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Biochemistry

A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis

(cholesterol ester/fatty acid/high density lipoprotein/prostaglandins/leukotrienes)

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ABSTRACT **We report herein the cDNA cloning of a novel rat acyl-CoA synthetase (ACS) that preferentially uses arachidonate and eicosapentaenoate. This newly identified ACS (designated ACS4) contains 670 amino acids and is 68% identical to rat ACS3, a previously characterized ACS that is highly expressed in brain. ACS4 was overproduced in *Escherichia coli* and the resulting enzyme was purified to homogeneity. The purified enzyme utilizes arachidonate and eicosapentaenoate most preferentially among C_8–C_{22} saturated fatty acids and C_{14}–C_{22} unsaturated fatty acids. Kinetic analyses revealed that the enzyme has a high affinity for arachidonate and eicosapentaenoate and low affinity for palmitate. ACS4 transcripts are detectable in a wide range of tissues, with the highest level in adrenal gland. Immunoreactivity to ACS4 was detected in the zona fasciculata and reticularis of adrenal gland, in the corpus luteum and stromal luteinized cells in ovary, and in the Leydig cells of testis.**

In mammals, fatty acid utilization is initiated by activation of fatty acid, catalyzed by acyl-CoA synthetase (ACS, EC 6.2.1.3). Acyl-CoA, produced by ACS from fatty acid, ATP, and CoA, is a key intermediate in various metabolic pathways including membrane biogenesis, energy production, and fat deposition. This enzyme reaction is essential in fatty acid metabolism, since mammalian fatty acid synthase contains a specific thioesterase to produce free fatty acid as the final reaction product (1, 2). Inhibition of ACS by specific inhibitors, triacsins, profoundly reduces the synthesis of cellular phospholipids, thereby blocking the proliferation of mammalian cells (3).

There are several types of ACS in mammals and these are expressed to various degrees in different tissues. ACS1 is the well-characterized ACS abundant in liver, adipose tissue, and heart and exhibits a broad fatty acid specificity (4–6). In rat brain, the expression of ACS1 mRNA is very low despite the extremely high lipid content of brain (4). A brain-specific subtype of ACS, designated ACS2 (previously referred as to BACS), was revealed by molecular characterization of a brain cDNA (7). ACS1 and ACS2 are structurally closely related and exhibit similar acyl-chain specificity. In addition to ACS1 and ACS2, we have recently found another type of ACS, designated ACS3 (8). Although ACS3 has a structural architecture common to ACS1 and ACS2, the amino acids in ACS3 are poorly conserved in ACS1 and ACS2 (approximately 30% amino acid identities). ACS3 is highly expressed in brain and uses laurate and myristate most efficiently among C_8–C_{22} saturated fatty acids and arachidonate and eicosapentaenoate among C_{16}–C_{22} unsaturated fatty acids.

In arachidonate metabolism, ACS plays a key role in esterification of free arachidonate into membrane phospholipids. Most of the arachidonate present in cells is esterified predominantly in phospholipids (9). After its release by the action of calcium-dependent phospholipases (10), arachidonate can be converted to prostaglandins and thromboxanes (11), hydroxy eicosatetraenoic acids (12) and leukotrienes (13), or epoxy eicosatrienoic acids (14) via the cyclooxygenase, lipoxygenase, or cytochrome P450 pathways, respectively, depending on the cell type. The products of these reactions are generally released from the cell and exert their effect in an autocrine or paracrine fashion. Free arachidonate released from the plasma membrane is believed to be rapidly converted to arachidonoyl-CoA and reesterified into phospholipids to prevent constant synthesis of potent eicosanoids (15–17). Although the presence of an ACS specific for arachidonate has been shown by enzymatic characterizations (16), the exact nature of this enzyme remains unclear.

To further extend our studies on ACS in the metabolism of arachidonate, we have isolated a cDNA that encodes a new type ACS distinct from ACS1, ACS2, and ACS3. Purification of this newly identified ACS revealed that the enzyme prefers arachidonate and eicosapentaenoate. We describe herein the primary structure, enzymatic properties, and tissue expression of this newly identified ACS, designated ACS4.

EXPERIMENTAL PROCEDURES

Standard Procedures. Standard molecular biology techniques were used (18). DNA sequencing was performed by the dideoxynucleotide chain-termination method (19) manually or on an Applied Biosystems model 373A DNA sequencer. For Northern blot analysis, total RNA prepared by the guanidinium thiocyanate/CsCl method (20) was fractionated in a 1.5% agarose gel, transferred to a Zeta-Probe nylon membrane (Bio-Rad), and hybridized with ^{32}P-labeled probes.

cDNA Cloning of Rat ACS4. A rat liver cDNA library constructed in a λZapII vector (Stratagene) was screened with a 2.2-kb *Sac*I–*Xba*I fragment from pACS3 as a probe. By screening of 5.0×10^5 clones, we obtained four positive clones, and one representative clone containing the largest cDNA insert (pACS4) was further characterized.

Construction of an ACS4 Expression Plasmid. To overproduce ACS4 in *Escherichia coli*, we used a bacterial expression vector pTV118N in which a *lac* promoter directs the expression of a large amount of a foreign protein. To place the second

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Abbreviations: ACS, acyl-CoA synthetase; HDL, high density lipoprotein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. D85189).

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A

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MAKRIKAKPT SDKPGSPYRS VTHFDSLAVI  30
DIPGADTLDK LFDHAVAKFG KKDSLGTREI  60
LSEENEMQPN GKVFKKLILG NYKWINYLEV  90
NCRVNNFGSG LTALGLKPKN TIAIFCETRA 120
EWMIAAQTCF KYNFPLVTLY ATLGKEAVVH 150
GLNESEASYL ITSVELLESK LKAALLDINC 180
VKHIIYVDNK TINRAEYPEG LEIHSMQSVE 210
ELGSKPENSS IPPSRPTPSD MAIVMYTSGS 240
TGRPKGVMMH HSNLIAGMTG QCERIPGLGP 270
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AAVPEIMDRI YKNVMSKVQE MNYIQKTLFK 360
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IGQGYGLTES CGAGTVTEVT DYTTGRVGAP 450
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YVSLGKVEAA LKNCPLIDNI CAFAKSDQSY 570
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B

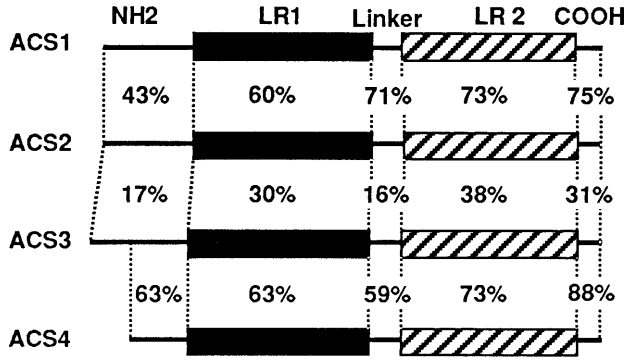

FIG. 1. Structure of rat ACS4 cDNA. (*A*) Deduced amino acid sequence of rat ACS4 cDNA. Amino acids are numbered on the right. (*B*) Schematic comparison of the amino acids in rat ACS4 with the other three known rat ACSs. The percentage amino acid identities are given between each region of adjacent proteins: rat ACS1 (6) vs. rat ACS2 (7), rat ACS2 vs. rat ACS3 (8), and rat ACS3 vs. rat ACS4. Luciferase-like regions 1 (LR1) and 2 (LR2) are indicated by solid and hatched boxes, respectively.

codon of the ACS4 cDNA adjacent to the initiator ATG of pTV118N, PCR (21) was carried out with sense primer 1 (5′-ACGCCATGGCAAAGAGAATAAA-3′) and antisense primer 2 (5′-TCATTTTCTTCGCTCAGGATCT-3′). The PCR products were digested with *Nco*I and the resulting 172-bp fragment was inserted into pTV118N. This intermediate plasmid was digested with *Sma*I and *Xba*I and ligated with a 1.9-kb *Sma*I–*Xba*I fragment of pACS4 to create pTV-ACS4.

Assay of ACS Activity. ACS activity was determined by the isotopic method or the spectrophotometric method (5); the spectrophotometric method was used only for the purified enzyme. The protein content of the cell extract was measured by the Lowry method (22) with bovine serum albumin as the standard.

Table 1. Summary of the purification of recombinant ACS4

Step	Total protein, mg	Total activity, μmol/min	Specific activity, μmol per min per mg	Yield, %
Crude extract	1360	883	0.65	100
Triton X-100 extract	1040	698	0.67	79
Red-Toyopearl	144	630	4.37	71
Blue-Toyopearl	8.4	178	21.1	20

A 500-ml culture of *E. coli* cells carrying pTV-ACS4 was used to prepare the crude extract. Enzyme activity was measured by the isotopic method with palmitate as a substrate.

Purification of Recombinant ACS4. A typical purification is described. All steps were carried out at 4°C and the enzyme was monitored by ACS activity.

The cell pellet from a 500-ml culture was suspended in 90 ml of buffer A [50 mM potassium phosphate, pH 7.4/1 mM EDTA/1 mM dithiothreitol/10% (wt/vol) glycerol] containing lysozyme (0.5 μg/ml) and 1 mM phenylmethylsulfonyl fluoride. This mixture was allowed to stand for 1 h, followed by sonication (five 1-min pulses at 30 W in an ice bath) and centrifugation at 8000 × *g* for 20 min to remove cell debris. The supernatant represents the crude extract and was mixed with 0.1 vol of 10% (wt/vol) Triton X-100. This mixture was gently stirred for 1 h and was then centrifuged at 105,000 × *g* for 30 min. The resulting supernatant was applied to a Red-Toyopearl (Tosoh, Tokyo) column (2.6 × 18 cm) equilibrated with buffer B (buffer A containing 1% Triton X-100). The column was washed with buffer B containing 10 mM ATP and material was eluted with buffer B containing 10 mM ATP and 1 M KCl.

The enzyme solution from the Red-Toyopearl chromatography step was diluted with 10 vol of buffer B and applied to a Blue-Toyopearl (Tosoh, Tokyo) column (1.5 × 6.5 cm) equilibrated with buffer B containing 0.1 M KCl. The column was washed with the equilibration buffer and material was eluted with a salt gradient from 0 to 2 M KCl in the equilibration buffer. The enzyme emerged in a single peak and the fractions exhibiting high enzyme activities were combined.

Immunohistochemistry and Immunoblot Analysis. A polyclonal antibody against rat ACS4 was produced by immunizing rabbits with purified ACS4 by using standard procedures (23). The specificity of the antibody was evaluated by immunoblot analysis of lysates of COS cells transfected with ACS1, ACS2, ACS3, and ACS4 expression plasmids. The antibody specifically detected a 74-kDa protein only in COS cells transfected

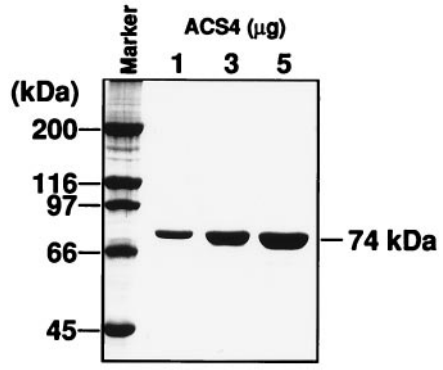

FIG. 2. SDS/polyacrylamide gel electrophoresis of the purified recombinant ACS4. The purified enzyme at 1, 3, and 5 μg was subjected to electrophoresis on an SDS/8% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Molecular size markers are indicated on the left.

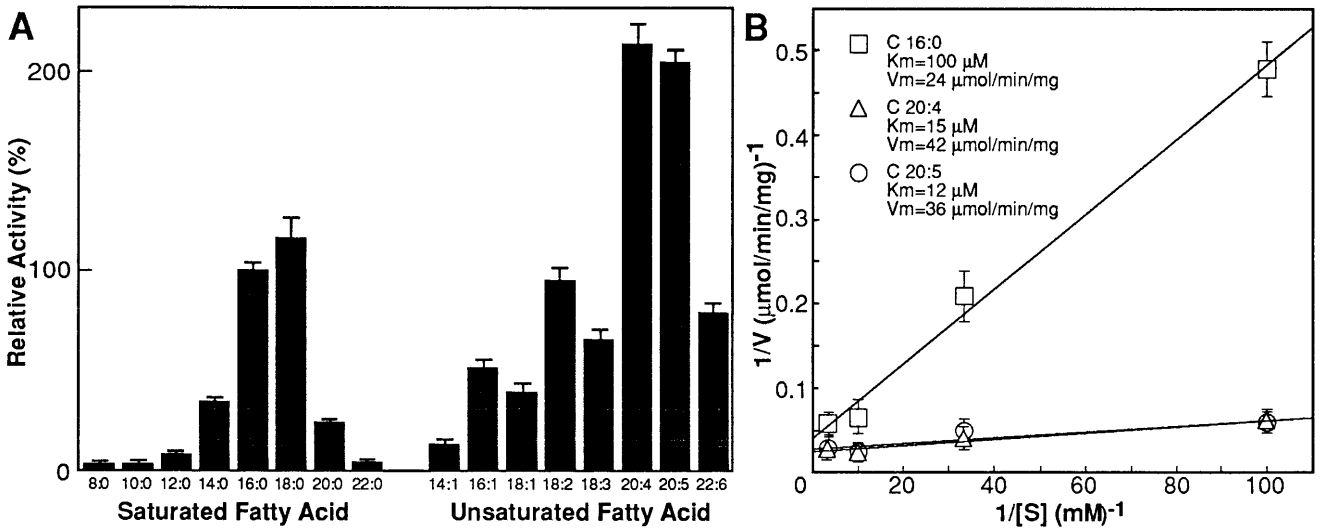

FIG. 3. Fatty acid specificity of the purified recombinant ACS4. (*A*) Enzyme activity was determined by the spectrophotometric method with 1 μg of the purified enzyme and the standard reaction mixture, except that various saturated and unsaturated fatty acids (final concentration = 0.1 mM) were used. Enzyme activity is expressed as a percentage of that obtained with palmitate as a substrate (21.1 μmol per min per mg). The data are the mean ± SD of triplicate experiments. Saturated fatty acids: 8:0, octanoic acid; 10:0, decanoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 20:0, arachidic acid; 22:0, docosanoic acid. Unsaturated fatty acids: 14:1, myristoleic acid; 16:1, palmitoleic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid. (*B*) Lineweaver–Burk plots of ACS activity for arachidonate, eicosapentaenoate, and palmitate. Assays were performed with the indicated fixed concentrations of arachidonate, eicosapentaenoate, and palmitate, as described above. Data points represent the mean of three determinations obtained in one experiment.

with ACS4 expression plasmid. Immunohistochemical analysis was performed on routinely processed, formalin-fixed, paraffin-embedded sections with the biotin-streptavidin Histofine immunostaining system (Nichirei, Tokyo), as described (24).

RESULTS

A cDNA encoding a new type of ACS, designated ACS4, was isolated from rat liver cDNA library by using a previously characterized ACS3 cDNA (8). This newly isolated cDNA (pACS4) is 4.9 kb long and includes a 2010-bp open reading frame encoding a protein of 670 amino acids with a calculated molecular weight of 74,325 (Fig. 1*A*).

Fig. 1*B* compares the amino acid sequence of ACS4 deduced from the cDNA with that of ACS3. The two proteins share 68% of their amino acids, and the identities extend throughout the proteins excluding the NH_2-terminal 50 amino acids present in ACS3.

To overproduce ACS4, a bacterial expression plasmid containing a *lac* promoter and the entire coding region of the ACS4 cDNA was created. The expression of the plasmid was induced by isopropyl β-D-thiogalactopyranoside and the resulting enzyme was purified to homogeneity. The purification procedure involved treatment of the crude extract with Triton X-100 and chromatography on Red- and Blue-Toyopearl (Table 1). The overall purification was 33-fold with a yield 20%. The specific activity of the purified ACS4 was 21.1 μmol per min per mg when assayed with palmitate as a substrate. The purified enzyme was essentially homogenous, as shown by SDS/polyacrylamide gel electrophoresis, with an apparent molecular mass of 74 kDa (Fig. 2); a value that agrees well with the calculated molecular mass from the deduced amino acid sequence of the cDNA.

Fig. 3*A* shows the fatty acid specificity of the purified ACS4. It utilizes arachidonate and eicosapentaenoate most preferentially among C_8–C_{22} saturated fatty acids and C_{14}–C_{22} unsaturated fatty acids. To further characterize the fatty acid specificity of the purified ACS4, kinetic analyses were carried out by using arachidonate, eicosapentaenoate and palmitate as substrates. The double reciprocal plots in Fig. 3*B* reveal that the purified enzyme has a high affinity for arachidonate (K_m = 15 μM) and eicosapentaenoate (K_m = 12 μM) and a low affinity for palmitate (K_m = 100 μM).

Northern blot analysis of RNA from various rat tissues revealed hybridization to a major transcript 4.9 kb long, corresponding to pACS4 (Fig. 4). The ACS4 transcripts are expressed in a wide range of tissues, with the highest level in adrenal gland; relatively high levels in epididymis, brain, seminal vesicle, lung, ovary, and liver; and lower levels in adipose tissue, kidney, and testis. A trace amount of the

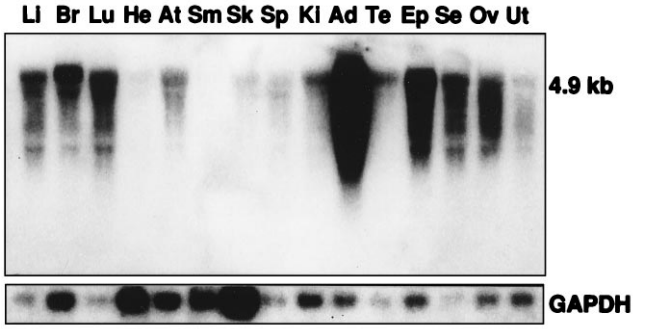

FIG. 4. Northern blot analysis of ACS4 mRNA in various rat tissues. Total RNA prepared from the indicated rat tissue was subjected to electrophoresis on a 1.5% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a ^{32}P-labeled 2.2-kb *Sac*I–*Xba*I fragment of pACS4 under stringent conditions. The filter was washed in 0.1× standard saline citrate (SSC) containing 0.1% SDS at 65°C for 30 min and exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 48 h. The lanes were loaded with 15 μg of total RNA isolated from liver (li), brain (br), lung (lu), heart (He), adipose tissue (At), small intestine (Si), skeletal muscle (Sk), spleen (Sp), kidney (Ki), adrenal gland (Ad), testis (Te), epididymis (Ep), seminal vesicle (Se), ovary (Ov), and uterus (Ut). The same samples in *A* were subsequently hybridized with a control probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CLONTECH) and exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 20 h. The autoradiograph shown is a representative of five experiments that gave essentially identical results.

mRNA was detected in skeletal muscle, spleen, uterus, small intestine, and heart.

To localize cells expressing the enzyme, immunohistochemical analysis was carried out with tissue sections prepared from rat adrenal gland, ovary, liver, lung, spleen, and testis. Consistent with the abundant expression of the mRNA, immunoreactivity for the enzyme was observed in adrenal gland and ovary. We also detected immunoreactivity in testis but other tissues were negative. Fig. 5 shows the immunoreactivity to ACS4 in adrenal gland and ovary. In adrenal gland, immunoreactivity was detected most intensely in the zona fasciculata and reticularis (Fig. 5 *A* and *B*). Immunoreactivity in the zona glomerulosa was relatively weak and no significant immunoreactivity was observed in the capsule or adrenal medulla. In the ovary, significant immunostaining was detected in the large cells but was not observed in the small cells of the corpus luteum (Fig. 5*C*). It is also present in stromal luteinized cells of the ovary (Fig. 5*D*), but no significant immunoreactivity was observed in nonluteinized stromal cells, surface epithelium, or the great majority of ovarian follicles. In testis, immunoreactivity was observed exclusively in the Leydig cells but not in cells of the seminiferous tubules (Fig. 5*E*). In control experiments, tissue sections were stained with preimmune IgG or normal rabbit IgG. In both cases, no significant staining was detectable in any tissues (data not shown).

DISCUSSION

In the current studies, we have isolated a cDNA encoding a novel ACS that preferentially uses arachidonate and eicosapentaenoate *in vitro*. ACS4, like other mammalian ACSs, consists of five regions: an NH_2 terminus, luciferase-like regions 1 and 2, a linker connecting the two luciferase-like regions, and a COOH terminus (see Fig. 1*B*). Between ACS3 and ACS4, the amino acids in luciferase-like region 2 and in the COOH terminus show the highest similarities. These amino acids are also highly identical in ACS1 and ACS2 (7), suggesting that the two regions are crucial for the catalytic reaction of ACS. ACS4 lacks 50 amino acids that correspond to the NH_2 terminus of ACS3: these amino acids may contribute to the difference in fatty acid preference of the two ACSs (see below).

Despite the structural similarity of ACS3 and ACS4, the two enzymes exhibit different fatty acid specificity. ACS4 uses arachidonate and eicosapentaenoate most preferentially, whereas ACS3 uses a rather wide range of fatty acids including laurate, myristate, arachidonate, and eicosapentaenoate. Although the apparent K_m value of the purified ACS4 for arachidonate is approximately equal to those of purified ACS1 and ACS3 (10–15 μM), it has a lower affinity (K_m = 100 μM) for palmitate than ACS1 and ACS3: the apparent K_m values of the purified ACS1 and ACS3 toward palmitate and arachidonate were in the same range as those toward arachidonate (10–15 μM) (5, 8). This lower affinity of ACS4 toward palmitate may relate to the short NH_2 terminus of the enzyme: ACS4 has the shortest NH_2 terminus of the four ACSs. Although many factors (including the physical chemical properties of fatty acids, fatty acid binding proteins, acyl-CoA binding proteins, and intracellular proteins) may be involved in the fatty acid specificity of ACS4 *in vivo*, the high

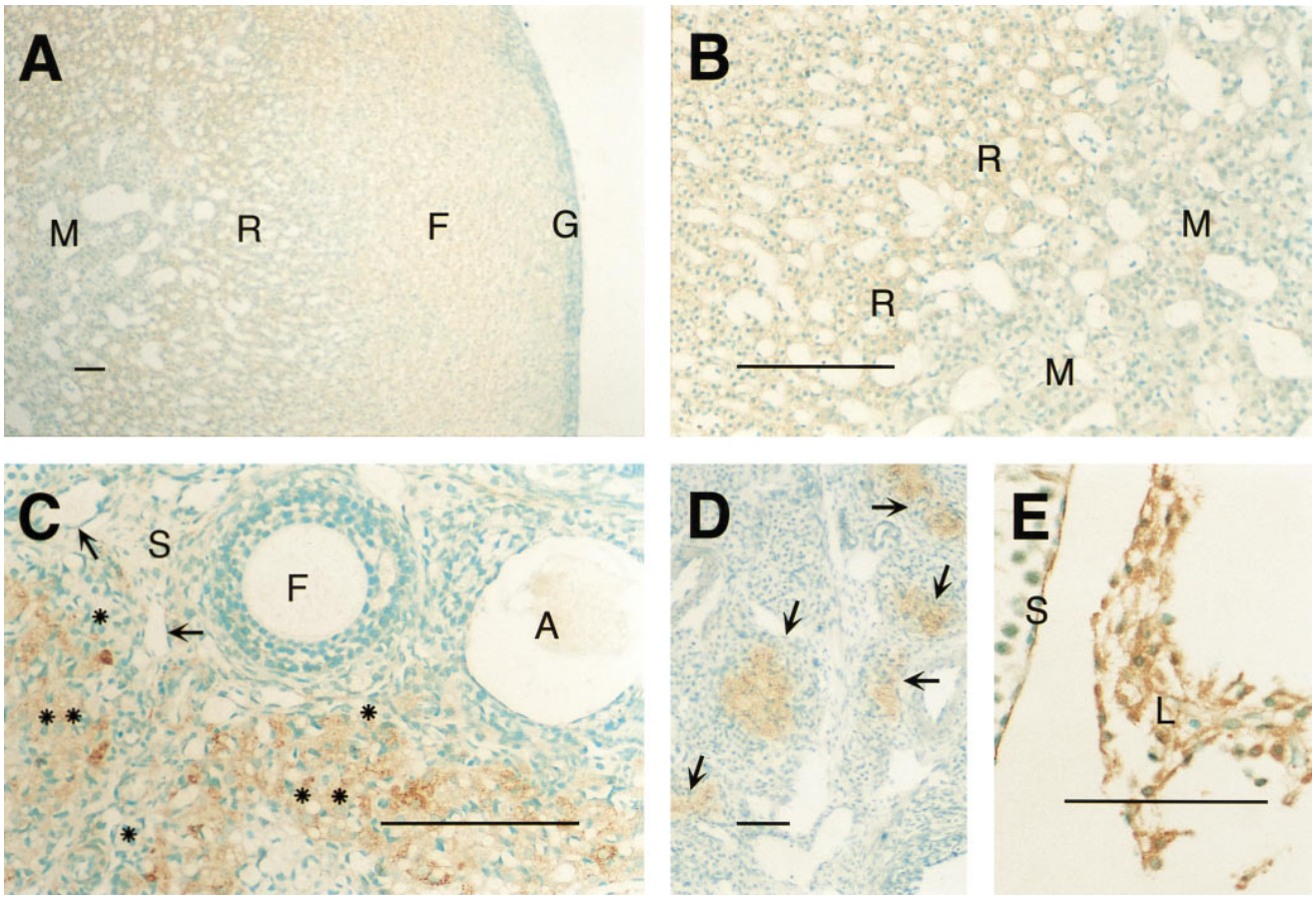

FIG. 5. Immunohistochemical analysis of ACS4. Tissue sections from rat adrenal gland (*A* and *B*), ovary (*C* and *D*), and testis (*E*) were analyzed with rabbit anti-ACS4 IgG. In *A* and *B*, the intensity of immunostaining in adrenal gland is highest in the zona fasciculata (F) and reticularis (R), relatively weak in the zona glomerulosa (G), and not detected in the capsule or medulla (M). In *C*, note significant immunoreactivity in the large cells (indicated by **), but not in the small cells (indicated by ∗) of the corpus luteum. Immunoreactivity was not undetected in theca or granulosa cells of the growing follicle (F), theca cells of atretic follicle (A), or vascular endothelial cells (indicated by arrows). In *D*, immunoreactivity is present in stromal luteinized cells (arrows). In *E*, Immunoreactivity is present in Leydig cells (L) but not in the cells of the seminiferous tubules (S). (Bars = *A–D*, 200 μm; *E,* 100 μm.)

affinity of ACS4 for arachidonate and eicosapentaenoate and low affinity for palmitate suggest that the enzyme plays a unique role in the metabolism of these biologically important fatty acids.

The mRNA for ACS4 is expressed in various tissues including adrenal gland, epididymis, brain, seminal vesicle, lung, ovary, and liver. In contrast, ACS3 mRNA is highly expressed in the brain; expressed to a much lower extent in lung, adrenal gland, kidney, small intestine, and adipose tissue; and is not detected in heart or liver (8). There was an apparent discrepancy between the distribution of the enzyme mRNA and that of cells stained by the antibody. This discrepancy may be due to differences in a population of cells expressing high levels of ACS4 protein. As shown in Fig. 5, cells stained by the antibody in ovary and testis are relatively sparse. Alternatively, it may be due to differences in sample preparation and sensitivity of the assay system or to property of the antibody: whereas no significant staining was detected in rat liver as revealed by immunohistochemistry, the antibody detected a 74-kDa immunoreactive protein in the liver by immunoblot analysis (data not shown).

The striking feature of ACS4 is its abundance in steroidogenic tissues, especially adrenal gland and ovary. ACS4 immunoreactivity was detected in the zona fasciculata and reticularis of the adrenal cortex, in the corpus luteum and stromal luteinized cells of the ovary, and in the Leydig cells of the testis. In these cells, cholesterol is actively metabolized to produce steroid hormones. The major source of this cholesterol is derived from cholesterol esters in plasma high density lipoprotein (HDL) in rats (25). HDL-cholesterol esters are selectively taken up *in vivo* in several species (26–28) and in perfused organs (29, 30) and *in vitro* in a number of cell types (31–34) via a direct nonendocytic pathway. In rat adrenocortical cells, HDL enhances steroid production and increases cellular cholesterol ester content (34). The cholesterol esters derived from HDL are hydrolyzed extralysosomally and converted to steroid hormones (35). Rat HDL contains a high content of arachidonate in its cholesterol ester fatty acids (36, 37): approximately half of the cholesterol ester fatty acids is arachidonate. Fleisher *et al.* (37) have shown that human and rat HDL stimulate prostacyclin synthesis in arterial endothelial cells, suggesting that HDL may be providing arachidonate to the endothelial cells for synthesis of prostacyclin.

Despite the high content of arachidonate in cholesterol esters and the presence of the prostaglandin production system, no detectable prostaglandins are produced in bovine fasciculoreticulata adrenocortical cells with or without the presence of corticotropin (38). This suggests the presence of an active reesterification mechanism for the arachidonate released from cholesterol esters. Based on the predominance in steroidogenic cells and high preference for arachidonate, we suggest that ACS4 actively converts free arachidonate derived from HDL-cholesterol esters to its CoA thioester for the reesterification of arachidonate, to prevent the synthesis of potent prostaglandins and leukotrienes in steroidogenic cells.

In this report, we have shown the structure, fatty acid specificity, and tissue expression of a unique ACS. Although we suggest the enzyme participates in the reesterification of arachidonate into phospholipids, the exact biological function of this enzyme remains to be clarified. The availability of the cDNA will allow us to produce a mouse strain lacking this enzyme that may reveal its biological role in the metabolism of arachidonate and eicosapentaenoate.

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