Molecular Characterization and Expression of Rat Acyl-CoA Synthetase 3*

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Isolation and characterization of a rat brain cDNA identified a third acyl-CoA synthetase (ACS) designated ACS3. The deduced amino acid sequence of the cDNA revealed that ACS3 consists of 720 amino acids and exhibits a structural architecture common to ACSs from various origins. ACS3 expressed in COS cells was purified to near homogeneity. The purified ACS3 resolved by SDS-polyacrylamide gel electrophoresis into two major proteins of 79 and 80 kDa. Cell-free translation of a synthetic mRNA encoding the entire region of ACS3 revealed that the two isoforms were derived from the same mRNA. The purified ACS3 utilizes laurate and myristate extensively among C8-C22 saturated fatty acids and arachidonate and eicosapentaenoate among C18-C20 unsaturated fatty acids. Northern blot analysis revealed that ACS3 mRNA is most abundant in brain and, to a much lesser extent, in lung, adrenal gland, kidney, and small intestine. During the development of the rat brain, expression of ACS3 mRNA reached a maximum level at 15 days after birth and then declined gradually to 10% of the maximum in the adult brain.

Activation of fatty acids catalyzed by acyl-CoA synthetase (ACS, EC 6.2.1.3) is an initial reaction of fatty acid metabolism in eukaryotic cells. This reaction is indispensable in fatty acid utilization; thereby ACS plays a key role in lipid metabolism. Acyl-CoA produced by this enzyme is a key intermediate for fatty acid utilization; thereby ACS plays a key role in lipid metabolism. Acyl-CoA is utilized in numerous reactions, including reesterification and nuclear thyroid hormone receptor (4).

Although the brain contains two types of ACS, the presence of ACS2 is predominantly expressed in the brain (18). Although the brain contains two types of ACS, the presence of other brain types of ACS specific for arachidonate (19), docosahexaenoate (20, 21), and lignocerate (22–24) have been shown by enzymatic characterization. However, none of these enzymes have been fully characterized by purification or cDNA cloning.

To further extend our studies on ACS in brain, we have isolated a cDNA that encodes a new brain-type ACS distinct from ACS1 and ACS2. We describe here the primary structure, expression, and regulation of this brain-type ACS designated ACS3. We also describe the enzymatic properties of the purified enzyme from COS cells transfected with ACS3 cDNA.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated, all restriction and DNA-modifying enzymes were from Takara Shuzo Corp. (Kyoto, Japan). ACMS; luciferase family includes acetyl-CoA synthetases from Neurospora crassa and Aspergillus nidulans (7), 4-coumarate:CoA ligase 1 from parsley (8), Aspergillus nidulans (7), 4-coumarate:CoA ligase 1 from parsley (8), 4-coumarate:CoA ligase 1 from parsley (8), 4-coumarate:CoA ligase 1 from parsley (8), and nuclear thyroid hormone receptor (4).

The brain is one of the most lipid-enriched organs in the body. Most brain lipids are actively synthesized in the brain itself and deposited in large amounts during the early phase of development of the nervous system (16, 17). Despite the importance of lipids in the brain, relatively little is known about lipid metabolism in the brain.

In a previous study, we have shown the presence of a brain-specific isozyme designated brain ACS (hereafter referred to as ACS2) by cDNA cloning (18). ACS2 resembles the well characterized ACS (hereafter referred to as ACS1) in amino acid sequence (5, 6); approximately 65% of the amino acids in the two enzymes are identical. Although ACS1 and ACS2 are structurally similar and exhibit similar fatty acid specificity, the patterns of tissue expression of the two enzymes are completely different. ACS1 mRNA is most abundant in liver, heart, and adipose tissue and, to a much lesser extent, in brain (5). In contrast, ACS2 is predominantly expressed in the brain (18).

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To further extend our studies on ACS in brain, we have isolated a cDNA that encodes a new brain-type ACS distinct from ACS1 and ACS2. We describe here the primary structure, expression, and regulation of this brain-type ACS designated ACS3. We also describe the enzymatic properties of the purified enzyme from COS cells transfected with ACS3 cDNA.

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The abbreviations used are: ACS, acyl-CoA synthetase; kb, kilobase(s); GST, glutathione S-transferase; LS regions, luciferase similar regions; PCR, polymerase chain reaction.
Acyl-CoA Synthetase 3

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Poly(A) RNA was prepared from adult rat brain total RNA using oligo(dT)-T-lutex (Takara Shuzo) as described (30). A mixture of oligo(dT)-primed cDNAs was synthesized from 3 μg of brain poly(A) RNA using oligo(dT)T25 (1.5 μg), 25 units of RNasin, and 400 units of Superscript (Life Technologies, Inc.) in 30 μl of standard reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 7.5 μM dithiothreitol, and 1 mM of each deoxynucleotide triphosphate) at 37 °C for 1 h. The reaction products were then subjected to replacement synthesis of the second strand cDNA (31). The resulting double-stranded cDNA was precipitated in ethanol and dissolved in 20 μl of 100 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Five μl of the cDNA were amplified (28 cycles with Tyr(Pr)4 ddTTP) using two highly degenerate primers derived from the most highly conserved amino acid sequences among ACS1, ACS2, and dick beetle luciferase (18). The sequences of the sense primer and the antisense primer were 5′-GG(A/G/C/T)GA(A/G/C/T)GA(A/G/C/T)AA(A/G/C/T)3′ and 5′-GA(C/T)GG(A/G/C/T)GA(A/G/C/T)CT(A/G/C/T)3′, respectively. PCR amplification was carried out using 1 μl of each primer and 0.75 unit of Taq DNA polymerase in 100 μl of standard PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, and 0.2 μM of each deoxynucleotide triphosphate). The thermal profile used was 94 °C for 30 s, 54 °C for 1 min, and then 72 °C for 2 min. After 30 cycles, the PCR products were separated by electrophoresis on a 5% acrylamide gel. Maxishield reaction products (0.5–1.0 kb) were eluted from the gel, subcloned into T-vectors (33), and sequenced. By sequencing 50 clones, we obtained a cDNA encoding a new type of ACS (pOCT5-5).

Isolation of cDNAs—Rat brain cDNA library (18) constructed in Okayama-Berg vector (34) was screened using a 290-base pair cDNA fragment from pOCT5-5 as a probe. By screening of 3 × 106 clones, we obtained eight positive clones; one representative clone containing the largest cDNA insert (pACS3) was further characterized.

DNA Transfection—COS-7 cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with 10 μg of plasmid DNA by the DEAE-dextran method (35). Three days after DNA transfection, the cells were harvested for measurement of ACS activity.

Assay of ACS Activity—ACS activity was determined at 37 °C either by the isotopic method (19) or by the spectrophotometric method (25). The latter was used only for the purified enzyme. The ratio of the activity measured by the spectrophotometric method (with the standard mixture) to that measured by the isotopic method (with the standard mixture) was 0.75. All assays were carried out within the range where the reaction proceeded linearly with time and the initial rate of reaction was proportional to the amount of enzyme added. The protein content of cell extract was measured by the Lowry method (36) with bovine serum albumin as the standard. The reaction product of the enzyme reaction was subjected to immunoprecipitation with an antibody against the fusion protein of glutathione S-transferase (GST) and a fragment of ACS3 (see below) and analyzed by SDS-polyacrylamide gel electrophoresis.

Antibodies—A fusion protein of GST and a fragment of ACS3 was used to immunize rabbits. To produce the GST-ACS3 fusion protein, a cDNA fragment encoding ACS3 amino acids 72–223 was ligated in-frame to a pGEX bacterial expression vector (Pharmacia). The fusion protein was induced in Escherichia coli JM109 with isopropyl-β-D-thiogalactopyranoside for 4 h. E. coli cells were sonicated in a lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100 (w/v), 0.1% sodium deoxycholate (w/v), and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,000 × g for 10 min at 4 °C. The pellet was resuspended with the lysis buffer containing 5 M urea and centrifuged at 12,000 × g for 20 min at 4 °C. The fusion protein recovered in the pellet was then dissolved by boiling in the SDS-sample buffer, purified by SDS-polyacrylamide gel electrophoresis, and used as an antigen.

RESULTS AND DISCUSSION

Isolation of ACS3 cDNAs from Rat Brain—A new type of ACS designated ACS3 was isolated through PCR-mediated DNA amplification by using two degenerate oligonucleotide primers corresponding to the conserved amino acid sequences among ACS1, ACS2, and dick beetle luciferase (18). Fifty clones isolated by PCR amplification were sequenced, and the sequence of one of them (pOCT5-5) showed 25% amino acid identity with the corresponding regions of ACS1 and ACS2. A new type of ACS designated ACS3 was isolated through PCR-mediated DNA amplification by using two degenerate oligonucleotide primers corresponding to the conserved amino acid sequences among ACS1, ACS2, and dick beetle luciferase (18). Fifty clones isolated by PCR amplification were sequenced, and the sequence of one of them (pOCT5-5) showed 25% amino acid identity with the corresponding regions of ACS1 and ACS2.

To obtain a cDNA encoding a complete coding region, an adult rat brain cDNA library was screened by hybridization with a 32P-labeled insert of pOCT5-5 under high stringency conditions. Screening 5 × 106 clones, we obtained eight positive clones. A representative clone (designated pACS3) containing the largest cDNA insert was sequenced. The insert of the cDNA includes a 2160-base pair open reading frame encoding a protein of 720 amino acids with a calculated molecular weight of Mr 80,456 (Fig. 1A). The putative initiator methionine was preceded by an in-frame stop codon present 39 nucleotides upstream.

In previous studies, we have shown that ACS1 and ACS2 consist of two regions, designated luciferase similar regions 1 and 2 (LS regions 1 and 2) that contain amino acids highly conserved among the members of the ACS/luciferase family (5, 6, 18). Between ACS1 and ACS2, the amino acids in the two LS
regions and the COOH terminus are highly conserved (18).
Similarly, the amino acids in the two LS regions and the COOH
terminus are most highly conserved between ACS2 and ACS3
(30–38%; Fig. 1B); the NH₂-terminal and a linker connecting LS regions 1 and 2 lacked amino acid
conservation. Like ACS1 and ACS2, the two LS regions of
ACS3 are most similar to the corresponding regions of click
beetle luciferase among the members of the ACS/luciferase
family (excluding ACSs) from various origins (Fig. 1B).

Expression and Purification of ACS3—To characterize the
fatty acid specificity and kinetic properties of the purified
ACS3, the cloned ACS3 cDNA was introduced into COS cells.
When measured with palmitate as a substrate, COS cells
transfected with the cDNA exhibited 8–10-fold increased ACS
activities as compared with those transfected with vector alone
(data not shown). Attempts to overproduce the recombinant
enzyme in E. coli cells were unsuccessful. In COS cells expressing
ACS3, most of the enzyme activity was found in the microsomal fraction (data not shown). Using the microsomal fraction of the
ACS3-transfected cells as a starting material, ACS3 was
purified to near homogeneity. The purification procedure in-
volved solubilization of the enzyme with Triton X-100 and
chromatography on Q-Sepharose and Blue-Sepharose (Table I).
When measured by the isotopic method using palmitate as a
substrate, the purified enzyme exhibited a specific activity of
4.62 \( \text{m mol/min/mg protein} \) at 37°C. This value is 5-fold lower
than that of the purified ACS1.

The purified ACS3 resolved by SDS-polyacrylamide gel elec-
trophoresis into two major proteins with 79 and 80 kDa (Fig.
2A). Consistent with the purification of the 79- and 80-kDa
proteins, cell-free translation of a synthetic mRNA encoding
the entire region of ACS3 produced 79- and 80-kDa proteins
(Fig. 1B).
that were precipitated with the anti-ACS3-GST antibody (Fig. 2B). This indicates that the two isoforms are derived from the same mRNA. Although the synthetic mRNA contains an in-frame stop codon 39 nucleotides upstream of the initiator AUG, a minor protein (87 kDa) copurified with the 79- and 80-kDa proteins was also detected in the cell-free translation. The nature of this 87-kDa protein is currently unknown.

Fatty Acid Specificity and Other Kinetic Properties—Fatty acid specificity of the purified enzyme was determined by the spectrophotometric method using various fatty acids. Fig. 3 compares the fatty acid specificity of the purified ACS3 with that of the purified ACS1. The purified ACS3 utilizes laurate and myristate most efficiently among C8–C22 saturated fatty acids and arachidonate and eicosapentaenoate among C16–C20 unsaturated fatty acids. This fatty acid specificity is completely different from that of the purified ACS1, which uses C10–C18 saturated fatty acids and C16–C20 unsaturated fatty acids with approximately equivalent activities. Unlike the purified ACS1, the purified ACS3 prefers C16–C20 unsaturated fatty acids; the relative activities of ACS3 for these fatty acids are 2·fold higher than those of ACS1. Although the purified ACS1 and ACS3 exhibited different fatty acid specificities, the apparent K_m values of the two enzymes for myristate and arachidonate were in the same range (10–15 μM).

The purified ACS3 has optimal activity at pH 7.0–8.5 and requires ATP (K_m = 0.5 ± 0.04 mM), CoA (K_m = 0.53 ± 0.08 μM), and fatty acids. The reaction product formed by the enzyme from [14C]palmitate was identified as palmitoyl-CoA by chromatographic analysis of its hydroxamic acid derivative. When adenylyl kinase was omitted from the reaction mixture for the spectrophotometric assay, no oxidation of NADH occurred, indicating that AMP was a reaction product.

Tissue Distribution and Developmental Expression of ACS3 mRNA—Northern blot analysis using a ACS3 cDNA probe revealed that ACS3 mRNA is expressed as a 3.3-kb transcript in normal adult rat tissues (Fig. 4A). The ACS3 mRNA is predominantly expressed in the brain and appears, to a much lesser extent, in lung, adrenal gland, kidney, small intestine, and adipose tissue but is not detected in heart or liver. Consistent with its predominant expression in the brain, ACS3 mRNA was detected in rat C6 glioma, rat glioma KEG1, and rat adrenal pheochromocytoma PC12 cells (Fig. 4B).

During the development of rat brain, ACS3 mRNA was detectable 5 days after birth, increased to a maximum level at 15 days, and then decreased gradually to 10% of its maximum level in the adult (Fig. 5A). In contrast, ACS2 mRNA increased gradually and reached a maximum level in the adult, and no change was observed in ACS1 mRNA levels during brain development (Fig. 5, B and C).

The presence in the brain of multiple forms of ACS with different fatty acid specificity is of considerable biological significance for controlling the synthesis of brain lipids. Although the exact nature of the enzyme remains to be elucidated, the
Northern blotting. Each lane represents total RNA (15 μg) from rat brains at the indicated postnatal age (days) were analyzed by Northern blotting using a 32P-labeled 2.0-kb fragment from ACS2 cDNA (pBACS9) and left to expose a Kodak XAR-5 film with an intensifying screen at 280°C for 72 h. Volume of total RNA in each lane was confirmed with ethidium bromide staining (data not shown). The autoradiograph shown is representative of five independent experiments that gave essentially identical results. The availability of the cDNA will allow us to establish a mouse strain lacking this enzyme. This may reveal the role of the enzyme in the brain.

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