Abstract

Obesity and non-insulin-dependent diabetes favor storage of fatty acids in triacylglycerol over oxidation. Recently, individual acyl-CoA synthetase (ACS) isoforms have been implicated in the channeling of fatty acids either toward lipid synthesis or toward oxidation. Although ACS1 had been localized to three different subcellular regions in rat liver, endoplasmic reticulum, mitochondria, and peroxisomes, the study had used an antibody raised against the full-length ACS1 protein which cross-reacts with other isoforms, probably because all ACS family members contain highly conserved amino acid sequences. Therefore, we examined the subcellular location of ACS1, ACS4, and ACS5 in rat liver to determine which isoform was present in peroxisomes, whether the ACSs were intrinsic membrane proteins, and which ACS isoforms were up-regulated by PPARα ligands. Non-cross-reacting ACS1, ACS4, and ACS5 peptide antibodies showed that ACS4 was the only ACS isoform present in peroxisomes isolated from livers of gemfibrozil-treated rats. ACS4 was also present in fractions identified as mitochondria-associated membrane (MAM). ACS1 was present in endoplasmic reticulum fractions and ACS5 was present in mitochondrial fractions. Incubation with troglitazone, a specific inhibitor of ACS4, decreased ACS activity in the MAM fractions 30–45% and in the peroxisomal fractions about 30%. Because the signal for ACS4 protein in peroxisomes was so strong compared to the MAM fraction, we examined ACS4 mRNA abundance in livers of rats treated with the PPARα agonist GW9578. Treatment with GW9578 increased ACS4 mRNA abundance 40% and ACS1 mRNA 25%. Although we had originally proposed that ACS4 is linked to triacylglycerol synthesis, it now appears that ACS4 may also be important in activating fatty acids destined for peroxisomal oxidation. We also determined that, unlike ACS1 and 5, ACS4 is not an intrinsic membrane protein. This suggests that ACS4 is probably targeted and linked to MAM and peroxisomes by interactions with other proteins. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Acyl-CoA synthetase; Liver subcellular fractions; Peroxisome proliferator; PPARα; Fatty acid metabolism

Metabolic disorders such as obesity and non-insulin-dependent diabetes exhibit altered fatty acid metabolism which favors storage in triacylglycerol over oxidation. On a cellular level, the partitioning of fatty acids between storage and oxidation is regulated by insulin and counter-regulatory hormones, transcription of degradative and lipogenic enzymes, and acute regulation of carnitine palmitoyltransferase-1. The role of long-chain acyl-CoA synthetases (ACS) in fatty acid partitioning has only recently been considered [1–4].

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; ACO, acyl-CoA oxidase; ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; MAM, mitochondrial-associated membrane; PEMT, phosphatidylethanolamine methyltransferase; PPAR, peroxisome proliferator-activated receptor; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase.
ACS catalyzes the activation of long-chain fatty acids to fatty acyl-CoA thioesters by one of a family of long-chain acyl-CoA synthetases [5]. In rat, five ACS isoforms, each the product of a different gene, have been cloned. The tissue distribution of each ACS differs and, although each will use a broad range of fatty acid substrates encompassing both saturated and unsaturated fatty acids of 14–22 carbons, ACS3 and ACS4 are distinguished by a marked preference for 20:4 and 20:5. ACS1, ACS2, and ACS5 have 60% homology to each other, whereas members of a second subfamily, ACS3 and ACS4, have about 30% homology with ACS1 and about 60% with each other.

Studies using the inhibitors troglitazone and triacsin C and D with cultured cells strongly suggest that individual ACSs provide acyl-CoAs for specific pathways of fatty acid metabolism [6,7]. In hepatocytes, these compounds markedly inhibit the incorporation of labeled fatty acid into triacylglycerol and phospholipid via the de novo synthetic pathway from glycerol 3-phosphate, but have no effect on phospholipid reacylation and little effect on fatty acid oxidation [2]. Specificity of the inhibitors for particular pathways and the hypothesis that different ACSs might synthesize acyl-CoAs destined for different metabolic pathways was strengthened by our studies of the three ACS isoforms expressed in liver. Using purified recombinant rat ACSs we demonstrated that triacsin C inhibits ACS1 and ACS4 but not ACS5 and that the thiazolidinediones inhibit only ACS4 [3]. Further, we found that each of these ACS isoforms has a unique subcellular location, again suggesting linkage to specific metabolic pathways [4].

Because it had been reported that ACS1 was present in three locations, ER, mitochondria, and peroxisomes [8], but we were unable to identify immunoreactive ACS1 in mitochondria [2,4], we used non-cross-reacting peptide antibodies to examine purified peroxisomal fractions for ACS expression. We found that ACS4 protein is highly expressed in peroxisomes and investigated the peroxisome proliferator-activated receptor α (PPARα) regulation of ACS4. Furthermore, although ACS4 is found in subcellular membrane fractions, transmembrane prediction algorithms for ACS4 fail to identify membrane-spanning regions. Therefore, we revisited the dogma that all ACSs are intrinsic membrane proteins.

Experimental procedures

Materials

[14C]Palmitate was from Amersham Life Sciences CoA, ATP, and BSA (essentially fatty acid free) were from Sigma. Troglitazone was the gift of Dr. Steven Jacobs, GlaxoSmithKline. Antibodies against ACS1, 4, 5, and glycerol-3-phosphate acyltransferase (GPAT) have been described previously [4]. Antibody against phosphatidylethanolamine methyltransferase (PEMT) was the gift of Jean E. Vance, University of Alberta.

Animals and tissue isolation

Animal protocols were approved by the University of North Carolina at Chapel Hill, San Diego State University, and GlaxoSmithKline Institutional Animal Care and Use Committees. Female (150 g) Sprague–Dawley rats were housed on a 12 h/12 h light/dark cycle with free access to water. Liver microsomes and mitochondria were isolated by differential centrifugation [9] with the following modifications for isolating salt-washed microsomes and mitochondria. After the initial isolation of mitochondria and microsomes, the mitochondrial or microsomal pellets were resuspended in half the original volume of homogenization buffer and divided into two equal aliquots. An equal volume of homogenization buffer or homogenization buffer plus 1.0 M KCl was added to each aliquot prior to homogenization and centrifugation.

To separate peroxisomal fractions from ER and mitochondria, a postnuclear fraction prepared by differential centrifugation of liver from rats maintained on chow plus 0.2% gemfibrozil for 17 days was further purified by equilibrium density centrifugation on a linear (20–40%) Optiprep gradient. Salt-washed peroxisomes were obtained by pooling fractions 15–20 and diluting in homogenization buffer or homogenization buffer plus KCl (final conc. 0.5 M). Following homogenization, peroxisomal membrane pellets were obtained by centrifugation at 100,000g.

Protein concentrations were determined by the BCA method (Pierce) using BSA as the standard.

Northern analyses

For PPARα activation experiments, frozen livers from rats that had been fed a diet supplemented with either the vehicle 5% methylcellulose (n = 4) or 0.3 mg/kg GW9578, a PPARα agonist (n = 4), for 7 days before sacrifice were obtained from Deborah Winegar, GlaxoSmithKline. Total RNA was isolated using Trizol reagent (Life Technologies) and a modification of the method of Chomczynski and Sacchi [10]. Total RNA (20 μg) was denatured in a solution containing 15 mM formamide, 2.7 M MOPS, and 0.06% ethidium bromide, separated on a 1.1% agarose–formaldehyde gel, and transferred to a nylon membrane. Probes against ACS1, 4, 5, acyl-CoA oxidase (ACO) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with [32P]CTP by random priming (Boehringer Mannheim). The blot was exposed to a Phosphor screen and the signal was quantified with a Molecular Dynamics Storm 840 system. After probing for ACS or ACO, blots were
stripped and reprobed for GAPDH. The ACS or ACO signal intensity was normalized to the intensity of the corresponding GAPDH bands.

**Immunoblotting**

Proteins were separated on an 8% polyacrylamide gel containing 1% SDS, transferred to a PVDF membrane (Bio-Rad), and incubated with antibody against ACS1, ACS4, ACS5, GPAT, or PEMT. For chemiluminescent detection, the immunoreactive bands were visualized by incubating the membrane with HRP-conjugated goat anti-rabbit IgG and PicoWest reagents (Pierce).

**Enzyme assays**

Acyl-CoA synthetase was assayed at 37°C for 5 min with 5 mM ATP, 250 μM CoA, and 20 μM [14C]palmmitate [11]. Activities measured initial rates. Optiprep fractions were assayed for esterase, glutamate dehydrogenase (GDH), and catalase [12] to identify fractions containing ER, mitochondria, and peroxisomes, respectively. Adenylate kinase [13] and NADPH cytochrome c reductase [14] were measured as described.

**Results**

**Acyl-CoA synthetase 4 is located in peroxisomes**

We have previously shown that in rat liver, ACS1 is located in ER, mitochondrial-associated membrane (MAM), and cytosol, ACS4 is located in MAM, and ACS5 is located in mitochondria [4]. MAM is an ER fraction defined by its close association with mitochondria, the presence of PEMT-2, and high specific activities of acyl-CoA:cholesterol acyltransferase (ACAT), diacylglycerol acyltransferase (DGAT) [15], and phosphatidylserine synthase-1 and -2 [35]. Depending on the cell fractionation method employed, MAM, identified by the presence of PEMT-2, is found in crude mitochondrial or microsomal preparations [4]. Because ACS1 had been identified in peroxisomes [8], a site for acyl-CoA metabolism through β-oxidation, we looked for the presence of the ACS isoforms by analyzing rat liver homogenate fractionated over an Optiprep gradient by Western blot with non-cross-reacting peptide-specific antibodies against ACS1, 4, and 5.

We found that ACS4 (74 kDa) was highly expressed in fractions 15–20 (Fig. 1). The signal on the Western blot was so strong that only 1/10 the amount of total protein loaded for other Western blot analyses (10 μg vs 100 μg) was required. Fractions 15–20 contain the highest catalase activity, indicating that they are highly enriched in peroxisomes (Fig. 2C). These fractions contain no esterase or GDH activity, indicating lack of contamination with ER or mitochondria (Figs. 2A and B). We also confirmed our previous finding that ACS4 (74 kDa) is located in MAM, fractions 5–10 and 15–18 which contain PEMT, a specific marker for MAM (Fig. 1) [15]. Fractions 6–10 also contain a small peak of catalase (Fig. 2C), representing soluble catalase that was released from broken peroxisomes. The ACS4 protein in fractions 8–9, however, was present only in the pellet after recentrifugation at 100,000 g for 1 h (data not shown), indicating that that they remained with non-peroxisomal organelles. Interestingly, we also detected a larger 80 kDa species of ACS4 in fractions 7–9 which may correspond to a 4500-Da larger isoform that uses an alternative start site 117 nucleotides upstream [16].

In contrast to the presence of ACS4 protein, total ACS activity (Fig. 2D) was present in three approximately equal peaks corresponding to the esterase marker (Fig. 2A) for endoplasmic reticulum, the GDH marker (Fig. 2B) for mitochondria, and the catalase marker (Fig. 2C) for peroxisomes. The ACS activity measured is the sum of the three long-chain ACSs known to be expressed in liver and possibly other, as yet undiscovered, ACS isoforms.

We assayed the Optiprep fractions for ACS activity in the absence and presence of troglitazone, a specific inhibitor of ACS 4 [3], to confirm the presence of ACS4 in fractions 6–10 and 15–20. Troglitazone (50 μM) specifically inhibited ACS activity in fractions that expressed ACS4 (Fig. 3). Troglitazone inhibited ACS activity 30–
45% in the MAM-containing fractions 8–10, consistent with our previous findings [4]. In the peroxisomal fractions, 15–20, 50 μM troglitazone inhibited ACS activity about 30%.

ACS1 was detected in fractions 8–10 (Fig. 1) which have high esterase activity (Fig. 2A), indicating an enrichment in endoplasmic reticulum. This result agrees with our previous finding of ACS1 in microsomes [4]. We also detected ACS1 in the very light fractions 3–7. ACS1 was not detected in peroxisomes, even though it had been previously reported in this fraction using an antibody raised against full-length ACS1 [8].

ACS5 at 76 kDa was detected in fractions 11–14 (Fig. 1) which are enriched in mitochondria, as evidenced by high GDH activity (Fig. 2B) and the presence of GPAT (Fig. 1), an outer mitochondrial membrane protein. ACS5 was also detected in light fractions 3–7 which also contain GPAT, consistent with the presence of broken outer mitochondrial membranes. The presence of ACS5 in fractions enriched in mitochondria and the absence of ACS5 from peroxisomal fractions was a clarifying result, since our previous liver mitochondrial preparation may have been contaminated with peroxisomes [4]. The ACS5 antiserum also detected a 73-kDa protein in MAM fractions 8–9 (Fig. 1), as we previously reported [4].

**Acyl-CoA synthetase 4 mRNA is up-regulated by the PPARα agonist GW9578**

Because ACS4 protein was highly abundant in liver peroxisomes from rats maintained on a diet of 0.2% gemfibrozil for 17 days, we examined the regulation of ACS4 expression by PPARα [17,18]. We obtained livers from rats given vehicle (5% methylcellulose; n = 4) or 0.3 mg/kg GW9578 [19] (PPARα agonist; n = 4) for 7 days, isolated total RNA, and measured the abundance of ACS1, 4, and 5 mRNA by Northern blot analysis. Treatment with GW9578 increased ACS4 mRNA 42% and ACS1 mRNA 25% (Fig. 4), but ACS5 mRNA levels were not altered. ACO mRNA levels, which have been shown to increase after treatment with peroxisome proliferators [20], were three-fold greater in GW9578-treated rats.

**Acyl-CoA synthetase 4 is not an intrinsic membrane protein**

Although ACS1, 4, and 5 are all detected in membrane fractions and have 30–60% amino acid identity, the transmembrane prediction algorithms TMPred [21], DAS [22], SOSUI [23], and TMHMM [24] all predict that ACS1 and 5 each have at least one transmembrane
domain but that ACS4 contains no transmembrane segments. Comparison of Kyte–Doolitle hydrophathy plots [25] generated for ACS1, 4, and 5 also clearly shows that ACS4 does not have any regions with the hydrophobic character required for a transmembrane segment. On the other hand, ACS1 and ACS5 each have at least one predicted hydrophobic segment. If ACS4 does not contain a predicted transmembrane domain, it is likely to be a membrane-associated protein linked to MAM and peroxisomal membranes via specific protein–protein interactions.

To determine whether ACS 4 is an intrinsic membrane protein or a membrane-associated protein, we tested whether ACS4 could be removed from peroxisomes and MAM with a salt wash. Liver peroxisomes (Optiprep fractions 15–20) and microsomes (which contain the MAM fraction) were incubated and homogenized in the absence or presence of high salt levels and then recentrifuged. The presence of 0.5 M KCl removed a majority of the ACS4 protein from the peroxisomal membrane and a stronger ACS4 signal was detected in the KCl supernatant fraction (Fig. 5A). High salt levels completely washed ACS4 from the microsomal membranes and ACS4 immunoreactive protein appeared in the KCl wash fraction (Fig. 5B). These data indicate that ACS4 is not an intrinsic membrane protein, consistent with the results from transmembrane prediction algorithms.

Also consistent with these transmembrane predictive programs, we found that ACS1 and the 73-kDa ACS5 remain bound to microsomes following KCl treatment (Fig. 5B). In mitochondria, ACS5 (76-kDa) was also not removed by the KCl treatment (Fig. 5C), and a smaller 73-kDa ACS5 band was present only in the wash fractions. The inability of 0.5 M KCl to remove ACS1 and 5 from membranes suggests that these are intrinsic membrane proteins.

Although the separate activities of the individual ACS isoforms cannot be distinguished, we measured the total ACS activity in microsomes, KCl-washed microsomes, mitochondria, KCl-washed mitochondria, and supernatant fractions. After treatment with 0.5 M KCl, the total ACS activity recovered in the pellet fraction decreased 60% (Fig. 6A). The decrease in ACS activity was not due to inactivation of ACS by KCl, since incubation of untreated microsomes for 1.5 h with 0.5 M KCl had no effect on total ACS activity (data not shown). Further, the KCl treatment did not affect recovery of the microsomes themselves, because there was no difference in NADPH cytochrome c reductase activity between control and salt-washed microsomal pellet fractions (Table 1).
Because the loss of ACS activity from the microsomal pellet fraction is consistent with our finding that ACS4 is removed from microsomal membranes following KCl treatment, we expected to recover the ACS activity in the KCl supernatant fraction that contained immuno-reactive ACS4. However, although ACS activity increased in the supernatant fraction after KCl treatment (Fig. 6B), the total activity recovered was only 0.6 mmol/min/total protein, whereas 1.6 mmol/min/total protein was lost from the microsomal fraction. Further, the small increase in ACS activity in the KCl supernatant fraction was not due to the presence of additional microsomes, because NADPH cytochrome c reductase activity did not increase in the KCl supernatant fraction (Table 1). We were also unable to measure an increase in ACS activity in the peroxisomal supernatant fraction collected after high salt wash (data not shown).

In mitochondrial pellets collected after treatment with 0.5 M KCl, total ACS activity increased 17% (Fig. 6A), consistent with assaying a more highly purified membrane fraction from which peripheral protein had been removed. Incubating untreated mitochondria with 0.5 M KCl for 1.5 h had no effect on ACS activity (data not shown). As expected, ACS activity was unchanged in the mitochondrial supernatant fractions (Fig. 6B). That the 0.5 M KCl wash did not disrupt mitochondria is shown by the absence of differences in adenylate kinase activity in untreated and treated mitochondria and in the control and KCl supernatant fractions (Table 1).

**Discussion**

The presence of individual ACS isoforms in specific subcellular fractions suggests that they play separate and defined roles in providing long-chain acyl-CoAs for particular metabolic pathways. Understanding the subcellular locations of the specific ACS isoforms would help in clarifying their roles. Previous data localizing

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**Table 1**

<table>
<thead>
<tr>
<th>Activities of ER and mitochondrial marker enzymes</th>
<th>NADPH cytochrome c reductase</th>
<th>Adenylate kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (mol/min)²</td>
<td>Total activity (mol/min)³</td>
</tr>
<tr>
<td>Microsomal pellet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−KCl</td>
<td>3.12 ± 0.56</td>
<td>0.048 ± 0.01</td>
</tr>
<tr>
<td>+KCl</td>
<td>2.48 ± 0.18</td>
<td>0.035 ± 0.001</td>
</tr>
<tr>
<td>Microsomal supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−KCl</td>
<td>0.06 ± 0.02</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>+KCl</td>
<td>0.03 ± 0.003</td>
<td>0.018 ± 0.006</td>
</tr>
<tr>
<td>Mitochondrial pellet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−KCl</td>
<td>0.30 ± 0.06</td>
<td>0.302 ± 0.016</td>
</tr>
<tr>
<td>+KCl</td>
<td>0.18 ± 0.01</td>
<td>0.274 ± 0.015</td>
</tr>
<tr>
<td>Mitochondrial supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−KCl</td>
<td>0.37 ± 0.05</td>
<td>0.046 ± 0.002</td>
</tr>
<tr>
<td>+KCl</td>
<td>0.45 ± 0.05</td>
<td>0.050 ± 0.004</td>
</tr>
</tbody>
</table>

² Total activity in each fraction.
³ Percentage of total activity in all fractions. Activities do not add up to 100% because the activity in cytosol is not reported.
ACS1 to three different subcellular regions in rat liver, endoplasmic reticulum, mitochondria, and peroxisomes, was the result of a carefully performed study that used an antibody raised against the full-length ACS1 protein [8]. We now know that an antibody raised to a complete ACS1 protein will also detect both ACS4 and ACS5, most likely because all ACS family members contain highly conserved amino acid sequences that probably retain similar epitopes [4]. Use of an antibody raised to an ACS1 peptide that has little homology to ACS4 or ACS5 showed that ACS1 is not, in fact, located in mitochondria [4]. Thus, we decided to investigate purified peroxisomal fractions to determine which ACS isoform was present. Not only was ACS4 the only isoform present in peroxisomes, it appeared to be present in amounts greater than previously observed in the MAM fraction [4]. In rats fed GW9578, a PPARγ activator, ACS4 mRNA expression increased 42%, ACS1 increased 25%, and ACS5 was unchanged. As had been previously observed, ACS4 was also present in the MAM fractions identified by the presence of immunoreactive PEMT, ACS1 was present in ER fractions identified by the presence of esterase, and ACS5 was found in mitochondrial fractions that also contained GDH and immunoreactive GPAT [4].

ACS activity was first demonstrated in peroxisomes in 1966 [26]. Peroxisomes were reported to contain 7% of the total palmitoyl-CoA synthetase activity in liver, and total liver ACS activity increases two- to three-fold after rats are treated with the PPARγ activator clofibrate [27]. Part of this increase in ACS activity is likely due to up-regulation of ACS1 [28] and ACS4 mRNA expression. In the gemfibrozil-treated rats, ACS4 protein is much more highly expressed in peroxisomes than in the MAM fractions (Fig. 1), yet the activities are comparable (Fig. 3), suggesting that much of the peroxisomal ACS4 may be inactive under the isolation and assay conditions used in this study.

Peroxisomal ACS activity is located on the cytosolic face of the peroxisomal membrane [29]. Of note is the fact that several very long-chain ACS isoforms are also present on the peroxisomal membrane and these may overlap in fatty acid specificity with the long-chain ACSs [30,31]. Our data show that troglitazone, a specific inhibitor of ACS4 [3], inhibits about 30% of the ACS activity in liver peroxisomal fractions from gemfibrozil-treated rats; the resistant activity may represent an as yet uncharacterized ACS or the combined activities of the several VLACS present. Whether TZDs inhibit any VLACS isoforms is not known.

Finding ACS4 in peroxisomes is puzzling because previous data strongly suggested that ACS4 is linked to triacylglycerol synthesis [4]. ACS4 is specifically inhibited by triacsin C, an alkyl-N-hydroxytriazene fungal metabolite that is a fatty acid analog and competitive inhibitor [32,33], and by the thiazolidinedione drugs, troglitazone, rosiglitazone, and pioglitazone, which exhibit a complex inhibition that has noncompetitive, uncompetitive, and mixed aspects depending on which of the three ACS substrates is tested [3]. Because in rat primary hepatocytes, triacin C and troglitazone strongly inhibit incorporation of fatty acid into triacylglycerol but have weaker effects on incorporation into phospholipid [34], we hypothesized that ACS4 might be linked to triacylglycerol synthesis, perhaps by synthesizing a pool of acyl-CoAs that is channeled toward this pathway. This hypothesis was strengthened by finding ACS4 in the MAM fraction which contains microsomal triglyceride transfer protein, apoB, PEMT-2, high specific activities of ACAT and DGAT [15], and phosphatidylserine synthase-1 and -2 [35]. Vance’s group has hypothesized that the function of MAM may involve VLDL assembly [15] or the import of lipids into mitochondria [36].

Finding ACS4 in peroxisomes of gemfibrozil-treated rats led us to hypothesize that ACS4 might be up-regulated by peroxisome proliferators as has been reported for ACS1 [28,37]. GW9578 is cited as the most potent PPARγ agonist known to date [19]. Treatment of rats for 7 days with GW9578 resulted in a 42% increase in ACS4 mRNA (Fig. 4). Message for ACS1, which has a PPRE, was increased to a similar degree (25%), although previous studies reported that 0.5% fenofibrate treatment for 14 days increased ACS1 mRNA to a similar degree (25%), and that 14-day treatment with 0.1% Wy-14,643 did not alter ACS1 mRNA expression [38]. Since we expected a more robust response in ACS1 message, we performed a control experiment for the efficacy of GW9578. We measured changes in the expression of ACO, a peroxisomal β-oxidation enzyme, following GW9578 treatment. Consistent with previous studies using peroxisome proliferators [20], GW9578 resulted in a 3-fold increase in ACO expression (Fig. 4). Although no PPRE has been reported in the ACS4 promoter [39], our data suggest that ACS4 is modestly up-regulated by the PPARγ agonist GW9578, to a degree similar to that of ACS1. Other PPARγ agonists may be more potent up-regulators of ACS4, as observed for ACS1. Our new findings that ACS4 protein is highly expressed in peroxisomes and may be up-regulated by a PPARγ ligand suggest that ACS4 may also play a role in the activation of polyunsaturated fatty acids destined to be oxidized in peroxisomes. This is consistent with the 2-fold preference that ACS4 exhibits toward arachidonic acid as compared to palmitate [40]. In this light, it is of interest that ACS4 +/− female mice have decreased fertility and marked accumulation of uterine prostaglandins [41], suggesting that a defect in ACS4 allows arachidonic acid to increase in uterine cells, thereby promoting the synthesis of prostaglandins.

Another finding was that, unlike ACS1 and ACS5, ACS4 is not an intrinsic membrane protein. Confirming
the hydropathy plot and transmembrane prediction algorithms, ACS4 was readily washed from liver peroxisomes and microsomes by 0.5 M KCl (Figs. 5A and B), indicating that ACS4 is not an integral membrane protein. Consistent with this result, high salt treatment caused total ACS activity in microsomes to decrease 60% (Fig. 6A). Although we detected an increase in the ACS4 immunoreactive band in microsomal and peroxisomal supernatant fractions after KCl treatment (Figs. 5A and B), we did not measure an increase in ACS activity (Fig. 6A and data not shown). This suggests that the salt treatment may have removed a microsomal component required for activity. Taken as a whole, the data indicate that ACS4 is a peripheral membrane protein and suggest that it is probably targeted and linked by interactions with other proteins to its two very specific membrane locations.

ACS4 may provide two seemingly different and even contradictory functions within liver. Under conditions of energy surplus, it may provide acyl-CoAs destined for peroxisomal oxidation. Studies to test these predictions will require the use of specific inhibitors for ACS4 and for mitochondrial vs peroxisomal oxidation.

Acknowledgments

This work was supported by GlaxoSmithKline, by grants HD 56598 (R.A.C.), HD 08431 (T.M.L.), and DK58238 and DK58040 (S.K.K.) from the National Institutes of Health, and by a grant from the North Carolina Institute of Nutrition. We thank Dr. Maria Gonzalez-Baró for helpful discussions.

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