it is possible that phosphorylation on other sites might have been missed. In order to test whether ACSL5 was phosphorylated at serine residues, we performed immunoprecipitation followed by immunoblot with anti-phosphoserine (anti-PS) antibody. Western blot with anti-PS detected serine phosphorylated ACSL5FLAG (Figure 4.3 C). However, it seems that hormone treatment did not change the phosphorylation state, suggesting that this serine phosphorylation might be constitutive. An limitation of this study is that sample without hormone treatment is lacking.

**Potential acute regulation of ACSL1 in 3T3-L1 cells?** Our finding of phosphorylated ACSL5 prompted us to examine whether ACSL1 was also phosphorylated, since previous studies from other groups showed that microsomal ACS activity was regulated rapidly by hormones in adipocytes, where ACSL1 was the predominant ACSL isoform. 3T3-L1 CARΔ1 preadipocytes were used because of their very low endogenous ACS activity and lack of expression of ACSL1 (48,60). These cells are infected with adenovirus at higher efficiency because they express modified coxsackie and adenovirus receptor (CAR) (168,169). We overexpressed rat ACSL1 by recombinant adenovirus (Chapter III & Appendix) and checked ACS activity after different hormone treatment for 10 – 30 min. In
these cells, ACS activity was increased by insulin slightly but not changed by glucagon (Figure 4.4 A). Conversely, 8-Br-cAMP, a permeable analog of cAMP, decreased ACS activity (Figure 4.4 B).

ACSL1 has been identified in lipid droplet fractions in 3T3-L1 adipocytes. When lipid droplets were isolated from 3T3-L1 adipocytes by a fractionation technique, proteomic analysis of lipid droplet associated proteins by mass spectrometry identified ACSL1 (89). Since some contaminant proteins such as fatty acid translocase (CD36) from plasma membrane, and ATP synthase from mitochondria, were also identified in the lipid droplet fractions, the validity of the ACSL1 localization needs to be further proved. The role of ACSL1 in lipid droplets is unknown. Based on previous studies and current study, we hypothesized that lipolytic hormones might inhibit ACSL1 activity by phosphorylation and that lipogenic hormones might stimulate ACSL1 activity by dephosphorylation (Scheme 4.2). In this proposed model, when lipid is mobilized, lipolytic hormones, such as glucagon and epinephrine, phosphorylate ACSL1 and HSL, which results in inhibition of ACSL1 but stimulation of HSL. PKA also phosphorylates perilipin A, which facilitates the association of HSL to lipid droplets and hydrolysis. Inhibition of ACSL1 while HSL is active would prevent the re-activation of released fatty acids and their re-esterification back to glycerolipids, hence preventing a futile cycle and energy waste (2 ATP/fatty acid activation). Conversely, during lipogenesis, lipogenic hormones might cause dephosphorylation of ACSL1, HSL and perilipin A, and activate ACSL1 while inhibiting HSL, thus providing
Figure 4.4.  Effects of hormones on ACS activity in 3T3-L1 preadipocytes infected with Ad-ACSL1.  A) 3T3-L1 ∆CAR preadipocytes (60%~70% confluent) were infected by Ad-ACSL1 (MOI 10) for 14 h.  Cells were treated with hormones (1 µM Insulin x 30 min or 1 µM glucagon x 30 min).  B) 3T3-L1 ∆CAR preadipocytes (60%~70% confluent) were infected by Ad-ACSL1 (MOI 10) for 12 h and then stimulated by 8Br-cAMP (Sigma B 7880) at 1 mM or 2 mM for 50 min.  Homogenates were collected in MedI + DTT (10 mM Tris pH 7.4, 250 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol) supplemented with protease inhibitor and phosphatase inhibitor, and ACS specific activity was measured.  ACS activity was measured with 0.5, 1.0, 1.5 µg protein.  The experiment was repeated twice.
Scheme 4.2. *Proposed model of hormonal acute regulation of ACSL1 in adipocytes.*

**A)** During lipid mobilization, lipolytic hormones (glucagon, epinephrine) bind to GPCR and activate AC, which produces cAMP and release the catalytic subunits of PKA. Activated PKA phosphorylates perilipin A, ACSL1 and HSL, which results in inhibition of ACSL1, but stimulation of HSL. Phosphorylated perilipin A facilitates the translocation of HSL from the cytoplasm to the lipid droplet, where efficient TAG hydrolysis occurs (161). ACSL1 might be located on lipid droplets (89). Active HSL releases free fatty acids from TAG and inactive ACSL1 prevents fatty acid re-esterification, thus facilitating the release of fatty acids to plasma. The inhibition of ACSL1 while HSL is active would prevent a futile cycle of TAG hydrolysis and fatty acid re-esterification.

**B)** During lipogenesis, lipogenic hormones (insulin) activate phosphodiesterase and phosphatase. Phosphodiesterases hydrolyze cAMP and prevent the release of PKA catalytic subunits. Phosphatases dephosphorylate ACSL1 and HSL, resulting in activation and inactivation, respectively. Active ACSL1 provides acyl-CoA substrates for TAG synthesis, which will be stored in lipid droplets. AC, adenylate cyclase; ACSL1, long chain acyl-CoA synthetase 1; C, PKA catalytic subunit; Des, phosphodiesterase; FFA, free fatty acids; Gs, stimulatory G protein; GPCR, G protein-coupled receptor, HSL, hormone sensitive lipase; Peri A, perilipin A; PKA, protein kinase A; R, PKA regulatory subunit.
acyl-CoA substrates for TAG synthesis. Unphosphorylated perilipin serves as a blocker against HSL and thus inhibit hydrolysis. It has been reported that lipid futile cycles exist in adipocytes (174) but the role of ACSL1 has not been studied. Our model thus also provides a potential role of ACSL1 in regulation of futile cycle and fatty acid release under different nutritional status.

To test this hypothesis, we tried several methods. Our data were disappointing because the results showed no difference or were inconsistent. First, rat ACSL1FLAG was purified by affinity chromatography method as described (Appendix 6.2), and incubated with PKA at different doses, immediately followed by ACS enzyme assays. Our data did not show a consistent ACS activity change by PKA, nor were apparent 2nd bands found by Western blot with anti-FLAG antibody (data not shown). Second, to examine whether hormone treatment affected ACS activity in 3T3-L1 adipocytes, we treated differentiated 3T3-L1 adipocytes with insulin, dibutyryl-cAMP, AICAR, DiC8 (a cell permeable PKC activator), and isoproterenol. We did not find a reliable change in ACS activity as previously reported with rat primary adipocytes. One possibility could be that different regulation and kinase responses exist between 3T3-L1 adipocytes and primary adipocytes. Third, we immunoprecipitated ACSL1FLAG from Ad-ACSL1 infected 3T3-L1 CARΔ1 adipocytes, followed by Western blot with anti-PS antibody. Although serine phosphorylated ACSL1 was detected, no difference in protein amount or size was found in adipocytes treated with
lipogenic or lipolytic hormones (Figure 4.5).

**Insight for future research.** The data presented in this chapter show that ACSL can be phosphorylated. This post-translational modification might be isoform specific since the phosphorylation site identified on ACSL5 is not conserved on other ACSL isoforms. It is likely that hormones elicit tissue-specific effects on different ACSL isoforms, depending on the physiological roles of the tissues and ACSL isoforms. Although we did not detect marked changes on ACS activity by hormone treatments, regulation systems in 3T3-L1 adipocytes might differ from those in primary adipocytes. It is also possible that, phosphorylation might cause other effects, such as subcellular translocation or a conformational change of the ACSL isoform that alters their interaction with other proteins.

Based on the data observed, future research might exploit other methods or models to study the significance of acute regulation by phosphorylation. First, sample preparation for mass spectrometry needs to be optimized, probably by in solution digestion, with digestible detergents that can be removed from membrane proteins before mass spectrometry (172). Second, site-directed mutagenesis can be used to change potential phosphorylation sites, followed by examination of enzyme kinetics. For example, the potential serine phosphorylation sites for PKA in ACSL1 can be replaced with alanine (S74A and S411A) to block phosphorylation, or with glutamate (S74E and S411E) to mimic constitutive phosphorylation. Third, site-specific antibodies can be made against phosphorylated ACSL.
Figure 4.5. *Phosphorylated ACSL1 in adipocytes.* Ad-ACSL1 infected 3T3-L1ΔCAR adipocytes were treated with hormones (0.5 mM AICAR x 30 min, 2 mM dibutyryl-cAMP x 60 min, or 40 nM Insulin x 20 min). Lysates were collected for immunoprecipitation by anti-FLAG agarose, followed by Western blot with anti-phosphoserine antibody, which was then reprobed with anti-FLAG antibody. Reprobing with anti-FLAG confirmed that detected phosphoserine bands were ACSL1FLAG.
Then the effects of different hormones can be studied specifically rather than with the general phosphoserine antibody. Moreover, primary adipocytes model is worthy to consider although they need high dose of adenovirus for efficient infection.
CHAPTER V

SYNTHESIS

This dissertation describes three independent projects that examined the function and regulation of rat ACSL isoforms. The results provide strong evidence supporting our hypothesis that individual ACSL isoforms provide fatty acyl-CoA to specific downstream metabolic pathways. We also showed, for the first time, that ACSL isoforms could be phosphorylated, which might be an important regulatory mechanism of fatty acid channeling by ACSL. This chapter will summarize results from these projects and discuss how our finding may contribute to future research and clinical implication for treatment of obesity and diabetes.

5.1 Different capacities of rat ACSL isoforms in replacing bacterial ACSL

Using a bacteria strain with its own ACSL mutated (fadD), we transformed individual rat ACSL isoforms to examine whether they are different in their capacities in replacing this sole ACSL in bacteria. We demonstrated that only ACSL5 was able to support growth in the medium with oleate as the sole energy and carbon source, although the in vitro specific activities of all isoforms were greater than that of the endogenous FadD. ACSL isoforms variably and partially restored the capacities of exogenous fatty acids transport, β-oxidation,
and PL synthesis. This study added to the evidence that ACSL isoforms are not redundant, but functionally different. Consistent with our finding, rat ACSL isoforms also differ in their capacities in replacing the ACSL in yeast ($\text{faa1}\Delta\text{faa4}\Delta$) (6). In the yeast model, rat ACSL1, 4, and 6, but not ACSL5 rescue fatty acid transport activity and TAG synthesis. The different complementation between the prokaryotic and eukaryotic model systems suggests that membrane composition and protein-protein interactions may be critical for function of ACSL. The bacterial inner membrane composition is more similar to the mitochondrial membrane in mammalian cells where ACSL5 is located (64), whereas the yeast plasma membrane is more similar to mammalian plasma membranes, where ACSL1 is located (6,64).

An interesting question derived from the observation in $E.\ coli$ study is the importance of membrane location for function of ACSL. Different organelles adopt distinct membrane characteristics. For example, the mitochondria membrane contains cardiolipin (175) and plasma membrane contains different PL on the outer and inner leaflets (176). Since all ACSL isoforms, except ACSL4, which is a peripheral membrane protein, are predicted to have at least one transmembrane domain (ExPASy topology prediction, http://www.expasy.org/tools/#topology), the subcellular location is important for their function. We do not know how ACSL isoforms are targeted to distinct membranes, and whether their re-localization would affect their functions. Future studies to explore this question would require sequence truncated ACSL and ACSL with synthetic peptide signals.
that relocate to specific organelles.

5.2 Fatty acid channeling by ACSL isoforms

Our study replacing *E. coli* FadD with rat ACSL isoforms also indicated different roles among them. Thus, the next step was to examine the function of an individual isoform. We found that overexpression of ACSL1 in rat primary hepatocytes channeled fatty acids towards DAG and PL synthesis, and away from CE synthesis and medium TAG. ACSL1 overexpression did not change oleate incorporation into TAG or β-oxidation. These results are different from the effects of ACSL5 overexpression in rat hepatoma McArdle-RH7777 cells in a previous study from our lab (22). Overexpression of ACSL5 increases fatty acid incorporation into DAG and TAG, but does not affect fatty acid used for β-oxidation, PL, CE, or medium TAG. These two studies together provide direct evidence for our hypothesis that different ACSL isoforms have distinct functions in lipid metabolism.

We found that ACSL1 overexpression did not increase TAG synthesis in primary hepatocytes, unlike what occurs in heart-specific ACSL1 transgenic mice (86). This leads to two interesting questions: What will be the effects of overexpression of ACSL1 *in vivo*? Does ACSL1 or/and other isoforms have tissue specific functions in lipid metabolism? Overexpression of ACSL1 *in vivo* by adenovirus infection or a transgenic technique would provide a more physiological model for studying the role of ACSL1 in lipid metabolism, and also address questions about VLDL secretion, hepatic insulin sensitivity, and response to
nutritional status change. Since adenovirus mainly targets liver, to answer the second question of tissue-specific function, more practical methods would be in vitro studies using different cell types.

The pressing question arising from what we found is, what are the mechanisms behind the fatty acid channeling by ACSL isoforms? The different results from replacement studies in E. coli and yeast, and overexpression studies of ACSL1 and ACSL5, suggest that protein-protein interactions might play a role in fatty acid channeling into different downstream pathways. Although it has been reported that a physical association between ACSL and fatty acid transport proteins, such as Faa1p with Fat1p in yeast (8) and ACSL1 with FATP1 in adipocytes (20), is important for fatty acid uptake, no study has shown interaction between individual ACSLs with intracellular proteins. Our preliminary studies found that a protein of bigger size co-immunoprecipitated with ACSL1 (data not shown). Moreover, mass spectrometry analysis of protein bands that co-immunoprecipitated with overexpressed ACSL5 in hepatocytes identified proteins such as pyruvate carboxylase. We believe that ACSL is involved in protein complexes within subcellular locations, and interaction between different isoforms with downstream enzymes might facilitate fatty acid partitioning. Interaction of ACSL with different proteins available in specific tissues might mediate tissue-specific functions of ACSL. Potential interacting proteins include fatty acid binding proteins that might provide substrates to ACSL, ACBP that bind acyl-CoA, which might release the product inhibition on ACSL, downstream acyltransferases that use
acyl-CoAs for different pathways, such as CPT1 for oxidation, GPAT for LPA synthesis, and ACAT for CE synthesis, and transcription factors that use acyl-CoAs as ligands. To address this question, a yeast two-hybrid system might be used first to screen for potential interacting proteins at a wide scale. Co-immunoprecipitation can then be exploited to confirm the interaction between ACSL1 and potential interacting proteins. Mass spectrometry of proteins that are co-immunoprecipitate with ACSL1 would also be a good way to identify proteins that interact with ACSL1 under different conditions, such as with hormone treatment. However, due to the potential artificial effects (false positive interactions) when proteins are overexpressed (177), multiple methods should be tried to confirm an interaction.

5.3 Acute regulation of ACSL isoforms

Acute regulation of ACSL has been relatively ignored in the literature, partly because ACS activity did not always respond to nutritional status (80) and because of the difficulty in interpreting ACSL activities which are comprised of several ACSL isoforms. We hypothesized that ACSL isoforms could be regulated by phosphorylation, based on the reported rapid microsomal ACS activity change by hormones (insulin, epinephrine, glucagon) in rat adipocytes (101,102). In addition, on a Western blot with anti-ACSL1 antibody, we observed a 2nd band in 3T3-L1 adipocytes (data not shown). Since ACSL1 is predominant in 3T3-L1 adipocytes, and is associated with lipid droplets together with HSL and perilipin A (89), which are both regulated by phosphorylation, we studied potential phosphorylation on ACSL1 in these cells. Moreover, we studied ACSL5 in hepatocytes because ACSL5 in liver
might provide acyl-CoAs for mtGPAT1, which is regulated by AMPK (166), and because potential AMPK sites exist in rat ACSL5.

This study is the first one to identify phosphorylated forms of ACSL. Mass spectrometry following immunoprecipitation identified ACSL5 with a phosphorylated threonine 192, from hepatocytes that had not been treated or had been treated with AICAR. This threonine 192 exists only in ACSL5, but not in the other four isoforms, suggesting that phosphorylation could elicit isoform specific effects. Western blot with an anti-phosphoserine antibody also showed phosphorylated bands after immunoprecipitation of ACSL5 from hepatocytes and ACSL1 from adipocytes, suggesting that ACSL5 could be phosphorylated at different sites. The limit of this study, however, is that we were not able to solve the physiological significance of phosphorylation regulation. Our studies with different hormone stimulation showed no change of either ACS activity or the density of phosphorylated bands. Since multiple isoforms contribute to the total ACS activity, it is possible that opposite effects on individual isoforms resulted in no net change of activity. Alternatively, phosphorylation might elicit changes other than activity, for example, association of ACSL with other proteins. Since the identified threonine 192 in ACSL5 is not a consensus site for common protein kinases such as PKA, PKC and AMPK, but located in a FHA binding motif to which proteins with an FHA domain might bind, it is possible that this phosphorylation is linked to signaling processes.
Further study to examine the mechanism and physiological effects of phosphorylation would require better methods of sample preparation for mass spectrometry, since our extraction of peptides after in-gel digestion was only 32% - 36% and phosphorylation on other sites might have been missed. After identification of phosphorylation sites, site-specific antibodies would detect endogenous phosphorylated proteins under different hormone treatment, and can be used for samples from \textit{in vivo} study. Further, site-directed mutagenesis can be exploited to study the importance of the phosphorylation sites. Effects of phosphorylation might not be limited to activity change, although phosphorylation frequently affects enzyme activities.

5.4 Future direction and studies

The most pressing and interesting questions that remain to be answered are varied. In addition to studying in vivo effects of ACSL1, interaction of ACSL with other proteins, phosphorylation regulation, and tissue-specific functions as mentioned above, the following aspects would be important for us to better understand the roles of ACSL isoforms, and hence enable us to find potential therapeutic targets for diseases such as obesity, hepatic steatosis, insulin resistance, and possibly, cancer.

5.4.1 \textit{ACSL1 knockout mice as a model for treatment} Since ACSL partitions fatty acyl-CoAs to specific downstream pathways, and the balance of anabolic and catabolic pathways determines the lipid accumulation in cells, the specific isoform that channels
Acyl-CoAs to TAG synthesis will then be a potential therapeutic target for obesity and dyslipidemia. ACSL1 has been shown to be associated with TAG synthesis in adipocytes and heart cells when overexpressed, but the potential benefits of inhibiting ACSL1 await studies on knockout models.

Knock out models are a powerful method to examine directly the role of ACSL in fatty acid channeling. This is particularly true when multiple ACSL isoforms contribute to high endogenous ACS activity, which is difficult to be increased by overexpression of one isoform. Knock out models will then be more revealing than an overexpression approach in the tissues with high ACSL expression, such as liver and adipose tissues. Following the study of overexpressing ACSL1 in hepatocytes, which showed differences in lipid metabolism from overexpressing ACSL1 in heart, we hypothesized that the role of ACSL1 might be tissue-specific. That is, ACSL1 might provide acyl-CoAs for TAG synthesis in adipose tissue, whereas might provide acyl-CoAs for PL synthesis in liver. Currently we are making liver and adipose tissue-specific ACSL1 knockout mice, which will be useful models for us to determine 1) whether ACSL1 is essential for lipid synthesis in adipocytes, 2) whether deletion of ACSL1 in liver would affect PL and CE synthesis, and VLDL assembly and secretion, and 3) whether an ACSL1 deletion will change the response to diet challenge or diet induced obesity, as well as insulin sensitivity.

If, as hypothesized, ACSL1 has tissue-specific functions, caution is needed to target
ACSL1 as a treatment for obesity. Decreased PL or disturbance of PL species in liver might affect membrane and organelle functions, signal transduction, and assembly of VLDL, thus changing the morphology and function of liver cells.

5.4.2 Effects on gene expression An interesting aspect will be to define the role of ACSL isoforms in affecting gene expression, because acyl-CoAs might be natural ligands for transcription factors (23,24,38-40). Both ACSL protein and activity has been detected in nuclei of mammalian cells, and acyl-CoAs exist in nuclei fractions (41). We found that LPA, which is the product of GPAT from acyl-CoA and glycerol-3-phosphate, might be a ligand for PPARγ (Stapleton CM and Coleman RA, unpublished). Thus, if a specific ACSL isoform provides acyl-CoA to GPAT, overexpression or knockout of this isoform would elicit effects on gene expression by increasing or decreasing the LPA ligand for PPARγ. Other potential transcriptions factors that might use acyl-CoAs are PPARα (38,39) and HNF-4α (40). Both overexpression and knockout models would be useful to examine the effects of ACSL on the target genes of these transcription factors, and then on a broad range of processes including glucose and lipid homeostasis.

5.4.3 ACSL in apoptosis and cancer It has been proposed that free fatty acids and several lipid species initiate or promote apoptosis (1). For example, ceramide, the product of saturated fatty acid, induces apoptosis (178). Therefore, as the key enzyme for fatty acid activation and metabolism, ACSL may play a role in apoptosis and related diseases. In fact,
ACSL isoforms have been found to change in certain tumors. Some colon adenocarcinomas and colon carcinoma are associated with increases of ACSL4 or ACSL5 mRNA (70,73). Overexpression of ACSL4 in ECR 293 cells decreased apoptosis 25%, whereas triacsin C, which can inhibit ACSL4, increased apoptosis 9-fold in HT29 cancer cells (70). It has been proposed that increasing the C20:4 preferring ACSL4 might decrease free C20:4, which is important for apoptosis (70). Following the observation that C20:4 signals apoptosis, several studies have been conducted on the role of cyclooxygenase-2 in apoptosis, but the enzyme that transfers C20:4 to acyl-CoA, ACSL4, was ignored. The association between ACSL and apoptosis is also supported by the data from ACSL1 heart transgenic mice, which showed increased cytochrome c release, ceramide content and apoptosis (86). Moreover, we also observed decreased cell viability and changed cell morphology when primary hepatocytes were infected with high dose of Ad-ACSL1. The cells became dense and round, formed membrane blebs, and lost their shape and contact with neighboring cells. This did not occur in cells that were infected with Ad-GFP, thus excluding the possibility of toxicity from adenovirus infection itself.

Since ACSL isoforms are critical in fatty acid channeling, specific ACSL isoforms might mediate apoptosis. For example, the mitochondrially located ACSL5 might provide fatty acyl-CoAs for cardiolipin remodeling which would decrease cytochrome c release and apoptosis. Conversely, ACSL1 might provide acyl-CoA for ceramide synthesis, which would increase apoptosis. Studies of the effects of ACSLs on apoptosis and cancer
development would thus provide insight into potential targets for cancer.

Taken together, the more we learn, the more questions we have. The current studies further our understanding of function and regulation of ACSL isoforms, and prompt us to explore the mechanisms and application beyond the observations. In the long term, we would be able to utilize the knowledge of specific functions of individual ACSL isoforms, and target individual isoforms for therapeutic aims.
APPENDIX I:
DETAILED METHODS

6.1 Construction of adenovirus carrying rat ACSL1

We used the method in Dr. Newgard’s lab (Duke University) to make an adenovirus carrying a cDNA of rat ACSL1 with a C-terminal FLAG epitope (131). First, a full-length rat ACSL1 cDNA with a C-terminal FLAG epitope (DYKDDDDK) was subcloned from a previously constructed pTRE2hyg plasmid (pTRE2hyg-ACSL1) into a shuttle vector, pACCMVpLpA at the Bam HI and Sal I sites (Scheme 6.1). The inserted ACSL1FLAG in resulted construct (pACCMV-ACSL1) was confirmed by restriction enzyme digestion and DNA sequencing at the UNC DNA sequencing facility. Then this pACCMV-ACSL1 plasmid was transiently transfected into CHO cells via FuGene 6 (Roche) for 24 h, with empty vector and the original pTRE2hyg-ACSL1 as negative and positive controls, respectively. ACS enzyme assay showed increased activity (2.9-fold) by pACCMV-ACSL1 (Figure 6.1 A) and protein size was confirmed by Western blot with anti-FLAG primary antibody. Second, the shuttle vector pACCMV-ACSL1FLAG and the adenovirus vector pJM17 were amplified in bacteria and purified using MaxiPrep (QIAGEN). The plasmids were co-transfected into 293 cells by calcium phosphate co-precipitation. When a homologous recombination event occurs, the resulting recombinant adenovirus caused cell lysis and the formation of plaques of dead cells (Scheme 6.2). Seven single plaques were picked and 293 cells were infected. Lysates were collected and used to infect COS-7 cells, using uninfected cells and empty vector infected cells as controls. The ACS enzyme assay
showed 49% - 89% activity increase from lysates # 1, 3, 5, 6 and 7, with #3 the highest increase (89%) (Figure 6.1 B). Third, lysates #3 was sent to the UNC Vector Core Facility for further purification and amplification. 3T3-L1 preadipocytes were infected with purified adenovirus carrying ACSLFLAG (Ad-ACSL1) (1.3 x 10^8 pfu/µl). At MOIs 5, 10, 20, 50, and 100, Ad-ACSL1 increased ACS activity 3-, 10-, 19-, 40-, and 60-fold, respectively, showing a linear increase with dose-dependence. Complete inhibition by triacsin C indicates that the increased ACS activity is due to the overexpressed ACSL1 (Figure 6.1 C).

6.2 Different substrate preferences among rat ACSL isoforms

When we constructed recombinant pFLAG-CTC-ACSL plasmids for E. coli FadD complementation study (Chapter 2.1), we identified a novel splice variant of ACSL6, which arises from alternative slicing of exon 13 instead of exon 14. The newly identified rnACSL6_V2 cDNA has been deposited in the GenBank database (accession number AY625254). This variant encodes an ACSL6 protein with 12 different amino acids (termed ACSL6_v2, previously reported termed ACSL6_v1). Our previous studies purified rat ACSL1, 4 and 5, the three main isoforms in liver, and found distinct difference in enzyme kinetics. Since ACSL3 and ACSL6 predominate in brain and their substrate preferences might be important for neuronal lipid metabolism and brain development, the current study was to directly examine the preference of these ACSL isoforms for several fatty acids important in brain metabolism. Data described in this chapter was part of the work
published as a co-author (51).

**Expression of recombinant ACSL3-, ACSL4-, ACSL6_v1-, and ACSL6_v2-FLAG proteins in E. coli.** Recombinant plasmids for the expression of ACSL3, ACSL4 and ACSL6 with a C-terminal FLAG epitope were constructed (Chapter 2.1) and expressed in *E. coli* BL21-CondonPlus(DE3)-RIL which was grown at 25 °C with shaking at 250 rpm in LB broth supplemented with 50 µg/mL carbenicillin. When the bacteria were in mid-log growth (*A*<sub>600</sub> = 1.0), production of recombinant ACSL-Flag proteins was induced with 1 mM IPTG. After a 15-h induction, cells were harvested by centrifuging at 5000 rpm for 10 min in a Sorvall HS-4 rotor. The cell pellet was resuspended in 10 mM HEPES (pH 7.8), 0.5 mM EDTA, and 100 µg/mL lysozyme, and incubated for 30 min on ice. The cells were then sonicated with six 10-s bursts with a 10-s rest period between each burst. Sonicated samples were centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was layered over a 2 mL cushion of 55% (w/w) sucrose topped with 0.5 mL of 5% (w/w) sucrose in HEPES/EDTA buffer. After centrifugation in a SW41 rotor at 35000 rpm for 3 h at 4 °C, the membrane fraction was removed with a 19-gauge needle and syringe.

**Purification of recombinant ACSL3-, ACSL4-, ACSL6_v1-, and ACSL6_v2-FLAG proteins.** ACSL3-, ACSL4-, ACSL6_v1-, and ACSL6_v2-FLAG were purified by Flag M2 column chromatography. The anti-Flag M2 antibody affinity matrix (1 mL) was activated with 0.1 M glycine (pH 3.5) followed by equilibration with 50 mM Tris (pH 7.4), 150 mM
NaCl (TBS) buffer. BL21 membrane fractions containing recombinant ACSL3-, ACSL4-, ACSL6_v1-, and ACSL6_v2-FLAG were solubilized in TBS containing 1% Triton X-100 and passed over the affinity column four times. The column was washed twice with 10 mL of TBS (pH 7.4), and then eluted with five 1 mL aliquots of 100 µg/mL Flag peptide dissolved in TBS buffer. Lysates, cell extracts, membranes and elutes were electrophoresed on a 10% acrylamide gel containing 0.1% SDS and stained with Gel Code blue stain reagent (Pierce) (Figure 6.2).

**Competition ACSL Assays.** A competition ACS assay was conducted with 50 µM [14C]palmitate as described in Chapter 2.1, in the absence or presence of unlabeled C20:4, C20:5, C22:6, C18:2, or C18:3 at varying concentrations (0.5 µM – 100 µM). The unlabeled fatty acids competed with labeled palmitate as substrates for ACSL isoforms. Thus, by measuring the labeled palmitoyl-CoA, we were able to directly examine the preferences of ACSL for different fatty acids. The assay was initiated with 0.2 µg of purified protein and ACS activity was measured at 37 °C after a 5-min incubation (Figure 6.3). When assayed in the presence of increasing concentrations of unlabeled C20:4, ACSL4 activity was strongly inhibited with an IC₅₀ of 0.5 µM C20:4 (Figure 6.3 C). In contrast, ACSL6_v1 and ACSL3 did not have a preference for C20:4 compared with C16:1 (IC₅₀ ~45 µM), whereas ACSL6_v2 preferred C16:1 slightly more than C20:4 (IC₅₀ = 90 µM). Indeed, ACSL4 strongly preferred all of the PUFAs tested to C16:1, with IC₅₀ values ranging from 0.5 µM to 5 µM. Since PUFAs are important precursors for eicosanoids and
brain phospholipids, and thus critical for brain development and function, it is possible that lipid metabolism change plays a role in the ACSL4 mutation-linked mental retardation (54). Of the isoforms tested, only ACSL4 strongly preferred C20:4 and C20:5 to C16:1. C22:6 was strongly preferred by both ACSL4 and ACSL6_v2 (IC$_{50}$ = 3 µM and 5 µM, respectively). C18:2 and C18:3 were strongly preferred to C16:1 by ACSL4 and ACSL6_v1. Thus, the splice variants of ACSL6 display different substrate preferences despite only 1.7% difference in sequence. These data increased our understanding of enzyme kinetics among ACSL isoforms abundant in brain.

6.3 Nutritional regulation of ACSL1 and ACSL4 in rat liver

We previously showed that proteins levels of ACSL1, 4 and 5 in rat liver changed differently by nutritional status (64,80). Both ACSL1 (95%) and ACSL4 (64%) increase in rats that are refed a high sucrose diet after 48 h fasting, whereas ACSL5 increases 82% after fasting 48 h. To examine whether the response of protein was concordant with the change of mRNA, we compared the mRNA and protein levels of ACSL1 and ACSL4 in rat liver, under chow fed diet, fasting 48 h, and refeeding with a high sucrose diet 24 h after 48 h fasting. Consistent with previous data, fasting decreased and refeeding increased ACSL1 protein, and refeeding increased ACSL4 protein in liver (Figure 6.4 A and B). However, the change of mRNA was almost the opposite to that of protein. Fasting 48 h increased ACSL1 mRNA around 25%, which was decreased to only half by refeeding a high sucrose diet (Figure 6.4 C). The mRNA change of ACSL4 showed a similar pattern to ACSL1
mRNA. Our data showed dissociation of the change in mRNA level and the change in protein abundance under nutritional change. It is likely that ACSL isoforms are under regulation at post-transcriptional and post-translational levels. These data also suggest that, to understand the function of ACSL and other enzymes in metabolism, determination of products from different pathways is necessary in addition to enzyme abundance.

This is a part of work in a paper published as a co-author (61). This paper compared tissue distribution of all ACSL isoforms in rat, and the response to dietary change in adipose tissues, skeletal muscle, and liver. Isoform-specific distribution and responses supported our hypothesis that ACSL isoforms channel acyl-CoAs into specific pathways, and can be regulated differently under nutritional conditions.
Scheme 6.1. **Construction of shuttle vector containing ACS1FLAG** A full-length rat ACS1 cDNA with a C-terminal FLAG epitope (DYKDDDDK) was subcloned from a previously constructed pTRE2hyg plasmid into a shuttle vector, pACCMVpLpA at the Bam HI and Sal I sites. Expression of the inserted ACS1FLAG cDNA is driven by the cytomegalovirus promoter (CMV). The inserted ACS1FLAG in the pACCMV-ACSL1FLAG construct was verified by restriction enzyme analysis and confirmed by DNA sequencing at the UNC DNA sequencing facility.
Scheme 6.2. General strategy for preparing recombinant adenovirus by homologous recombination. The shuttle vector pACCMV-ACSL1FLAG and virus vector pJM17 are co-transfected into 293 cells by calcium phosphate co-precipitation for homologous recombination to form recombinant adenovirus carrying ACSL1FLAG cDNA (Ad-ACSL1). Adenovirus causes lysis of cells and lysates are collected and transfected to COS7 cells for ACS activity check (Figure 6.1B). Virions from selected lysate are further purified and amplified (131). Purified adenovirus is infected into 3T3-L1 preadipocytes for ACS activity check (Figure 6.1C).
Figure 6.1. **ACS activity check during recombinant adenovirus production.**  
A) Plasmids were transfected into CHO cells and ACS specific activity was measured. The shuttle vector pACCMV-ACSL1FLAG ([Scheme 6.2](#)) increases ACS activity, close to original pTRE2hyg transfected cells.  
B) Lysates from 293 cells after co-transfection were infected in COS7 cells, with uninfected and EV infected cells as controls. Lysate #3 increased ACS specific activity the most, and thus was used for virus purification and amplification.  
C) Purified adenovirus carrying ACSL1FLAG (Ad-ACSL1) was infected in 3T3-L1 preadipocytes at indicated doses. ACS enzyme assay was conducted in the presence (filled bar) or absence (open bar) of Triacsin C at 10 µM.
Figure 6.2. Expression and purification of recombinant ACSL3-, ACSL4-, ACSL6_v1-, and ACSL6_v2-FLAG proteins. Different fractions in the purification were electrophoresed on a 10% acrylamide gel containing 0.1% SDS and stained with Gel Code blue stain reagent (Pierce). The molecular weight markers are on the left (Lane M). The arrow indicates the position of the ACSL-FLAG protein. Fractions: Lys, lysate; CE, cell extract; Me, membrane fraction; Sol, soluble fraction; F, flow through; 1a, 1b, 2, 3 are eluted fractions. ACSL6_v1, ACSL6_v2 and ACSL3 are partially purified, as multiple bands indicate.
Figure 6.3. **Different substrate preferences among rat ACSL isoforms.** Flag-purified ACSL6_v1-Flag (�性), ACSL6_v2-Flag (性), ACSL3-Flag (性), and ACSL4-Flag (性) were assayed for ACS activity with 50 \( \mu \text{M} \) [\(^{14}\text{C}\)palmitate in the absence or presence of unlabeled A) linoleic, B) linolenic, C) arachidonic, D) eicosapentaenoic, and E) docosahexaenoic acid (0.5 \( \mu \text{M} \) to 100 \( \mu \text{M} \)). F) Relative IC\textsubscript{50} for each fatty acid (51).
Figure 6.4. Expression of ACSL1 and ACSL4 under chow diet, fasting, and refeeding after fasting. Rats (130g, male) were fed a chow diet, fasted for 48 h, or fasted for 48 h and then refed a high sucrose diet (60%) for 24 h. A) Liver were homogenized in buffer containing 250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol on ice. Homogenates were subjected to SDS-PAGE and Western blot with primary antibodies against ACSL1 and ACSL4, with antibody against β-actin as loading control. B) Densitometry analysis of ACSL1 and 4 immunoblots normalized to β-actin. C) RNA was extracted (Qiagen RNeasy) and quantified by Applied Biosystems 7700 sequence detection system. Data represent means ± SEM (n = 4). * p < 0.05 and ** p < 0.01 vs. chow-fed; # p < 0.05 and ## p < 0.01 vs. fasted as determined by Student’s t-test (61).
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