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Alternative Translation Initiation Generates Acyl-CoA Synthetase 3 Isoforms with Heterogeneous Amino Termini[1]

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ACS3 is a recently identified acyl-CoA synthetase (ACS) isozyme that preferentially utilizes laurate, myristate, arachidonate, and eicosapentaenoate among saturated and unsaturated long chain fatty acids. The ACS3 purified from COS cells transfected with the ACS3 cDNA was separated by SDS-PAGE into two major forms of 79 and 80 kDa. We report here that alternative translation initiation from ACS3 mRNA gives rise to these two isoforms of ACS3. *In vitro* mutagenesis of the ACS3 cDNA revealed that the translation of the 80-kDa and 79-kDa isoforms started from the first and second in-frame AUGs, respectively. The two isoforms of ACS3 expressed in COS cells exhibited similar levels of ACS activities toward palmitate and myristate. Immunocytochemistry of intact COS cells transfected with various ACS3 expression vectors suggested that the two forms are localized in the extranuclear compartment, where they exhibit a reticular pattern. In rat cerebrum, the 80-kDa isoform of ACS3 was detected mainly in the microsomal fraction. Only a trace amount of the 79-kDa isoform was detected in rat cerebrum, whereas both forms were detected in rat glioma cell line KEG1 cells.

Key words: acyl-CoA synthetase, alternative translation initiation, brain, fatty acid, subcellular localization.

In mammals, activation of fatty acids catalyzed by acyl-CoA synthetase (ACS) [EC 6.2.1.3] is an essential reaction in fatty acid metabolism, since mammalian fatty acid synthetase contains a specific thioesterase to produce a free fatty acid as the final reaction product (*1, 2*). Inhibition of ACS by specific inhibitors, triacsins, drastically reduces the incorporation of fatty acids into cellular phospholipids, thereby blocking the proliferation of mammalian cells (*3*). There are multiple types of ACS in mammals, which are expressed to varying degrees in different tissues. ACS1 is the well characterized ACS abundant in liver, adipose tissue and heart (*4*), and exhibits relatively broad fatty acid specificity (*5, 6*). In rat brain, expression of the ACS1 mRNA is very low despite the extremely high lipid content of the brain (*4*). A brain-specific subtype of ACS1, designated as ACS2, was revealed on molecular characterization of a cDNA (*7*). ACS1 and ACS2 are structurally closely related and exhibit similar specificities with respect to saturated fatty acids (*8*). Among unsaturated fatty acids with 16-22 carbon atoms, ACS1 utilizes palmitoleate, oleate and linoleate the most efficiently, whereas ACS2 preferentially converts oleate, arachidonate, eicosapentaenoate and docosahexaenoate to their CoA-thioesters (*8*).

In a previous study, we demonstrated the existence of another type of ACS, designated as ACS3 (*9*). Although ACS3 has the structural architecture common to ACS1 and ACS2, their amino acid identities are much lower. This novel ACS is highly expressed in brain, and activates arachidonate, eicosapentaenoate and myristate with the highest efficiency. The expression of the two brain ACS mRNAs during brain development is completely different, suggesting that ACS1 and ACS3 play different roles in the brain. The ACS3 purified from COS cells transfected with ACS3 cDNA is detected as a doublet of 79- and 80-kDa polypeptides (*9*). Cell free translation of a synthetic mRNA encoding the entire region of ACS3 also gave 79- and 80-kDa proteins that were precipitated with an anti-ACS3 antibody (*9*). This suggests that the two isoforms are derived from the same mRNA. To further clarify the mechanism generating the two isoforms of ACS3, *in vitro* mutagenesis studies were carried out. In the current paper, we demonstrate that alternative translation initiation produces two forms of ACS3 protein with heterogeneous amino termini. We also describe the subcellular distribution of the two forms of ACS3 protein in COS cells transfected with various mutants and in rat cerebrum.

EXPERIMENTAL PROCEDURES

Materials and Standard Procedures—Unless otherwise

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Abbreviations: ACS, acyl-CoA synthetase; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; PBS, phosphate buffered saline.

indicated, all restriction and DNA modifying enzymes were from Takara Shuzo Corp. [1-^{14}C]Palmitic acid (≈50 Ci/mol) and [^{35}S]PRO-MIX (>1,000 Ci/mol [^{35}S]methionine and L-[^{35}S]cysteine) were obtained from Amersham. [1-^{14}C]Myristic acid (~50 Ci/mol) was a product of DuPont NEN. Standard molecular biology techniques were performed essentially as described by Sambrook *et al.* (*10*). Oligonucleotides were synthesized with an Applied Biosystems model 381A DNA synthesizer.

Metabolic Labeling and Immunoprecipitation of ACS3—COS-7 cells (1×10^6 cells per 10-cm Petri dish) were transfected with pACS3 by the DEAE-dextran method, as described previously (*1*). After 60 h incubation at 37°C, the cells were washed once with methionine/cysteine-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (medium A), and then labeled with medium A containing 750 μCi/ml [^{35}S]PRO-MIX. After 6 h labeling at 37°C, the cells were washed twice and then suspended in 50 mM potassium phosphate (pH 7.4), 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (w/v) glycerol, and 0.5% (w/v) Triton X-100. The cells were disrupted by two cycles of 20 s sonication and then centrifuged at 230,000×*g* for 30 min at 4°C. The resulting supernatant was incubated with 200 μg of the antibody raised against the fusion protein of glutathione *S*-transferase (GST) and a fragment of ACS3 (*9*) at 4°C for 1 h, followed by the addition of 25 μl of Protein A-Sepharose CL-4B beads (Pharmacia). After incubation on a shaker at 4°C for 30 min, the beads were collected by centrifugation and washed sequentially: once with 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1% (w/v) Nonidet P-40; four times with 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) Nonidet P-40, and 0.1% (w/v) SDS; and once with 10 mM Tris-HCl (pH 6.8) and 100 mM NaCl. The washed beads were resuspended in 50 μl of SDS loading buffer (*11*) and then boiled for 3 min. The immunoprecipitated proteins were then separated by 8% SDS-PAGE, followed by autoradiography.

Construction of Mutant Expression Vectors—To create a mutant ACS3 with substitution of the Met residue at amino acid position 12 with Leu (M12L), PCR (*12*) was carried out using a sense primer (5′-AGGATCCATGAATAACCAGTATCTTCAACACCGTCTACCTTGAAG-3′) and an antisense primer (5′-TGAATTCAGGCCTTCGGCTTCTGGCCC-3′). The plasmid DNA (0.1 μg) was amplified with 250 nM of each primer and 0.75 units of *Taq* DNA polymerase in a 100 μl volume of the buffer recommended by the supplier. The thermal profile used was 94°C for 30 s, 52°C for 30 s, and then 72°C for 2 min. After 25 cycles, the PCR products were digested with *Bam*HI and *Hinc*II, and the resulting 217-bp fragment was separated by electrophoresis on a 5% polyacrylamide gel. The 217-bp PCR amplified fragment and the 664-bp *Hinc*II/*Eco*RI fragment from pACS3 were inserted into the *Bgl*II (blunt-ended)/*Eco*RI sites of pACS3 to create pACS3M12L. To generate a 5′ deletion mutant lacking the initiator ATG, pACS3 was digested with *Acc*I, blunt-ended with Klenow enzyme, and then digested with *Eco*RI. The resulting 848-bp fragment was inserted into the *Bgl*II (blunt-ended)/*Eco*RI sites of pACS3 to create pACS3Δ11. pACS3Δ73, a 5′ deletion mutant lacking both the first and second in-frame ATGs, was created by insertion of the 456-bp *Hinc*II/*Eco*RI fragment of pACS3 into the *Bgl*II (blunt-ended)/*Eco*RI sites of pACS3.

Immunoblotting—Protein samples were denatured in SDS loading buffer (*11*), separated on an 8% SDS-polyacrylamide gel, and then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell) using a Trans-Blot apparatus (Bio-Rad) for 12 h at 10 V and 100 mA. The membrane was blocked by soaking for 24 h in 5% (w/v) non-fat dry milk in phosphate-buffered saline (PBS). After rinsing with PBS, the blocked membrane was incubated at room temperature for 2 h with an anti-GST-ACS3 IgG at 5 μg/ml. After washing five times with PBS containing 0.05% (w/v) Tween 20, it was incubated with an alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector) at a dilution of 1:1,000 for 1 h. After another five washings with PBS containing 0.05% (w/v) Tween 20, color was developed according to the manufacturer's instructions using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Immunocytochemistry—COS cells transfected with the wild-type and mutant ACS3 expression vectors were plated on an eight-chamber well slide (Lab-Tek™, Nunc). After incubation for 24 h, the cells were washed twice with PBS, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5) for 15 min, and then rinsed with PBS. The cells were then incubated with PBS containing 0.05% Triton X-100 for 15 min at room temperature, followed by three washes with PBS. The permeablized cells were presoaked with PBS containing 5% normal goat serum for 30 min and then incubated with either a nonimmune IgG or an anti-GST-ACS3 IgG (50 μg/ml in PBS containing 1% bovine serum albumin) for 1h at room temperature, followed by washing with PBS and exposure to a FITC-conjugated goat anti-rabbit IgG (20 μg/ml). The cells were washed twice at room temperature with PBS for 15 min and then mounted with glycerol/PBS (1 : 1, v/v) containing 0.1 ng/ml propidium iodine. Fluorescence microscopy was performed with a Zeiss Axioskop microscope using a 40× oil immersion objective and an FITC filter package.

Subcellular Fractionation of Rat Cerebrum—Subcellular fractionation of rat cerebrum was carried out by the methods described by Mizoguchi *et al.* (*13*). All the procedures were carried out at 4°C. Briefly, 10 g of rat cerebrum was homogenized in 40 ml of buffer A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride) by 12 strokes at 1,000 rpm in a glass/Teflon homogenizer. The homogenate was centrifuged at 1,400×*g* for 10 min and the resulting pellet (P_1) was washed once with buffer A. The supernatant from the initial centrifugation was centrifuged at 13,800 ×*g* for 10 min to obtain a crude mitochondrial fraction (P_2). The supernatant was further centrifuged at 120,000×*g* for 1 h, yielding pellet P_3 and a supernatant fraction. The crude mitochondrial fraction, P_2, was suspended in 48 ml of buffer B (0.32 M sucrose and 1 mM $NaHCO_3$) and then homogenized in a glass/Teflon homogenizer (six strokes at 2,000 rpm). The homogenate was layered on top of a discontinuous sucrose density gradient consisting of 9.5 ml each of 0.85, 1.0, and 1.2 M sucrose containing 1 mM $NaHCO_3$, and then centrifuged at 82,500×*g* for 2 h to obtain the following subfractions: the band at the 0.32-0.85 M sucrose interface and the material in 0.85 M sucrose, designated as the P_2A fraction, containing mainly myelin and some contaminants from the P_2B fraction; the band at

the 0.85-1.0 M sucrose interface and the material in 1.0 M sucrose, designated as the P_2B fraction, containing a mixture of endoplasmic reticulum, Golgi complex and plasma membranes; the band at the 1.0-1.2 M sucrose interface and the material in 1.2 M sucrose, designated as the P_2C fraction, containing mainly synaptosomes; and the pellet, designated as P_2D, containing mainly mitochondria. Each fraction was analyzed by immunoblotting.

RESULTS

Comparison of the Sequences Surrounding the First and Second in-Frame ATGs of the ACS3 cDNA—Table I shows the sequences surrounding the first and second in-frame ATGs of the ACS3 cDNA aligned with the consensus sequence (5′-CCA/GCC**ATG**G-3′) proposed by Kozak: positions −3 and +4 are particularly important for ensuring a high efficiency of translation initiation (*14-16*). The sequence comparison revealed poor matching of the sequences around the first ATG (5/9; −3 and +4 not conserved), and moderate homology surrounding the second ATG (7/9; −3 conserved). Since the first in-frame ATG lies in an unfavorable context, it is likely that the two forms of ACS3 derived from the same mRNA are produced through alternative initiation at either the first or second in-frame AUG.

Expression of Wild-Type and Mutant ACS3 in COS Cells—To examine the possibility of alternative translation initiation from ACS3 mRNA, we constructed three mutant ACS3 expression vectors containing altered translation initiation codons (Fig. 1A). pACS3M12L was constructed by site-directed mutagenesis to replace Met 12 with Leu. pACS3⊿11 and pACS3⊿73 are 5′-deletion mutants: pACS3⊿11 lacks the first in-frame ATG, and pACS3⊿73 lacks both the first and second in-frame ATGs. The wild-type (pACS3) and mutant plasmids were each transfected into COS cells and then their expression was analyzed by immunoblotting using an anti-GST-ACS3 antibody. Consistent with the results of immunoprecipitation, the two isoforms of 79- and 80-kDa were detected on the immunoblotting of lysates of COS cells transfected with the wild-type plasmid (Fig. 1B, lane 1). The replacement of Met 12 with Leu by *in vitro* mutagenesis of the ACS3 cDNA prevented the synthesis of the 79-kDa isoform and instead increased the synthesis of a 72-kDa protein that is only weakly detected in COS cells transfected with the wild-type plasmid (Fig. 1B, lanes 2 and 5). The 72-kDa protein is most likely derived from the third ATG codon, since the mutant lacking both the first and second in-frame ATGs (pACS3⊿73) produced only this isoform (Fig. 1B, lane 4). In COS cells transfected with the deletion mutant lacking the first ATG (pACS3⊿11), both the 79- and 72-kDa proteins were detected (Fig. 1B, lanes 3 and 5).

We also analyzed the effects of these mutations in the ACS3 cDNA on ACS activity and fatty acid specificity. To

TABLE I. **Comparison of the sequences surrounding the first and second methionine codons in ACS3 cDNA (*9*) with the consensus sequence proposed by Kozak (*14-16*).**[a]

	−3 +4
Consensus	(GCC)GCCACC**ATG**G G
Met-1	(t t t)Ga g c a C**ATG**a
Met-2	(c Cg)t Ct ACC**ATG**a

[a]Methionine codons are underlined.

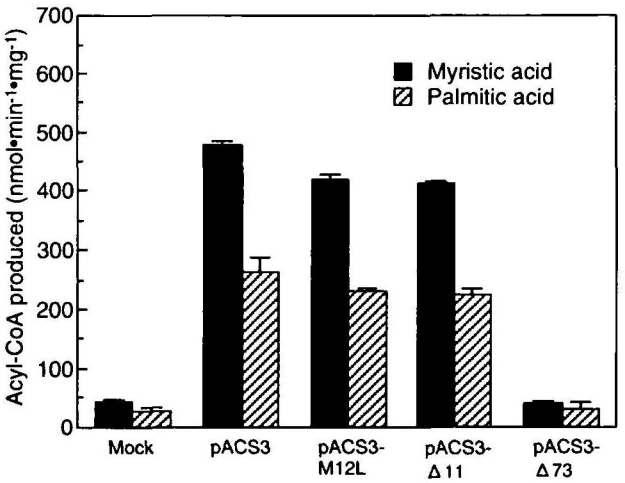

Fig. 2. **ACS activities in COS cells transfected with the wild-type and mutant ACS3 expression vectors.** ACS activities in COS were measured using ^{14}C-labeled palmitate and myristate as substrates. Means±SD for triplicate experiments.

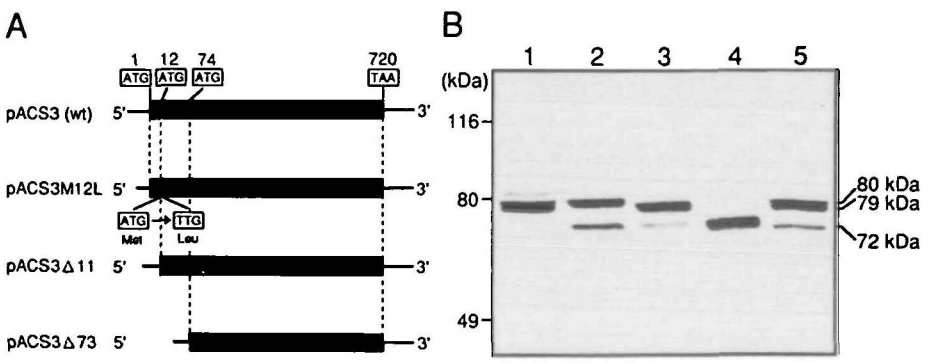

Fig. 1. **Immunoblot analysis of ACS3 in COS cells transfected with wild-type and mutant ACS3 expression vectors containing altered translation initiation start sites.** A: Schematic representation of the wild-type pACS3 and mutant (pACS3M12L, pACS3⊿11, and pACS3⊿73) ACS3 expression vectors. The M12L substitution mutant (pACS3M12L) was constructed by replacing the A of the second ATG (ATG2) with T. pACS3⊿11, and pACS3⊿73 are 5′ deletion mutants of pACS3 lacking the first ATG, and both the first and second in-frame ATGs, respectively. The thick lines indicate the coding regions, and the thin lines represent the 5′- and 3′-untranslated regions. B: Lysates of COS cells transfected with the wild-type or mutant ACS3 expression vectors were analyzed by immunoblotting. Each lane contained 10 μg protein and the blot was probed with an anti-GST-ACS3 IgG. Lane 1, cells transfected with the wild-type (pACS3); lane 2, pACS3M12L; lane 3, pACS3⊿11; lane 4, pACS3⊿73; and lane 5, a mixture of the lysates of COS cells individually transfected with pACS3M12L or pACS3⊿11.

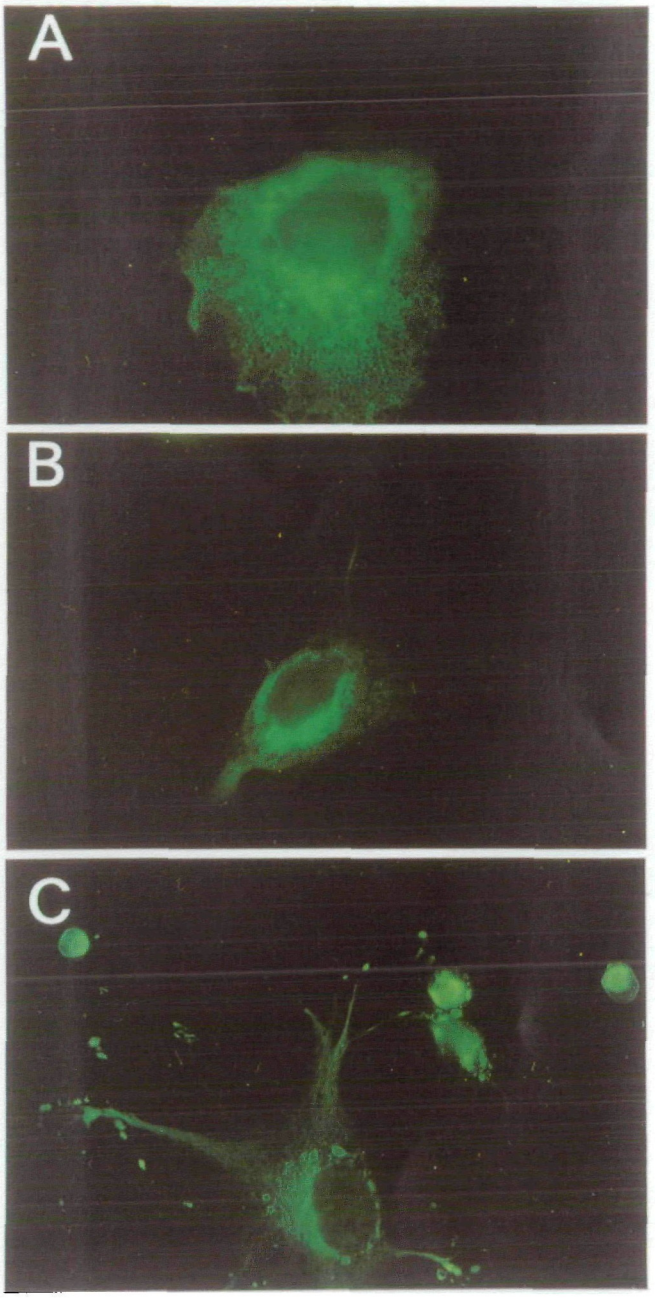

Fig. 3. **Indirect immunofluorescence localization of wild-type and mutant ACS3 in transfected COS cells.** COS cells transfected with (A) the wild-type (pACS3), (B) the M12L substitution mutant (pACS3M12L), or (C) the mutant lacking the first ATG (pACS3⊿11) were permeablized, and then immunofluorescence staining was carried out using an anti-GST-ACS3 IgG as the primary antibody and a FITC-conjugated goat anti-rabbit IgG as the second antibody. Magnification: 100×.

evaluate fatty acid specificity, palmitate and myristate were used as substrates in ACS assays. Figure 2 shows the ACS activities in COS cells transfected with the wild-type and mutant plasmids. COS cells transfected with the mutant lacking the first ATG (pACS3⊿11) and the M12L substitution mutant (pACS3M12L) exhibited similar ACS activity levels to those observed in COS cells transfected with the wild-type plasmid. The ratios of myristoyl- to palmitoyl-CoA synthetase activities in COS cells transfected with the wild-type, pACS3⊿11, and pACS3M12L were essentially the same. In COS cells transfected with the mutant lacking both the first and second in-frame ATGs (pACS3⊿73), the levels of ACS activities were similar to those in mock-transfected cells. Together with the production of the 72-kDa immunoreactive protein in COS cells transfected with the mutant lacking both the first and second in-frame ATGs, these results indicated that the sequence between Met 12 and Met 74 of ACS3 is required for the functional expression of ACS activity.

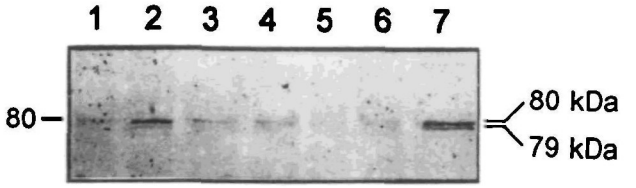

Fig. 4. **Immunoblot analysis of ACS3 in various subcellular fractions of rat cerebrum.** Fifty micrograms protein of each subcellular fraction and the total extract of KEG1 cells was electrophoresed, respectively, on an 8% SDS-polyacrylamide gel and then probed with an anti-ACS3-GST IgG. Lane 1, P_1 fraction; lane 2, P_3; lane 3, P_2A; lane 4, P_2B; lane 5, P_2C; lane 6, P_2D; lane 7, total extract of KEG1 cells. The results are representative of two independent experiments. (Fractions are defined in the text.)

Subcellular Localization of ACS3—To examine the localization of the 79- and 80-kDa isoforms of ACS3 in intact cells, we performed immunocytochemistry. The available antibodies were not sufficiently sensitive to detect ACS3 as to immunofluorescence in non-transfected cells, so we used COS cells transfected with the wild-type and mutant ACS3 cDNAs. Wild-type ACS3 was observed in the extranuclear compartment, where it exhibited a reticular pattern (Fig. 3A). Similar patterns were observed in COS cells transfected with pACS3M12L (Fig. 3B) and pACS3⊿11 (Fig. 3C).

To study the subcellular localization of the 79- and 80-kDa isoforms of ACS3, we performed a cell fractionation experiment on rat cerebrum. The homogenate of rat cerebrum was fractionated into a P_1 fraction containing nuclei and cell debris, a P_2 fraction containing synaptosomes, mitochondria and myelin, and a P_3 fraction containing microsomes. The P_2 fraction was further fractionated into the following four fractions: P_2A, comprising myelin and some contaminating membrane components from P_2B; P_2B, comprising a mixture of endoplasmic reticulum, Golgi complex and plasma membranes; P_2C, mainly comprising synaptosomes; and P_2D, mainly comprising mitochondria. Among these fractions, the 80-kDa isoform of ACS3 was detected mainly in the P_3 fraction (Fig. 4, lane 2), and weakly in the P_2A and P_2B fractions (Fig. 4, lanes 3 and 4). Only a trace amount of the 79-kDa isoform was detected in rat cerebrum, whereas both forms were detected in rat glioma cell line KEG1 cells (Fig. 4, lane 7).

DISCUSSION

In the present study, we found that two ACS3 isoforms are generated through alternative translation from the same mRNA using mutant ACS3 expression plasmids containing altered translation initiation codons. Our data clearly

indicate that the translation of the 80- and 79-kDa ACS3 isoforms starts from the first and second in-frame AUGs, respectively.

The production of the 79-kDa ACS3 isoform at the second in-frame AUG appears to depend on sequences surrounding the first AUG. The most plausible mechanism accounting for the alternative translation initiation in the case of ACS3 is the leaky ribosome scanning model proposed by Kozak (*17, 18*). The small subunit of the ribosome first recognizes the 5′-terminal cap structure of an mRNA and then scans the mRNA sequence in the 5′ to 3′ direction for potential AUG initiation codons. Often, but not always, the first AUG is utilized for the initiation of protein synthesis. Whether the first AUG is selected or ignored depends on the sequence context surrounding it. Since the first in-frame AUG of the ACS3 mRNA lies in an unfavorable context (Table I), the small subunit of the ribosome may not recognize the first in-frame AUG completely, allowing the translation initiation at the second in-frame AUG.

COS cells transfected with the mutant lacking the N-terminal 11 amino acids (expressing only the 79-kDa isoform) and the M12L substitution mutant (expressing only the 80-kDa isoform) exhibited similar levels of ACS activities toward myristate and palmitate, suggesting that the N-terminal 11 amino acids are not required for the functional expression of ACS3 in COS cells and do not drastically alter the fatty acid specificity of the enzyme.

Immunocytochemistry with an anti-ACS3 antibody showed that COS cells transfected with the wild-type and mutants lacking the N-terminal 11 amino acids, as well as the M12L substitution mutant exhibited similar patterns of extranuclear localization surrounding the nucleus and extending throughout the cytoplasm. This suggests that the N-terminal 11 amino acids have no effect on the cellular localization of the ACS3 protein. Consistent with the results of immunocytochemistry, the 79- and 80-kDa isoforms were also detected in the microsomal fraction of rat cerebrum, in which the 79-kDa isoform was a minor one. Despite the consistent production of the two ACS3 isoforms in KEG1 cells and COS cells transfected with the ACS3 cDNA, as well as cell-free translation of synthetic ACS3 mRNA (*9*), the 79-kDa isoform is a minor form in the brain. This may be a consequence of preferential degradation of the 79-kDa isoform or preferential translation initiation leading to production of the 80-kDa isoform in the brain. The 80-kDa isoform contains an extra dodecapeptide enriched with serine and threonine residues at its N-terminal (*9*). Soldati *et al.* have shown that multiple forms of chicken brain-type creatine kinase with different N-termini are produced through alternative translation initiation from the same mRNA (*19*). The amino terminal dodecapeptide in one of the creatine kinase isoforms contains potential sites for cAMP-dependent protein kinase and AMP-activated protein kinase (*19*). Similarly, the N-terminal dodecapeptide of the 80-kDa isoform of ACS3 contains a potential site for protein kinase C (*20, 21*) at amino acids 11–13 (Thr-Met-Lys), raising the possibility that phosphorylation of the amino terminal by protein kinase C modulates the enzyme activity of ACS3 or the stability of the enzyme in the brain. Although the biological significance of the generation of two ACS3 isoforms is not clearly understood at this point, further characterization will unveil the cellular mechanisms regulating the activation of fatty acids by ACS3.

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