Characterization of Recombinant Long-Chain Rat Acyl-CoA Synthetase Isoforms 3 and 6: Identification of a Novel Variant of Isoform 6

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ABSTRACT: The metabolism of long-chain fatty acids in brain and their incorporation into signaling molecules such as diacylglycerol and LPA and into structural components of membranes, including myelin, requires activation by long-chain acyl-CoA synthetase (ACSL). Because ACSL3 and ACSL6 are the predominant ACSL isoforms in brain, we cloned and characterized these isoforms from rat brain and identified a novel ACSL6 clone (ACSL6_v2). ACSL6_v2 and the previously reported ACSL6_v1 represent splice variants that include exon 13 or 14, respectively. Homologue sequences of both of these variants are present in the human and mouse databases. ACSL3, ACSL6_v1, and ACSL6_v2 with Flag-epitopes at the C-termini were expressed in *Escherichia coli* and purified on Flag-affinity columns. The three recombinant proteins were characterized. Compared to ACSL4, another brain isoform, ACSL3, ACSL6_v1, and ACSL6_v2 showed similarities in kinetic values for CoA, palmitate, and arachidonate, but their apparent K_m values for oleate were 4- to 6-fold lower than for ACSL4. In a direct competition assay with palmitate, all the polyunsaturated fatty acids tested were strong competitors only for ACSL4 with IC_{50} values of 0.5 to 5 μM. DHA was also strongly preferred by ACSL6_v2. The apparent K_m value for ATP of ACSL6_v1 was 8-fold higher than that of ACSL6_v2. ACSL3 and the two variants of ACSL6 were more resistant than ACSL4 to heat inactivation. Despite the high amino acid identity between ACSL3 and ACSL4, rosiglitazone inhibited only ACSL4. Triacsin C, an inhibitor of ACSL1 and ACSL4, also inhibited ACSL3, but did not inhibit the ACSL6 variants. These data further document important differences in the closely related ACSL isoforms and show that amino acid changes near the consensus nucleotide binding site alter function in the two splice variants of ACSL6.

Acyl-CoA synthetase catalyzes the formation of fatty acyl-CoA's from ATP, CoA, and long-chain fatty acids. After this essential activation step, the acyl-CoA's enter multiple degradative pathways including β-oxidation and fatty acid retroconversion, or multiple synthetic pathways including glycerolipid synthesis, phospholipid reacylation, and cholesterol ester formation (*3*, *4*). Five rat ACSL[1] isoforms, each encoded by a separate gene, have been described. These isoforms differ in fatty acid preference, subcellular location (*5*), and regulation (*6*). We previously characterized the liver isoforms, ACSL1, ACSL4, and ACSL5, and found that triacsin C inhibits only ACSL1 and ACSL4 and that thiazolidinediones inhibit ACSL4 exclusively (*7*). In human fibroblasts, triacsin C inhibits de novo PL and TAG synthesis 83% and 93%, respectively, but does not impair phospholipid reacylation (*8*). In primary hepatocytes triacsin C inhibits the de novo synthesis of triacylglycerol 73%, but inhibits β-oxidation by only 33% (*9*). Similarly in primary hepatocytes, troglitazone inhibits oleate incorporation into triacylglycerol and oxidation products 50% and 20%, respectively (*10*). These indirect data suggest that the individual ACSL isoforms channel fatty acids toward separate metabolic fates and, more specifically, that ACSL1 and ACSL4 are linked to de novo glycerolipid synthesis.

We did not include rat ACSL3 or ACSL6 (formerly ACS2[2]) in our previous study (*7*) because neither had been identified in liver. However, ACSL6 and ACSL3 are the predominant isoforms in rat brain (*11*, *12*) and may play roles in regulating brain fatty acid metabolism similar to those proposed for liver. Characterization of the brain ACSL isoforms, particularly their susceptibility to specific inhibitors, could well be important for studies of neuronal lipid metabolism (*13*) and contribute to our understanding of acyl-CoA channeling in Alzheimer disease, depression, and alcoholism (*14*). In this study we characterize three ACSL isoforms that we cloned from rat brain: ACSL3 and two splice variants of ACSL6, ACSL6_v1 and ACSL6_v2.

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[1] Abbreviations: ACSL, acyl-CoA synthetase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FACL, fatty acyl-CoA ligase; mLACS, mouse acyl-CoA synthetase; NEM, *N*-ethylmaleimide; PHAX, phytanoyl-CoA α-hydroxylase; PL, phospholipid; TAG, triacylglycerol.

[2] The nomenclature for the acyl-CoA synthetases has been changed. Rat ACS and human FACL, previously termed long-chain acyl-CoA synthetase or fatty acid CoA ligase, are now both termed ACSL. The numbering system has been changed so that rat ACS1, human FACL1, and human FACL2 are all now ACSL1, and the former rat ACS2 and human FACL6 are both ACSL6 (see http://www.gene.ucl.ac.uk/nomenclature/genefamily/acs.html).

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EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Palmitate, [1-^{14}C]oleate, and [1-^{14}C]-arachidonate were from New England Nuclear. Triacsin C was from Biomol. Rosiglitazone was from Alexis Biochemicals. The bacterial Flag expression system including pFlag-CTC vector, Flag M2 agarose beads, and Flag peptides, *N*-ethylmaleimide, coenzyme A, ATP, palmitate, oleate, arachidonate, and Triton X-100 were from Sigma. Trizol, M-MLV reverse transcriptase was from Invitrogen. *Escherichia coli* BL21-CondonPlus(DE3)-RIL was from Stratagene.

Construction of Recombinant pACS-Flag Plasmids. Animal protocols were approved by the University of North Carolina at Chapel Hill Animal Care and Use Committee. ACSL4 and ACSL5 were previously cloned from rat liver in our lab, and the recombinant plasmids for ACSL4- and ACSL5-Flag were described previously (*7*). Total RNA was isolated from Sprague−Dawley whole rat brain by the Trizol method. cDNA was synthesized using M-MLV reverse transcriptase. ACSL6_v1 and ACSL3 were cloned from rat brain, and the recombinant plasmids for expression of ACSL6_v1 (previously termed ACS2) and ACSL3-Flag were described (*15*).

ACSL6 was amplified by polymerase chain reaction as described (*15*). Two populations of clones were obtained, the previously reported ACSL6 (*11*) and a novel variant, ACSL6_v2 (*16*). The previously reported ACSL6 (accession number D10041) is termed ACSL6_v1. The rnACSL6_v2 cDNA has been deposited in the GenBank database (accession number AY625254). Amino acid multiple sequence alignments were performed using Clustal W software (*17*).

Expression of Recombinant ACSL6_v1-, ACSL6_v2-, and ACSL3-Flag Proteins in E. coli. Recombinant ACSL6_v1-, ACSL6_v2-, and ACSL3-Flag were 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_{600} = 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. Protein concentrations were determined by the BCA method (Pierce) with bovine serum albumin as the standard.

Purification of Recombinant ACSL6_v1-, ACSL6_v2-, and ACSL3-Flag Proteins. ACSL6_v1-, ACSL6_v2-, and ACSL3-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 ACSL6_v1-, ACSL6_v2-, or ACSL3-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. Eluates were electrophoresed on a 10% acrylamide gel containing 1% SDS and stained with Gel Code blue stain reagent (Pierce). Compared with the cell extracts, the activities of the partially purified proteins increased 3-fold for ACSL6_v1-Flag, 16-fold for ACSL6_v2-Flag, and 35-fold for ACSL3-Flag.

ACSL Assays. The acyl-CoA synthetase assay contained 50 μM [^{14}C]palmitate in 0.5 mM Triton X-100, 10 μM EDTA, 10 mM ATP, 250 μM CoA, 175 mM Tris (pH 7.4), 8 mM $MgCl_2$, and 5 mM dithiothreitol in a total volume of 200 μL (*18*). In some assays, [^{14}C]oleate or [^{14}C]arachidonate was substituted for [^{14}C]palmitate. In some assays, varying concentrations of unlabeled arachidonate, EPA, DHA, linoleic acid, or linolenic acid were added to the assay as a competitor. Rosiglitazone or triacsin C was added directly to the enzyme reaction mixture during inhibition assays. The assay was initiated with 0.4 μg of protein for ACSL6_v1, 0.2 μg of protein for ACSL6_v2, 0.1 μg of protein for ACSL3, and ACSL4, and 0.5−1.0 μg of protein for ACSL5. ACSL activity was measured after a 5-min incubation with substrates at 37 °C. The reaction was stopped with 2-propanol:heptane:1 M sulfuric acid (80:20:1) and the acyl-CoA products extracted from heptane with water. Specific activities were linear over an incubation range of 2 to 10 min. Substrate concentrations and incubation time allowed measurement of initial rates. The specific activities of ACSL6_v1-, ACSL6_v2-, ACSL3-, ACSL4-, and ACSL5-Flag in different assays were in the ranges of 115−159, 469−624, 1281−1921, 1238−2200, and 98−166 nmol/min/mg, respectively.

RESULTS

Identification of a Novel Splice Variant of ACSL6. While cloning ACSL6, we obtained a new cDNA, the sequence of which had not been previously reported. The variant ACSL6 differs from the previously reported ACSL6 (*11*) in nucleotides 916−993 within the coding region. This stretch corresponds to exon 13 of the recently sequenced rat ACSL6 gene (accession number NW_047334.1, nucleotides 26588438−26588515). The previously reported ACSL6 sequence includes exon 14 (nucleotides 26588573−26588650) instead of exon 13. Thus, in the rat, the ACSL6 gene is expressed as at least two different transcript variants that arise from alternative splicing of exons 13 or 14 (Table 1A). Analogously, homologous genes in human and mouse appear to encode the same two splice variants. The new rat variant is named ACSL6_v2 in this paper, whereas ACSL6_v1 is used for the previously reported ACSL6 (D10041) (*16*). These alternative exons are 78 base pairs long; within this region they differ by 32 nucleotides. These exons encode amino acids 306 to 331 in the predicted protein isoforms. Within this 26 amino acid region, 12 amino acids are different and 14 are identical for both isoforms. Among the identical amino acids, 7 are identical in all 6 rat ACSL isoforms and 6 others represent conserved substitutions (Table 1B).

Comparison of Amino Acid Sequence Identities in Rat ACSL Isoforms. ACSL isoforms share high sequence homology (Table 2) (*3*). ACSL1, ACSL6_v1, ACSL6_v2, and

Table 1: ACSL6_v1 and ACSL6_v2 Are Splice Variants[a]

A

Protein isoform		Sequence		Acc. number
Hs LACS5	(306)	SQWAPTCADVHISYLPLAHMFERMVQ	(331)	AAD47199
Mm ACSL6	(306)	SQWAPTCADVHFSYLPLAHMFERMVQ	(331)	AAH22959
Rn ACSL6_v1	(306)	SQWAPTCADVHFSYLPLAHMFERMVQ	(331)	AY625254
Rn ACSL6_v2	(306)	KVIFPRQDDVLISFLPLAHMFERVIQ	(331)	AAT41589
Hs KIAA0837	(306)	KVIFPRQDDVLISFLPLAHMFERVIQ	(331)	BAA74860
Mm LACS	(331)	KVIFPRQDDVLISFLPLAHMFERVIQ	(356)	AAO38689
		* ** :*:**********::*		

B

Protein isoform		Sequence		Acc. number
Rn ACSL6_v1	(306)	SQWAPTCADVHFSYLPLAHMFERMVQ	(331)	NP_570095
Rn ACSL6_v2	(306)	KVIFPRQDDVLISFLPLAHMFERVIQ	(331)	AAT41589
Rn ACSL1	(307)	SAFIASPEDVLISFLPLAHMFETVVE	(332)	NP_036952
Rn ACSL5	(291)	PIFQPTPEDVTISYLPLAHMFERLVQ	(316)	NP_446059
Rn ACSL3	(317)	---RLGEEDVYIGYLPLAHVLELSAE	(339)	NP_476448
Rn ACSL4	(267)	---GLGPKDTYIGYLPLAHVLELTAE	(289)	NP_446075
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[a] The amino acid sequence predicted from the newly cloned cDNA (ACSL6_v2) was aligned with rat ACSL6_v1 and the homologue sequences from human and mouse (A) and with all other rat isoforms (B). The region shown corresponds to amino acids 306 to 331 in ACSL6 (encoded by exon 13 or 14); (*) identity; (**:**) conserved substitutions; (**.**) semiconserved substitutions. Hs: *Homo sapiens*. Rn: *Rattus norvegicus*. Mm, *Mus musculus*.

Table 2: Comparison of Sequence Identities in Rat ACSL Isoforms[a]

rat ACSL	1	3	4	5	6_v1	6_v2
1	100	29.1	27.6	60.4	64.4	64.7
3	29.1	100	62.8	29.9	28.3	28.3
4	27.6	62.8	100	29.4	28.6	28.6
5	60.4	29.9	29.4	100	58.5	58.2
6_v1	64.4	28.3	28.6	58.5	100	98.3
6_v2	64.7	28.3	28.6	58.2	98.3	100

[a] Amino acid sequences were obtained from GenBank. Pairwise alignments were performed using the LALIGN program. (http://www.ch.embnet.org/software/LALIGN_form.html).

ACSL5 share approximately 60% sequence identity. Similarly, ACSL3 and ACSL4 share approximately 63% sequence identity with each other but only about 30% identity with ACSL1. Rat and human ACSL isoforms show amino acid identities of about 86% (ACSL1), 92% (ACSL3), 97% (ACSL4), 77% (ACSL5), and 92% (ACSL6) (*4*).

Kinetic Parameters for ACSL6_v1, ACSL6_v2, and ACSL3. To characterize the three ACSL isoforms cloned from rat brain, we studied the kinetics of the three substrates required for ACSL catalysis. For comparison, we include the previously reported kinetic values for the three ACSL isoforms cloned from rat liver (*7*) (Table 3). The apparent K_m values for CoA ranged between 2.4 and 6.4 μM, for palmitate between 3.6 and 8.6 μM, and for arachidonate between 6.5 and 10 μM, and were similar for all six ACSL isoforms. ACSL4 differed in its K_m for oleate, which was 4- to 6-fold higher than the corresponding K_m values for oleate of ACSL6_v1, ACSL6_v2, or ACSL3. The apparent K_m values for ATP were also disparate. ACSL4 had an apparent

Table 3: Kinetic Constants for Rat ACSL Isoforms[a]

	ATP	CoA	palmitate	oleate	arachidonate
ACSL6_v1					
K_m (μM)	12210	4.7	6.0	3.0	9.7
V_{max}[b]	301	130	208	153	139
V_{max}/K_m[c]			34	51	14
ACSL6_v2					
K_m (μM)	1480	2.4	3.6	5.3	6.5
V_{max}[b]	613	522	739	1088	1212
V_{max}/K_m[c]			205	190	186
ACSL 3					
K_m (μM)	402	3.0	4.3	5.1	8.9
V_{max}[b]	1844	2547	2306	2399	4022
V_{max}/K_m[c]			536	470	452
ACSL4[d]					
K_m (μM)	34	4.1	5.4	19.5	10
V_{max}[b]	1050	2092	2800	673	4200
V_{max}/K_m[c]			519	35	420
ACSL5[d]					
K_m (μM)	666	2.4	8.6	*e*	*e*
V_{max}[b]	287	75	130	*e*	*e*
ACSL1[d]					
K_m (μM)	649	6.4	5.0	*e*	*e*
V_{max}[b]	1012	1072	1695	*e*	*e*

[a] Recombinant, purified, Flag-tagged enzymes were assayed as described in Experimental Procedures. [b] Units: nmol/min/mg protein. [c] Units: nmol/min/mg protein (μM^{-1}). [d] Values for ACSL1, -4, and -5 were reported previously (*7*). [e] Not determined.

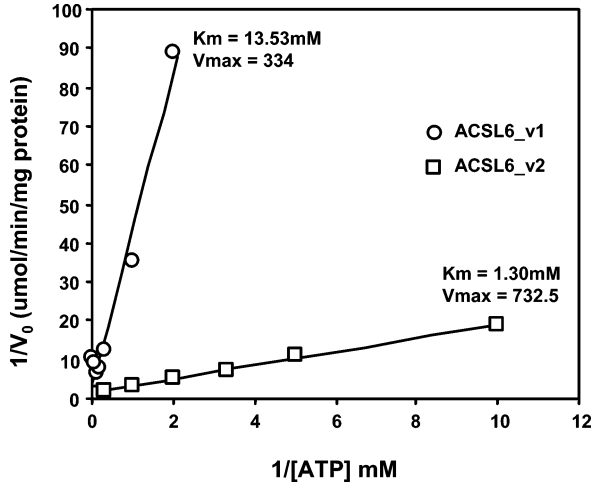

FIGURE 1: The apparent K_m for ATP is 8-fold higher for ACSL6_v1 than for ACSL6_v2. Flag-purified ACSL6_v1-Flag (○) and ACSL6_v2-Flag (□) was assayed for ACSL activity for 5 min at 37 °C with varying concentrations of ATP. This figure is representative of the results of 3 independent assays.

K_m value for ATP that was 12-fold lower than the K_m of ACSL3 for ATP. The two ACSL6 splice variants also had very different apparent K_m values for ATP; the K_m value for ACSL6_v1 was 8-fold higher than that for ACSL6_v2 (Figure 1).

V_{max}/K_m is the rate constant for the capture of substrate by an enzyme into a productive complex (*19*). In Table 3, the V_{max}/K_m data suggest that ACSL6_v1 interacts with oleate at a slightly higher catalytic efficiency than with palmitate or arachidonate. Conversely, ACSL4 interacts with oleate at a much lower catalytic efficiency than with palmitate or arachidonate. ACSL6_v2 exhibits similar catalytic efficiency with all three substrates. V_{max}/K_m values for the three fatty acid substrates with ACSL 3 are similar. Taken as a whole, the patterns of kinetic differences observed for the brain ACSL isoforms with regard to ATP, CoA, and the fatty acid substrates are consistent with our hypothesis of distinct functions.

Inhibition by Heating and Triton X-100. To further characterize the three brain isoforms, we measured ACSL activity after incubating purified recombinant enzyme at 43 °C for various times. ACSL3 was minimally more stable than ACSL6_v1 or -6_v2, with a half-life of 7 min compared to 5 min (Figure 2). In comparison, ACSL4 was much more sensitive to heat inactivation with a $t_{1/2}$ shorter than 2 min. The isoforms were also affected differently by Triton X-100. ACSL6_v2 was the most tolerant of Triton X-100 and maintained optimal activity up to 4 mM (Figure 3). ACSL6_v1 and ACSL3 were similar, maintaining activity in the presence of 2 to 3 mM Triton X-100. In contrast, ACSL4 was very sensitive to Triton X-100.

Inhibition by Rosiglitazone and Triacsin C. We previously reported that the insulin-sensitizing thiazolidinedione drugs, rosiglitazone, piaglitazone, and troglitazone, strongly inhibit ACSL4, but that ACSL1 and ACSL5 are unaffected by these agents (*7*). We now observe that ACSL6_v1, ACSL6_v2, and ACSL3 are also insensitive to rosiglitazone (Table 4). Thus, despite the high degree of amino acid identity between ACSL3 and ACSL4, rosiglitazone inhibited only ACSL4.

Although triacsin C, an alkenyl-*N*-hydrozytriazene fungal metabolite, is frequently described as an acyl-CoA synthetase

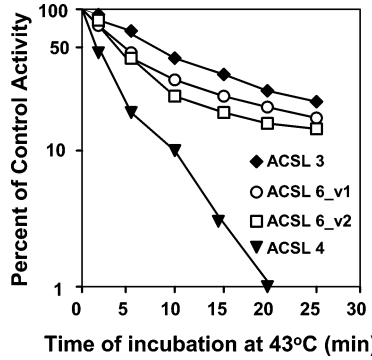

FIGURE 2: ACSL6_v1, ACSL6_v2, ACSL3, and ACSL4-Flag differ in thermolability. Flag-purified ACSL6_v1-Flag (○), ACSL6_v2-Flag (□), ACSL3-Flag (◆), and ACSL4-Flag (▼) were heated to 43 °C in TBS buffer. Samples were removed at the times indicated and assayed immediately at 37 °C. Initial activities were 115 for ACSL6_v1, 621 for ACSL6_v2, 1874 for ACSL3, and 1491 nmol/min/mg for ACSL4. This figure is representative of the results of at least 2 independent assays. ACSL4 data were previously reported (*7*).

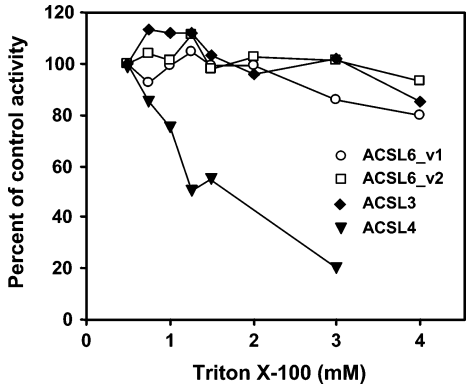

FIGURE 3: Triton X-100 inhibits ACSL4. Flag-purified ACSL6_v1-Flag (○), ACSL6_v2-Flag (□), ACSL3-Flag (◆), and ACSL4-Flag (▼) were assayed for ACSL activity after incubation in the assay with varying concentrations of Triton X-100. Activities with 0.5 mM Triton X-100 were 157 for ACSL6_v1, 818 for ACSL6_v2, 2097 for ACSL3, and 2220 nmol/min/mg for ACSL4 (*7*). This figure is representative of the results of 2 independent assays.

Table 4: IC_{50} Values of Rat ACSL Isoforms for Various Inhibitors[a]

	IC_{50} values, μM					
inhibitor	1[b,c]	6_v1[b]	6_v2[b]	3[b]	4[b,c]	5[b,c]
rosiglitazone	>10	>50	>50	>50	0.6	>10
triacsin C	4	>50	>50	5.5	7.5	>10

[a] Six rat ACSL isoforms were assayed for ACSL activity in the presence of varying concentrations of inhibitors. These numbers are representative of the results of at least 2 independent assays. [b] ACSL isoform. [c] Values for ACSL1, -4, and -5 were reported previously (*7*).

inhibitor, it inhibits only ACSL1 and ACSL4, but not ACSL5 (*7*). We now show that triacsin C also inhibits ACSL3. When triacsin C was added to the enzyme reaction, the IC_{50} values for ACSL1, ACSL3, and ACSL4 were 4, 5.5, and 7.5 μM, respectively (Table 4) (*7*). In comparison, ACSL6_v1 and ACSL6_v2 activities were not affected by triacsin C at concentrations as high as 50 μM.

ACSL Isoforms Have Different Preferences for Fatty Acids Important in Brain Metabolism. ACSL isoforms can activate a wide variety of fatty acids containing 12 to 22 carbons and 0 to five double bonds (*12*, *20*−*22*). Comparison of specific activities obtained with a single concentration of each

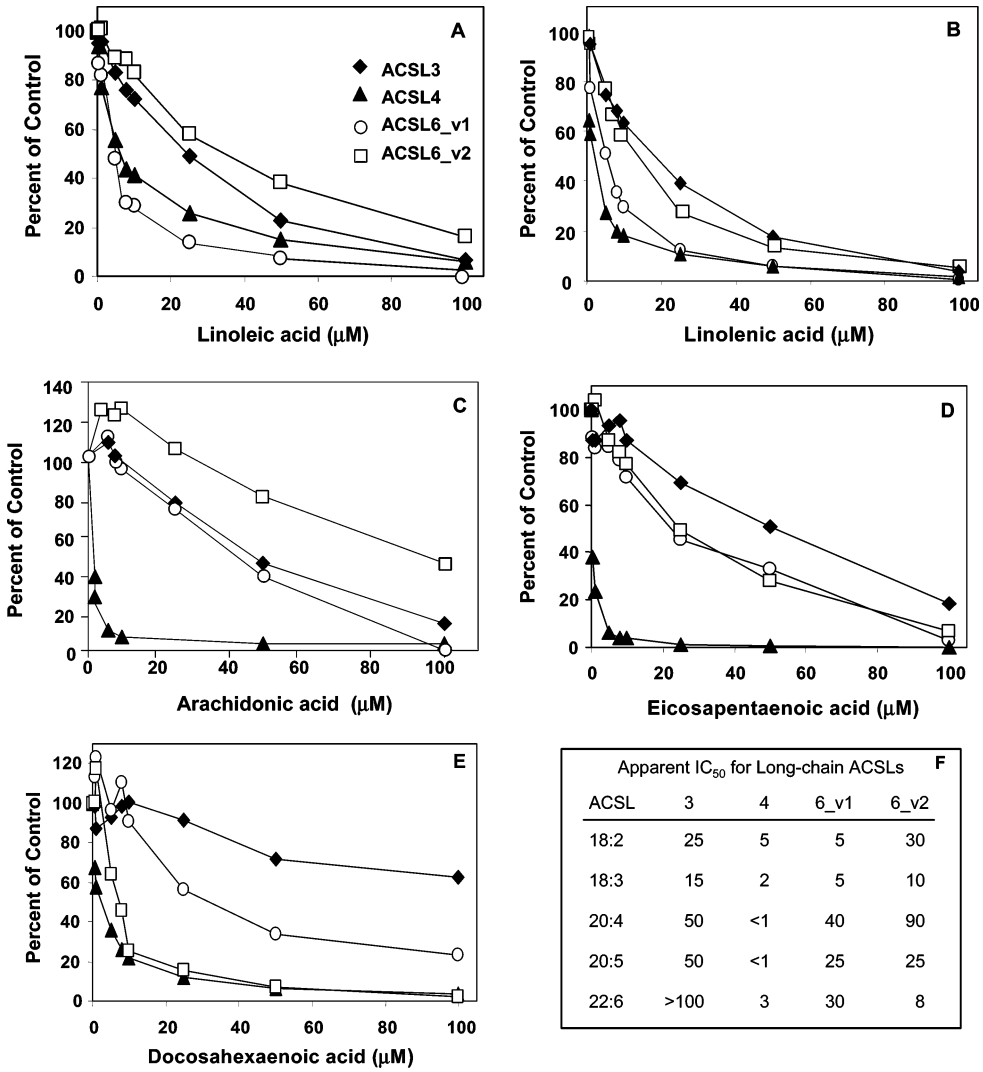

FIGURE 4: ACSL isoforms have different preferences for fatty acids important in brain metabolism. Flag-purified ACSL6_v1-Flag (○), ACSL6_v2-Flag (□), ACSL3-Flag (◆), and ACSL4-Flag (▲) were assayed for ACSL activity with 50 μM [^{14}C]palmitate in the absence or presence of unlabeled (A) linoleic, (B) linolenic, (C) arachidonic, (D) eicosapentaenoic, and (E) docosahexaenoic acid (0.5 μM to 100 μM). (F) Relative IC_{50} for each fatty acid. This figure is representative of the results of at least 3 independent assays that used different Flag-purified enzyme preparations.

fatty acid showed that ACSL4 activity was 2-fold higher with arachidonic acid than with 16:0, 18:0, 18:2, or 22:6 (*20*) and that ACSL3, which shares 63% amino acid identity with ACSL4, has a specific activity with arachidonate that is similar to the activity observed with other long-chain unsaturated fatty acids, but twice as high as with 16:0 and 18:0. These reported comparisons did not directly examine the preference of ACLS3 and ACSL4 for different fatty acids. In the present study, however, ACSL isoforms were assayed in the presence of 50 μM [^{14}C]palmitate with increasing concentrations of each of several fatty acids important in brain metabolism, arachidonic, DHA, EPA, linoleic, and linolenic acids (Figure 4). When assayed in the presence of increasing concentrations of unlabeled arachidonate, ACSL4 activity was strongly inhibited with an IC_{50} of 0.5 μM arachidonate (Figure 4C). In contrast, ACSL6_v1 and ACSL3 did not have a preference for arachidonate compared with palmitate (IC_{50} ~45 μM), whereas ACSL6_v2 preferred palmitate slightly more than arachidonate (IC_{50} = 90 μM). Indeed, ACSL4 strongly preferred all of the polyunsaturated fatty acids tested to palmitate, with IC_{50} values ranging 0.5 μM to 5 μM. Of the isoforms tested, only ACSL4 strongly preferred arachidonate and EPA to palmitate. DHA was strongly preferred by both ACSL4 and ACSL6_v2 (IC_{50} = 3 μM and 5 μM, respectively). Linoleic and linolenic acids were strongly preferred to palmitate by ACSL4 and ACSL6_v1.

DISCUSSION

The predominant ACSL isoforms in liver, ACSL1, -4, and -5, differ in fatty acid preference, subcellular location (*5*, *23*), and regulation (*6*). Our previous studies suggest that the three liver isoforms have independent functions and act to channel acyl-CoA's toward different pathways of lipid metabolism (*3*, *4*). In the current study we extend our characterization to the ACSL isoforms that predominate in rat brain, ACSL3 and -6, and compare them to the minor brain isoforms ACSL1 and -4 (*20*, *24*). ACSL5 transcripts are also present at low abundance in human brain but are expressed at increased levels in primary gliomas and glioma cell lines (*25*). However, although human and rat ACSL5 share 76% identity, ACSL5 mRNA is not found in rat brain (*21*).

By characterizing the brain ACSL isoforms, we hoped to find tools to facilitate the study of the different pathways of acyl-CoA metabolism in brain. As much as 50% of the dry weight of the brain is composed of phospholipids that are highly enriched in polyunsaturated fatty acids (*26*). DHA and arachidonate are quantitatively important in providing approximately 20% of the fatty acids of brain phospholipids (*14*), and are critical for normal brain physiology. Dysfunction of acyl-CoA metabolism occurs in several brain disorders, and abnormalities in brain polyunsaturated fatty acid metabolism have been implicated in Alzheimer disease, depression, and alcoholism (*14*). Although ACSL5 has not been identified in rat brain, increased levels of ACSL5 mRNA are expressed in human gliomas and may play a role in malignant glioma proliferation (*25*). In addition, a form of X-linked mental retardation results from a mutation in the human ACSL4 gene (*27*). In view of the preference of ACSL4 for polyunsaturated fatty acids, one might expect to find abnormalities in the fatty acid composition of brain phospholipids in ACSL4-linked mental retardation.

Both ACSL3 (*28*) and ACSL4 (*29*) are expressed as splice variants with different start sites. The present study reports the first characterization of an ACSL with an internal splice variation. The ACSL6_v2 isoform is 98.3% identical to the previously cloned ACSL6_v1 and differs by substituting exons 13 for 14, encoding a 26 amino acid region. In this region, 14 amino acids are identical, suggesting an important role for these residues in enzyme function. The region containing the altered amino acids lies in neither the putative fatty acid nor the adenine nucleotide binding sites; instead it comprises a region that begins approximately 21 amino acid residues downstream from the adenine nucleotide binding site. This region was not previously identified as playing an important functional role. Although we found no differences in the affinity of ACSL6_v1 and ACSL6_v2 for their fatty acid or CoA substrates, the exon substitution altered affinity for the third substrate, ATP. The apparent K_m value for ATP was 8-fold lower for ACSL6_v2 than for ACSL6_v1 (Figure 1). Thus, it appears that the substitution of exon 13 for 14 after the nucleotide binding site increases ATP affinity, perhaps because of the presence of several charged residues in ACSL6_v2. In the fed state, brain ATP is approximately 3 mM.[3] One might predict that ACSL6_v2 would retain function in the presence of diminished brain ATP levels. It will be interesting to determine whether the two isoforms are variably present in different regions of the brain or whether their expression is developmentally regulated. It is possible that less ACSL6_v2 than ACSL6_v1 is present in brain because the former variant was expressed in only 3 of 19 ACSL6 clones analyzed.

The function of the two major brain ACSL isoforms is not well understood. ACSL3 mRNA is developmentally expressed in rat brain, with peak expression occurring on postnatal day 15, after which levels decline, whereas ACSL6_v1 mRNA is first observed on postnatal day 10 and increases until adulthood (*12*). In contrast, ACSL1 mRNA abundance in brain remains constant. The mRNA for ACSL4 is upregulated by ACTH and suppressed by glucocorticoid in the adrenal gland and the ovary, suggesting that it plays a role in steroidogenesis; it is not known whether regulation in brain is similar. Although PPARβ upregulates ACSL6_v1, but not ACSL1 or ACSL3, in reaggregated rat brain cell cultures, the significance of this observation has not been elucidated (*24*).

ACSL3 appears to have 2 start sites, but transfection of these variant isoforms into COS cells does not affect specific activity or location in the reticular pattern believed to represent endoplasmic reticulum (*28*). When ACSL1 or ACSL6_v1 is overexpressed in the neuronal cell line PC12, both isoforms increase the rate of uptake and incorporation of 18:1, 20:4, and 22:6 fatty acids into TAG, but only ACSL6_v1 increases neurite outgrowth, suggesting that it diverts these fatty acids toward phospholipid synthesis (*30*).

In the mouse, the mRNA from the homologue of rat ACSL6_v2 (96.7% amino acid identity; Table 1A) increases in brain during the fetal period to reach a maximum on the 15th postnatal day (*31*). A yeast two-hybrid screen and immunoprecipitation assays showed that this mouse ACSL associates with phytanoyl-CoA α-hydroxylase (PHAX), a protein deficient in Refsum disease, that normally controls the α-oxidation of phytanic acid. The mouse ACSL6_v2 may deliver phytanoyl-CoA to PHAX or may convert the product of PHAX to pristanoyl-CoA.

All six purified ACSL-Flag isoforms had similar apparent K_m values for CoA, palmitate, and arachidonate, but differed in their affinities for ATP. Of the brain ACSL isoforms, ACSL6_v1 had the highest K_m for ATP at 12210 μM, ACSL3 had a lower K_m for ATP at 402 μM, and ACSL4 had the lowest K_m for ATP at 34 μM, suggesting that it could continue to function in the presence of extremely low ATP levels.

Although ACSL3 and ACSL4 have 62% identity and are more similar to each other than they are to any of the other isoforms, they exhibited a number of different kinetic properties. ACSL4 had a higher K_m for oleate than did ACSL3 and was also substantially more sensitive to heating. ACSL4 lost activity in the presence of low concentrations of Triton X-100, whereas ACSL3 and the ACSL6 variants maintained activity up to 2 to 4 mM. ACSL4 and ACSL3 also had markedly different preferences for arachidonic acid, DHA and EPA. In contrast to previous reports describing specific activities at one fixed concentration of each fatty acid species (*12*, *20*), when ACSL4 was assayed in the presence of both arachidonate and palmitate, arachidonate was activated more readily than palmitate. These differences suggest that specific regions of amino acid nonidentity critically alter several important properties of ACSL3 and ACSL4.

V_{max}/K_m has been described as the rate constant for the capture of substrate by an enzyme into a productive complex (*19*) and V_{max} as a measure of the rate of release of enzyme and product. Both rates together contribute to enzyme catalytic turnover. Surprisingly, the V_{max}/K_m value of ACSL4 for arachidonate is lower than that for palmitate even though the V_{max} is higher. In addition, Figure 4C suggests that ACSL4 prefers arachidonate to palmitate. This apparent discrepancy can be explained using Northrop's descriptions (*19*). By these criteria, the rate of release of product from ACSL4 using arachidonate as a substrate is faster than the corresponding rate for palmitate, whereas the rate of capture

[3] In the fed state brain ATP is 2.38 μmol/g wet wt (*1*). Since the water weight of brain tissue is 0.8 g of water/g of tissue (*2*), and assuming 1 g of water = 1 mL, brain ATP concentration is 3 mM.

of the substrate is similar for both fatty acids. On the other hand both V_{max}/K_m and V_{max} are much lower for oleate than palmitate or arachidonate, clearly suggesting a lower catalytic efficiency for this substrate. This information may help elucidate the mechanism by which different ACSL isoforms prefer different fatty acids substrates.

The activities of some of the ACSL isoforms were diminished by inhibitory agents. Triacsin C, previously identified as a potent inhibitor of ACSL1 and -4 (*7*), also inhibited ACSL3. Igal et al. showed that triacsin C inhibits the de novo synthesis of triacylglycerol in human fibroblasts, but not the reacylation of phospholipids (*8*). If this pathway inhibition pattern holds true for brain tissue, we might surmise that the ACSL6 variants are particularly important for phospholipid reacylation. Because reacylation is critical in brain which has a constant turnover of arachidonate and DHA (*14*), triacsin C may be a useful inhibitor for testing the function of the ACSL6 variants in cultured brain cells. For example, exposing PC12 cells to 5 μM triacsin for 4 days decreased the protein expression of the mouse homologue of ACSL6_v2 and decreased cell proliferation (*31*). The observation of decreased protein synthesis is surprising and suggests a novel effect of triacsin on transcription, perhaps via a transcription factor whose ligand is a fatty acid or an acyl-CoA.

The insulin-sensitizing thiazolidenedione drugs strongly inhibit ACSL4, but not ACSL1 or ACSL5 (*7*). We now show that rosiglitazone does not inhibit ACSL6_v1, ACSL6_v2, or ACSL3. The relative proportions of fatty acids activated by the several brain ACSL isoforms are unknown, but clinical use of thiazolidinediones might inhibit ACSL4 in brain, thereby altering the amount of arachidonate available for incorporation into phospholipids and changing the arachidonate/DHA balance that is critical for normal brain function (*14*).

We previously presented indirect evidence linking the different ACSL isoforms to distinct pathways of acyl-CoA metabolism in liver. Indirect evidence also supports ACSL-mediated channeling of acyl-CoA's in brain. Rapoport reported that only 10% of the arachidonate and DHA, but 50% of the palmitate that enters the brain proceeds through β-oxidation (*13*). These observations suggest that ACSL isoforms with greater preferences for arachidonate and/or DHA, such as ACSL4 and ACS6_v2, target arachidonoyl-CoA and DHA-CoA toward phospholipid synthesis rather than toward β-oxidation. Conservation of arachidonate and DHA would be important in brain because, as precursors for eicosanoids and brain phospholipids, these polyunsaturated fatty acids play critical roles in cell signaling as well as contributing to membrane structure. Fatty acids from plasma provide only 2−4% of the net fatty acids reesterified in brain, but deacylation/reacylation reactions are rapid with turnover rates as high as 20%/h (*13*). Thus, separate enzymes may contribute to each of these two distinct functions: one ACSL isoform may activate fatty acids originating from plasma and direct them toward β-oxidation, while another may activate fatty acids liberated by deacylation reactions. Further studies with ACSL inhibitors and cultured brain cells will be required to determine how the ACSLs present in brain direct acyl-CoA's toward different metabolic fates.

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