The Second Member of the Human and Murine “Bubblegum” Family Is a Testis- and Brainstem-specific Acyl-CoA Synthetase*

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Acyl-CoA synthetases that activate fatty acids to their CoA derivatives play a central role in fatty acid metabolism. ACSBG1, an acyl-CoA synthetase originally identified in the fruit fly mutant bubblegum, was hypothesized to contribute to the biochemical pathology of X-linked adrenoleukodystrophy. We looked for homologous proteins and identified ACSBG2 in humans, mice, and rats. Human ACSBG1 and ACSBG2 amino acid sequences are 50% identical. ACSBG2 expression was confined to the testis and brainstem. Immunohistochemistry and in situ hybridization studies further localized ACSBG2 expression to testicular Sertoli cells and large motoneurons in the medulla oblongata and cervical spinal cord. Full-length cDNA encoding human and mouse ACSBG2 was cloned. In transfected COS-1 cells, both human and murine ACSBG2 were detected as 75- to 80-kDa proteins by Western blot. A shift of the human ACSBG2 pH optimum to a more acidic pH and mutation of this histidine to arginine improved catalytic function at neutral pH by shifting the pH profile without affecting substrate specificity. Although the role of ACSBG2 in testicular and neuronal lipid metabolism remains unclear, the limited tissue expression pattern and limited substrate specificity rule out a likely role for this enzyme in X-linked adrenoleukodystrophy pathology.

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2 The abbreviations used are: ACS, acyl-CoA synthetase; h, human; m, mouse; r, rat; X-ALD, X-linked adrenoleukodystrophy; PBS, phosphate-buffered saline; EST, expressed sequence tag; Triacsin C, 1-hydroxy-3-(E,E,E,Z)2, A7- undecatrienylidine)-triazene.

3 P. A. Watkins, submitted for publication.

4 A uniform nomenclature for long-chain ACSs was approved by the Nomenclature Commission of the Human Genome Organization (35), and adaptation of this system for several other ACS families, including the “bubblegum” family, was recently approved. Approved gene names ACSBG1 and ACSBG2 are used to designate genes formerly called BG1 and BGR, respectively.

5 GenBankTM accession numbers: hACSBG2, NM_030924; mACSBG2, DQ250679; and rACSBG2, XM_236792.
tissue and cellular expression pattern. Furthermore, the native human protein has a naturally occurring mutation at a highly conserved position that affects its enzymatic function. We describe here some of the properties of human and mouse ACSBG2.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**—[1-14C]Lauric acid (C12:0), [1-14C]palmitic acid (C16:0), [1-14C]arachidonic acid (C20:4), [1-14C]docosahexaenoic acid (C22:6), and [1-14C]linolenic acid (C24:0) were obtained from Moravek, Inc. [1-14C]Oleic acid (C18:1) and [1-14C]linoleic acid (C18:2) were obtained from American Radiolabeled Chemicals. [1-14C]Stearic acid was obtained from Amersham Biosciences. Unlabeled fatty acids were from either Sigma or Cayman Chemical. Protein was measured by the method of Lowry et al. (10).

**Cloning of Full-length ACSBG2 cDNA**—Human testis total RNA was prepared by electrophoretic transfer as described previously (11). TM4 cells were transfected using Oligofectamine (Invitrogen). Statistical significance was calculated using Student’s t test.

**Animals and Their Care**—Wild-type 129SvEv mice were obtained from Taconic, Inc. (Germantown, NY). All mice used in these studies were approximately 3 months of age. Mice were housed in facilities of the Johns Hopkins University School of Medicine under controlled conditions, between 22 °C and 27 °C, on a 12-h light/dark cycle, with food and water ad libitum. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with all national and international laws and policies (ECC Council Directive 86/609, OL 358, 1 Dec 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

**Cloning of Full-length ACSBG2 cDNA**—Human testis total RNA (Ambion) was reverse-transcribed using the ThermoScript™ reverse transcription-PCR First Strand cDNA Synthesis kit (Invitrogen) to obtain testis cDNA. Full-length ACSBG2 cDNA was obtained by PCR amplification using the forward oligonucleotide primer 5’-CGCGGTTCTCCTGCACAACCTGGAAATGC-3’ (P15-1), which incorporates an EcoRI restriction site, and reverse primer 5’-ATACGCGCCGCCGCTT-TCATGTTACTATG-3’ (P15-6), which incorporates a NotI site, and testis cDNA as template. The 2016 bp PCR product was cloned into the EcoRI and NotI sites of the mammalian expression vector pcDNA3 as template. The resulting construct, which included the entire open reading frame and 13 bp of 5’-untranslated DNA but no additional 3’ sequence following the stop codon, was fully sequenced. The insert sequence was identical to that of GenBank™ accession number NM_030924, which incorporates an XhoI site. The sequenced DNA was exchanged back to PBS using a Centricon 30 (Millipore), 1% bovine serum albumin was added, and purified antibody was stored at 80 °C.

**Site-directed Mutagenesis**—The overlapping extension method was used to mutate histidine 511 of hACSBG2 to arginine (12). PCR was used to amplify two fragments of hACSBG2 that overlap and that each encodes the desired mutation (underlined in the oligonucleotide primers); both reactions used full-length hACSBG2 in pcDNA3 as template. The first reaction amplified a 1547-bp fragment encoding amino acids 1–511 using forward primer P15-1 and reverse primer 5’-TGGCGG-CGTTGACATAAGAGGAA-3’. The second reaction amplified a 482-bp fragment that encodes amino acids 508–666 using forward primer 5’-GCACCGGCGCATCAAAGAGA-3’ and reverse primer P15-6. The underlined CGC (R) codon replaced the wild-type CAC (H) codon. The two PCR products were gel-purified and used as template for addi-
for detection of array containing 76 tissues was obtained from Clontech. A cDNA probe were as previously described (13). A human multiple tissue expression
respectively. Conditions for probe labeling, hybridization, and detection
CAGTGTAGCCCAGGATGC-3
(Invitrogen). Twenty
pared from freshly harvested mouse tissues using the TRIzol reagent
membrane (Amersham Biosciences). A cDNA probe for detection of
Northern Blot and mRNA Dot Blot Analyses—Total RNA was prepared from freshly harvested mouse tissues using the TRizol reagent (Invitrogen). Twenty μg of RNA was electrophoresed on a 1% agarose gel at 4 V/cm for 2.5 h and then transferred overnight to a Hybond-N+
membrane (Amersham Biosciences). A cDNA probe for detection of
mAcshbg2 mRNA was prepared by PCR amplification of a 450-bp fragment using 5'-AGAGTCTCCAAGTGCACTC-3' and 5'-GGTGTA-
GATGCCAGTGCAAT-3' as forward and reverse primers, respectively, and mouse testis cDNA as template. For control, a 528-bp glyceraldehyde-3-phosphate dehydrogenase probe was similarly pre-
pared using 5'-ACCACCATGGAGAAGGCTGG-3' and 5'-CT-
CAGTGTAGCCCAGGATGC-3' as forward and reverse primers, respectively. Conditions for probe labeling, hybridization, and detection
were as previously described (13). A human multiple tissue expression array containing 76 tissues was obtained from Clontech. A cDNA probe for detection of hAcshbg2 mRNA was prepared by PCR amplification of a 1138-bp fragment using 5'-CACGATGGAGGAATTCTAGG-3' and 5'-CACTCTGACCAAGACTGATA-3' as forward and reverse primers, respectively, and hAcshbg2 cDNA as template.
Subcellular Fractionation and Western Blotting—Mouse tissues were homogenized in 5 volumes of buffer (0.25 M sucrose, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) containing protease inhibitor mixture (Complete, Roche Applied Science) using a Pellet Pestle homogenizer (Kimble/Kontes). Subcellular fractions were prepared essentially following the method of de Duve et al. (14) as previously described (5). For Western blots, cells were harvested by gentle trypsinization, washed with PBS containing protease inhibitor mixture, and solubilized in Laemmli sam-
ples were transferred to nitrocellulose membranes; after incubation with primary antibody and horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were detected using the SuperSignal West Pico chemiluminescent reagent (Pierce).
Acyl-CoA Synthetase Assays—Radiochemical assays of ACS activity in frozen/thawed COS-1 cell suspensions using [1-14C]fatty acid sub-
strates were performed essentially as described (16, 17). COS-1 cells, 3 days post-transfection with either ACsbg2 constructs or the empty pcDNA3 vector, were harvested by gentle trypsinization, washed with phosphate-buffered saline, and resuspended in 0.25 M sucrose containing 10 mM Tris (Cl−), pH 8.0, 1 mM EDTA, and protease inhibitor mixture (Complete, Roche Applied Science). Cell suspensions were subjected to at least one freeze/thaw cycle (−80 °C) prior to assay. In some cases, as noted in the text and figure legends, either KCl was added to the reaction mixture, or potassium phosphate buffer, pH 7.5, replaced Tris (Cl−), pH 7.5. When the inhibitor Triacsin C was used, the assay was modified to permit preincubation of enzyme with the inhibitor prior to adding the radiolabeled substrate. Triacsin C (final concentration, 10 μM) in ethanol was added to reaction mixes containing all com-
ponents except 14C-fatty acid. An equal volume of ethanol (final concentra-
tion, 1%) was added to control incubations. After 15 min at 37 °C, labeled substrate solubilized in α-cyclodextrin (16) was added and the reaction allowed to proceed for 20 min as usual. Fluorometric ACS assays were performed as previously described (5).
Indirect Immunofluorescence and Immunohistochemistry—For indirect immunofluorescence analysis, cells were fixed in 4% formaldehyde in phosphate-buffered saline and permeabilized with 1.0% Triton X-100 prior to incubation with primary and secondary antibodies as described previously (18). For immunohistochemistry, testis, brainstem, and spinal cord from 3-month-old mice were harvested, quickly frozen in liq-
uid nitrogen, and stored at −80 °C. Tissue sections (5–8 μm thick) were

![Alignment of hACSBG1 and hACSBG2 amino acid sequences. The predicted amino acid sequence of human ACsbg2 (666 amino acids) was aligned with that of hACSBG1 using the ClustalW program. The AMP-binding domain (solid line) and conserved motif 2 (dashed line) are indicated. An arginine residue (asterisk) within motif 2 that is invariant among all ACSs from all species and thought to be critical for catalytic activity was found to be a histidine residue in hACSBG2.](image)
cut using a cryostat and fixed with 4% paraformaldehyde; blocking, incubation with primary and secondary antibody, peroxidase-based detection, counterstaining, and mounting were performed as described previously (5).

**In Situ Hybridization**—The method of Giger et al. (19) was used with modification as previously described (20). Sense and antisense probes corresponding to full-length hACSBG2 cDNA were prepared and labeled with digoxigenin as previously described (20).

**RESULTS**

**Identification and Predicted Characteristics of ACSBG2**—The ACSBG1 gene encodes a protein found in several species, including fruit flies (4), mice (7), rats (21), and humans (2). Transfected cells expressing human, mouse, or rat ACSBG1 cDNA exhibited increased ACS activity (2, 7, 21). To determine whether mammalian genomes contained related proteins, we queried NCBI nucleotide and protein databases using the BLAST algorithm with the human (h) ACSBG1 sequence as probe. We identified one predicted human protein consisting of 666 amino acid residues that shared 50% identity and 69% similarity with hACSBG1 (Fig. 1). Two highly conserved amino acid motifs (solid and broken underlines in Fig. 1) characteristic of ACSs were present in this open reading frame, hereafter referred to as hACSBG2. Within motif 2 (broken underline), the sequence that facilitates segregation of ACSs into families of related proteins, hACSBG1 and the predicted protein share 68% identity and 89% similarity. The next most homologous human proteins identified in the BLAST search had <30% identity with hACSBG1 and were members of the long-chain ACS family. The gene encoding hACSBG2 had previously been referred to as “bubblegum-related” with the interim gene symbol BGR. The BLAST search also detected putative mouse (m) and rat (r) homologs of hACSBG2, each of which contains 667 amino acids (Fig. 2).

Human ACSBG2 has a theoretical molecular weight of 74,413 Da and a pI of 8.59 (us.expasy.org/tools/pi_tool.html). No signal peptide sequence, mitochondrial targeting signal, or peroxisomal targeting signals were evident. Potential N-glycosylation sites were detected at residues 19 and 246 by the NetNGlyc 1.0 program (available at www.cbs.dtu.dk/services/NetNGlyc/). No potential O-glycosylation sites were found. Hydropathy analysis of the hACSBG2 sequence using the algorithm of Kyte and Doolittle (window size = 19) suggested one potential transmembrane region near residue 125.

Inspection of motif 2 (Fig. 1) of hACSBG2 revealed a surprising feature. All known proteins with either documented or predicted ACS activity, from bacteria to human, contain within this region a highly
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FIGURE 3. Immunoblot analysis of ACSBG2 expressed in COS-1 cells. COS-1 cells were transfected by electroporation with full-length ACSBG2 cDNA or vector alone. Three days post-transfection, cells were harvested and examined for ACSBG2 expression by Western blot analysis as described under “Experimental Procedures.” 50 µg of cell protein was loaded onto each gel lane. A, pcDNA3 vector alone; B, native hACSBG2; C, the H511R HACSBG2 mutant; D, mACSBG2. The positions of molecular size markers are shown on the left. ACSBG2 is indicated by the arrow on the right.

conserved arginine residue (indicated by the asterisk in Fig. 1). This arginine is preceded by glycine (7 residues upstream) and a hydrophobic amino acid (3 residues upstream) and is followed by a hydrophobic amino acid (5 residues downstream) and glycine (9–10 residues downstream)3. This “invariant” arginine residue was found to be a histidine residue in hACSBG2 (H511). However, the homologous regions of mACSBG2 and rACSBG2 contained arginine (R511, indicated by the asterisk in Fig. 2).

Cloning and Expression of Full-length ACSBG2 cDNA—To investigate the properties of ACSBG2, we cloned full-length cDNA encoding hACSBG2 and mACSBG2 by PCR amplification using human or mouse testis cDNA templates, as described under “Experimental Procedures.” For investigation of molecular size and subcellular location of ACSBG2, a polyclonal antibody was raised against the C-terminal 315 amino acids of hACSBG2 and purified as described under “Experimental Procedures.” When expressed in COS-1 cells, both hACSBG2 and mACSBG2 migrated as proteins of 75–80 kDa on SDS-PAGE, in agreement with their theoretical molecular weight (Fig. 3, lanes B and D). In agreement with Northern blot analysis (below), no endogenous ACSBG2 was detected in the kidney-derived COS-1 cells when transfected with vector alone (Fig. 3, lane A).

Mouse and Human Tissue Expression Pattern of ACSBG2 mRNA and Protein—To determine in which mouse tissues Acsbg2 mRNA was expressed, we performed Northern blot analysis as described under “Experimental Procedures.” Using a 32P-labeled cDNA probe, a transcript of ~3.0 kb was detected only in testis (Fig. 4A). No signal was observed in brain, adrenal gland, lung, skeletal muscle, small intestine, liver kidney, spleen, or heart. A human RNA Multiple Tissue Expression Array was also probed for ACSBG2 mRNA expression as described under “Experimental Procedures.” Similar to the result of mouse Northern blot analysis, we detected expression only in human testis (Fig. 4B, position F8).

When the human expressed sequence tag (EST) data base was probed with the human ACSBG2 cDNA sequence, more than half of the EST clones with sequence identity were from testis libraries, in agreement with the Northern blot and RNA dot blot observations. However, ~10% of the EST clones were from medulla oblongata (brainstem) libraries; the remainder was primarily from mixed tissue libraries and tumor cells. Therefore, we examined ACSBG2 expression by Western blot in several mouse tissues (Fig. 4C). Robust ACSBG2 protein expression was seen in mouse testis. The protein was also clearly detected both in medulla and in spinal cord but not in liver, kidney, heart, lung, or whole brain (cerebral cortex plus cerebellum, without brainstem).

Identification of Mouse Cell Types Expressing Acsbg2—Both in situ hybridization and immunohistochemical analysis were used to identify the specific cell types that endogenously express ACSBG2 in testis. For in situ hybridization, a digoxigenin-labeled antisense RNA probe was used. In contrast to Acsbg1 mRNA, which is expressed primarily in the interstitial cells of Leydig (5), Acsbg2 mRNA appeared to be expressed mainly in the Sertoli cells (Fig. 5, A and B). No labeling was observed in testis sections incubated with the corresponding sense RNA probe (data not shown). A similar result was obtained when mouse testis sections were probed with affinity-purified ACSBG2-specific antibody (Fig. 5, C and D). We also used histochemistry to examine medulla oblongata and spinal cord ACSBG2 expression. In the medulla, ACSBG2 appeared to be highly expressed in large motor neurons of the medullary reticular formation and the hypoglossal nucleus (Fig. 5, F and E). Some motor neurons in both the anterior and posterior horns of the cervical spinal cord showed ACSBG2 labeling as well (Fig. 5, G and H), but few cells of the thoracic spinal cord showed evidence of ACSBG2 expression (data not shown).

Subcellular Location of ACSBG2—Western blot and indirect immunofluorescence approaches were used to determine the subcellular location of ACSBG2. In COS-1 cells expressing hACSBG2, the protein had a diffuse immunofluorescent staining pattern that appeared to be predominantly cytoplasmic (Fig. 6A). There was no evidence for association of ACSBG2 within any specific subcellular structure in these transfected cells. Because this observation was inconsistent with the single transmembrane domain predicted by amino acid sequence, we wanted to examine the subcellular location of endogenous ACSBG2. Therefore, we fractionated mouse testis by differential centrifugation and used Western blot analysis to detect ACSBG2 in the subcellular fractions. A prominent 75–80-kDa ACSBG2 band was seen mainly in the microsomal (P) fraction (Fig. 6C). Weaker cross-reacting bands of ~65 and...
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Acyl-CoA Synthetase Activity of ACSBG2—To assess the ability of ACSBG2 to catalyze activation of fatty acids, we expressed full-length hACSBG2 cDNA in COS-1 cells. Three days post-transfection, cells were harvested and assayed for ACS activity with various 14C-labeled fatty acid substrates. Surprisingly, human ACSBG2 exhibited ACS activity only when either oleic acid (C18:1ω9) or linoleic acid (C18:2ω6) were used as substrates (Fig. 7). The hACSBG2-specific rates of oleic acid and linoleic acid activation were, respectively, 28 and 31% higher than endogenous COS-1 cell rates. These increases, though modest, were statistically significant (p < 0.001 and p = 0.015, respectively). No evidence for significant activation of C12:0, C16:0, C18:0, C20:4ω6, C22:6ω3, or C24:0 by hACSBG2 was observed (Fig. 7 and data not shown).

Because the “invariant” arginine residue (Arg-565 of hACSBG1) within motif 2 of hACSBG2 is a histidine residue (His-511 of hACSBG2) (Fig. 1), and because this residue has been shown to be critical for catalytic activity in some ACSs (3, 23), we used site-directed mutagenesis to replace His-511 with arginine. Both wild-type hACSBG2 and hACSBG2 with the H511R mutation were expressed in COS-1 cells by Western blot analysis (Fig. 3, lanes B and C, respectively). However, ACS activity of the H511R mutant was lower, not higher, than that of the wild-type protein (Fig. 7). The hACSBG2/H511R-specific rate of oleic acid activation was only 3% higher than the endogenous COS-1 cell rate; this increase was not statistically significant. No evidence for linoleic acid activation by hACSBG2/H511R was noted. The lower activity of the H511R mutant was lower, not higher, than that of the wild-type protein (Fig. 7). The hACSBG2/H511R-specific rate of oleic acid activation was only 3% higher than the endogenous COS-1 cell rate; this increase was not statistically significant. No evidence for linoleic acid activation by hACSBG2/H511R was noted. The lower activity of hACSBG2/H511R versus native hACSBG2 was due to lower protein levels of the former, as both proteins were expressed equally well in COS-1 cells (Fig. 3, B and C).

As indicated earlier, mouse ACSBG2 retains an arginine residue (Arg-511) within motif 2 (Fig. 2). Therefore, we also expressed this protein in COS-1 cells and measured its ACS activity. The level of...
expressed mACSBG2 protein was similar to that of hACSBG2 and hACSBG2/H511R (Fig. 3D). The substrate specificity of mACSBG2 was similar to that of native hACSBG2 (Fig. 7). Activation rates for oleic acid and linoleic acid activation were, respectively, 30 and 35% greater than endogenous COS-1 cell rates, findings that were statistically significant (p < 0.0001 and p < 0.02, respectively). No evidence for mACSBG2 activation of C16:0, C18:0, or C24:0 was observed (Fig. 7 and data not shown).

Effects of Potassium Phosphate, KCl, and Tris on ACSBG2 Enzyme Activity—To investigate the pH dependence of ACSBG2 enzyme activity (described below), we modified the assay conditions by switching from Tris(Cl) buffer to potassium phosphate. This modification had a significant effect on the specific activity of the enzyme. The activity of endogenous COS-1 cell acyl-CoA synthetases (vector-transfected cells) was somewhat lower when assayed in potassium phosphate versus Tris buffer (compare Fig. 8A with Fig. 7, C18:1). However, the ability of both hACSBG2 and mACSBG2 to activate oleic acid in COS-1 cells overexpressing the enzymes was increased when assayed in potassium phosphate buffer versus Tris buffer (compare Fig. 8A with Fig. 7, C18:1). The activity of the hACSBG2 with the H511R mutation, which was inactive in Tris buffer, showed significant activity (88% increase over vector only) when assayed in phosphate buffer (Fig. 8A).

To understand the nature of this increase in ACSBG2 activity when potassium phosphate buffer was used, we investigated the effects of potassium ions and Tris on enzyme activity. When activation of oleic acid was assayed in vector-transfected cells or COS-1 cells overexpressing mACSBG2 in Tris buffer with increasing concentrations of KCl, no significant stimulation of activity was noted (Fig. 8B). When the same assay was performed in potassium phosphate buffer with increasing concentrations of Tris, a modest, but not statistically significant, inhibitory effect was observed (Fig. 8C). However, the magnitude of this inhibition was not sufficient to explain the differences observed between potassium phosphate buffer and Tris buffer.

Effect of Triacsin C on Endogenous COS-1 Cell and ACSBG2 ACS Activity—Triacsin C has been shown to inhibit long-chain fatty acid activation by ~60% in rat liver at a maximally effective concentration of 10 μM (24). We investigated the effects of Triacsin C on COS-1 cells expressing ACSBG2 cDNAs to determine (a) whether this compound affected ACSBG2 activity and (b) if the endogenous COS-1 cell ACS activity background could be reduced. COS-1 cells transfected with hACSBG2, the hACSBG2 H511R mutant, mACSBG2, or empty vector were preincubated with Triacsin C and then assayed. As shown in Table 1, when either C18:1 or C18:2 were used as substrate, the inhibitor had no effect on enzyme activity of any of the three ACSBG2 constructs. However, Triacsin C did reduce the endogenous COS-1 cell activation of C18:1 and C18:2 by 50 and 40%, respectively, increasing the -fold increase in enzyme activity catalyzed by all three expressed ACSBG2 constructs (Table 1).

We also assayed activation of C16:0 and C24:0 in the presence of Triacsin C. Although activation of C16:0 by endogenous COS-1 cell ACSs was reduced by 75%, none of the expressed ACSBG2 enzymes utilized this substrate (data not shown). Triacsin C had no effect on endogenous COS-1 cell activation of C24:0, and none of the expressed ACSBG2 constructs activated this fatty acid in the presence of inhibitor (data not shown). These observations are all consistent with the data presented in Figs. 7 and 8.

Effect on Catalytic Function of the Presence of a Histidine Rather Than Arginine Residue within Motif 2—The crystal structures of three ACSs, a bacterial and a yeast acetyl-CoA synthetase (25, 26) and a bacterial...
long-chain ACS (27), have been solved. The arginine residue within motif 2 is conserved within all three structures. The arginine side chain appears to form hydrogen bonds with the ribose 3'-hydroxyl group in the acyl-adenylate ACS reaction intermediate. We hypothesized that substitution of a histidine residue (which has a considerably lower pK" than arginine) would lower the pH optimum of wild-type hACSBG2, containing the histidine substitution, as compared with the wild-type mACSBG2, which has an arginine at this position. When overexpressed in COS-1 cells, the pH profile of hACSBG2 (assayed in potassium phosphate buffer) was consistently biphasic (Fig. 9). Optimal activity was seen at pH 6.5, but a lower secondary maxima was seen at pH 7.5. In contrast, the pH profile for mACSBG2 was monophasic with maximal activity at pH 7.0–7.5 (Fig. 9). However, when the human H511R mutant was expressed in COS-1 cells, the pH profile was also monophasic, and the pH optimum was shifted to a higher pH, similar to that seen with the mouse protein (Fig. 9). This experiment was repeated in the presence of 10 μM Triacsin C, although the background (endogenous COS-1 cell) activity was lower, the pH profiles of hACSBG2, mACSBG2, and the H511R mutant of hACSBG2 were unchanged (data not shown). At neutral pH (7.0–7.5), the histidine-containing human enzyme was considerably less active than either the mouse enzyme or the human H511R mutant. These data suggest that the substitution of histidine for arginine in the active site of hACSBG2 affects the catalytic efficiency of the human enzyme in vivo.

DISCUSSION

The potential importance of ACSBG1 (2, 5) in the biochemical pathology of X-ALD prompted us to search for homologous proteins that could also be relevant to our understanding of this disease. Not only was ACSBG1 expressed in tissues pathologically affected in X-ALD, it appeared to be substrates. These two fatty acids are abundant in normal brain for Northern blots such as that presented in Fig. 4A. No obvious signal was detected in medulla on the human RNA dot blot (Fig. 4B), although a weak signal may be present in fetal brain (Fig. 4B, position A11). Nonetheless, we clearly detected ACSBG2 protein in mouse medulla oblongata, as well as in mouse spinal cord. Of the fatty acids tested for activation by either human or mouse ACSBG2, C18:1 and C18:2 appeared to be substrates. These two fatty acids are abundant in normal mouse testis; C18:1ω9 and C18:2ω6 accounted for nearly 55% of total testis fatty acids.6 However, ACS activity of ACSBG2 was modest when compared with the robust activity of ACSBG1 when expressed in COS-1 cells. One possible reason for weak ACS activity is that the true

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**TABLE 1**

**Effect of Triacsin C on ACSBG2 acyl-CoA synthetase activity**

|        | No inhibitor | No inhibitor | +Triacsin C | +Triacsin C | -Fold increase |
|--------|--------------|--------------|-------------|-------------|---------------|
|        |              | (−pcDNA3)    | (pcDNA3)    | (pcDNA3)    |               |
| C18:1  |              |              |             |             |               |
| pcDNA3 | 33.2 ± 1.5   | 16.7 ± 1.0   |             |             |               |
| hACSBG2| 52.9 ± 3.3   | 19.7         | 37.2 ± 2.7  | 20.5         | 2.2           |
| mACSBG2| 62.5 ± 2.2   | 29.3         | 47.3 ± 3.1  | 30.6         | 2.8           |
| hACSBG2(H/R) | 67.5 ± 2.5 | 34.4         | 51.7 ± 2.6  | 35.0         | 3.1           |
| C18:2  |              |              |             |             |               |
| pcDNA3 | 11.2 ± 0.6   | 6.6 ± 0.6    |             |             |               |
| hACSBG2| 15.0 ± 1.0   | 10.8 ± 1.4   | 3.9         | 4.1         | 1.6           |
| mACSBG2| 15.8 ± 0.9   | 11.6 ± 1.3   | 4.7         | 5.0         | 1.8           |
| mACSBG2| 16.6 ± 1.2   | 12.2 ± 0.9   | 5.5         | 5.5         | 1.8           |

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6 A. B. Moser and P. A. Watkins, unpublished observations.
endogenous substrate for ACSBG2 has not yet been identified. An alternative explanation is that correct subcellular location of ACSBG2 is critical for its enzyme activity. In subcellular fractions of testis, ACSBG2 was primarily in the microsomal pellet, whereas the protein expressed in either COS-1 cells or TM4 Sertoli cells was predominantly cytoplasmic. This question cannot be resolved until a cell line in which ACSBG2 is endogenously expressed is identified.

The ACSBG2-dependent ACS activity of transfected COS-1 cells was increased when assayed in potassium phosphate buffer instead of our typical assay buffer, Tris. This could not be attributed to stimulation by the presence of potassium ions, because assays done in Tris with increasing concentrations of K⁺ up to 4-fold higher than that found in assays with potassium phosphate did not stimulate enzyme activity. We also considered the possibility that Tris might have an inhibitory effect on catalytic efficiency. In other enzymes, Tris has been shown to form hydrogen bonds with amino acid residues that would normally participate in substrate recognition (28). Although the presence of Tris in ACS assays conducted in potassium phosphate buffer was slightly inhibitory, the effect was not concentration-dependent, and maximal inhibition was only 11% (at 200 mM Tris for mACSBG2), a value that was not statistically significant.

Triacsin C, 1-hydroxy-3-(E,E,E-2’,4’,7’-undecatrienylidene)triazen, was initially described as an inhibitor of long-chain ACS activity (29). Recent work has established that three members of the long-chain ACS family, ACSL1, ACSL3, and ACSL4, are particularly sensitive to this compound (30, 31). ACSBG2, like some members of the very-long-chain ACS family (32, 33), was not inhibited by Triacsin C. Using this inhibitor, we were able to decrease the background of endogenous COS-1 cell ACS activity without affecting ACSBG2-specific ACS activity, thereby improving the signal-to-noise ratio. Results of assays done in the presence of Triacsin C thus confirmed that ACSBG2 preferentially activates C18:1 and C18:2.

A curious feature of the human ACSBG2 amino acid sequence was the presence of a histidine rather than an arginine (residue 511) in the C18:1 and C18:2. The presence of Triacsin C thus confirmed that ACSBG2 preferentially activates ACS activity without affecting ACSBG2-specific ACS activity, thereby stabilizing of ATP in its binding site facilitates the initial transformation from an open to the closed conformation and that this function is less efficient when His rather than Arg is present. Taken together, these findings suggest that ACSBG2 residue 511 is catalytically important and that there are likely other amino acid changes in the catalytic pocket of hACSBG2 that may compensate for the Arg → His switch.

Zheng et al. (34) recently described a transcriptional variant of ACSBG2 that they referred to as “BGR-like.” This variant is predicted to encode a protein of 497 amino acids that would retain both conserved sequence motifs but lack the N-terminal 187 residues of ACSBG2. Motif 1 would thus begin only 41 residues downstream of the putative start codon, raising questions regarding the ability of BGR-like to catalyze fatty acid activation. Using reverse transcription-PCR, these investigators concluded that BGR (i.e. ACSBG2) was expressed primarily in testis but also in pancreas, kidney, liver, and small intestine, while BGR-like was found almost exclusively in testis. In contrast, we found no evidence for ACSBG2 expression in tissues other than testis, brainstem (medulla oblongata), and spinal cord. Using as a guide the nucleotide sequence of the BGR-like gene, which lacks exon 5 of ACSBG2, we re-evaluated the relevant ESTs in the NCBI data base. 28 ESTs were informative, allowing a clear distinction between ACSBG2 and BGR-like. 19 ESTs, including 12 from testis, supported the ACSBG2 sequence. On the other hand, 9 ESTs (7 from testis) corresponded to BGR-like, supporting the existence of this transcript variant. Antibody raised against the C terminus of ACSBG2 should cross-react with BGR-like. Our Western blot analyses of unfractionated mouse tissues detected only a single band of 75–80 kDa (Fig. 4C), in agreement with the predicted size of ACSBG2. When testis was subjected to differential centrifugation to determine its subcellular distribution, however, the soluble (cytosolic) fraction contained a band of ~50 kDa (Fig. 6C), which is near the predicted size of BGR-like (53.5 kDa). Also using reverse transcription-PCR, Zheng et al. (34) reported that BGR-like expression was decreased or absent in fetal testis and in adults with azoospermia, suggesting a potential role for this gene/protein in male fertility. ACSBG2 expression in azoospermic testes was not determined in this study. Thus, it remains possible that ACSBG2 plays a role in spermatogenesis.

In summary, ACSBG2 was identified as the only mammalian ACS that shares a high degree of amino acid identity with ACSBG1. The
limited tissue expression pattern of ACSBG2, along with its limited substrate specificity, suggests that participation of ACSBG2 in X-ALD pathology is unlikely. In the testis, this protein may play an important role in spermatogenesis, but its function in the brainstem remains unclear.

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