Cloning, Expression, and Characterization of the Human Mitochondrial $\beta$-Ketoacyl Synthase

COMPLEMENTATION OF THE YEAST CEM1 KNOCK-OUT STRAIN

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A human $\beta$-ketoacyl synthase implicated in a mitochondrial pathway for fatty acid synthesis has been identified, cloned, expressed, and characterized. Sequence analysis indicates that the protein is more closely related to freestanding counterparts found in prokaryotes and chloroplasts than it is to the $\beta$-ketoacyl synthase domain of the human cytosolic fatty acid synthase. The full-length nuclear-encoded 459-residue protein includes an N-terminal sequence element of $\sim$38 residues that functions as a mitochondrial targeting sequence. The enzyme can elongate acyl-chains containing 2–14 carbon atoms with malonyl moieties attached in thioester linkage to the human mitochondrial acyl carrier protein and is able to restore growth to the respiratory-deficient yeast mutant cem1 that lacks the endogenous mitochondrial $\beta$-ketoacyl synthase and exhibits lowered lipoic acid levels. To date, four components of a putative type II mitochondrial fatty acid synthase pathway have been identified in humans: acyl carrier protein, malonyl transferase, $\beta$-ketoacyl synthase, and enoyl reductase. The substrate specificity and complementation data for the $\beta$-ketoacyl synthase suggest that, as in plants and fungi, in humans this pathway may play an important role in the generation of octanoyl-acyl carrier protein, the lipoic acid precursor, as well as longer chain fatty acids that are required for optimal mitochondrial function.

Over the last decade, it has become clear that fungal and plant mitochondria are capable of synthesizing fatty acids de novo (1–6). The enzymes involved in the pathway are freestanding, monofunctional proteins of the type II variety and are distinct from the multifunctional polypeptide type I FASs1 present in the cytosol of fungi and animals. Thus far, the preponderance of evidence suggests that these mitochondrial FAS pathways function to produce octanoyl-ACP, the precursor of lipoic acid (4) and longer chain-length fatty acids that may be utilized for the remodeling of mitochondrial membrane phospholipids (7). The presence of a similar mitochondrial FAS system in animals has been suspected for some time, based initially on the discovery of an ACP-like protein in animal mitochondria (8, 9). However, only recently have other components of a putative mitochondrial FAS been identified, cloned, and characterized; they include the human ACP and malonyl transferase (10) and enoyl reductase (11). In continuation of the search for other components, we have now identified and characterized a single candidate for a type II human mitochondrial $\beta$-ketoacyl synthase, the critical enzyme required for catalysis of the chain-elongating condensation reaction.

**EXPERIMENTAL PROCEDURES**

Cloning of the Human Mitochondrial $\beta$-Ketoacyl Synthase—Using the sequences of several authentic type I and type II $\beta$-ketoacyl synthases as probes to BLAST search the human genome sequence database, we identified a sequence (accession NP_060367) that also was represented in a human EST clone (IMAGE clone 3446492, ATCC MGC-4856) and appeared likely to encode a full-length human $\beta$-ketoacyl synthase, together with an N-terminal mitochondrial targeting sequence. A cDNA from this EST clone was used as template to generate a construct for expression in Escherichia coli. Specific primers (KSmitqET/B, Table I) with appropriate restriction sites for cloning were designed, and the cDNA encoding the putative $\beta$-ketoacyl synthase was amplified using PCR, essentially as described earlier (12). The E. coli expression construct carried a 37-residue N-terminal deletion that we deduced, by analysis of multiple sequence alignments, might represent a mitochondrial targeting sequence. The amplified fragment was purified using a QIAquick PCR purification kit (Qiagen), cleaved with appropriate restriction enzymes and cloned into the pQE80 L (QE) E. coli expression vector (Qiagen). To facilitate purification of the recombinant protein by affinity chromatography, a His$_6$-tag was encoded at the N terminus of the construct. The authenticity of the cloned, amplified fragments was confirmed by DNA sequencing.

Verification That the Putative N-terminal Mitochondrial Recognition Elements Direct the $\beta$-Ketoacyl Synthase into Mitochondria—A PCR-amplified fragment (primer set KSmit1–38gfpT/B, Table I) encoding the first 38 amino acid residues (putative targeting sequence) of the human mitochondrial $\beta$-ketoacyl synthase was directionally cloned into the EcoRI and KpnI sites of the pEGFP-N3 vector (Clontech) to facilitate expression as the N-terminal partner of a KSmit1–38-EGFP fusion protein. Authenticity of the cloned fragment was confirmed by DNA sequencing. HeLa cells, grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine fetal calf serum, were cotransfected with two plasmids using FuGENE 6 reagent; one plasmid encoded the KSmit1–38-EGFP fusion protein (or as a control the pEGFP-N3 parental vector), the other encoded pDsRed2-Mito (Clontech), which served as a mitochondrial targeting sequence. The amplified fragment was purified using a QIAquick PCR purification kit (Qiagen), cleaved with appropriate restriction enzymes and cloned into the pQE80 L (QE) E. coli expression vector (Qiagen). To facilitate purification of the recombinant protein by affinity chromatography, a His$_6$-tag was encoded at the N terminus of the construct. The authenticity of the cloned, amplified fragments was confirmed by DNA sequencing.

**Complementation of the Yeast Mitochondrial $\beta$-Ketoacyl Synthase Mutant, cem1, by the Human Mitochondrial Enzyme**—The human mi-
Human Mitochondrial β-Ketoacyl Synthase

TABLE I

| Primers | Sequence | Location* |
|---------|----------|-----------|
| E. coli | KS\textsubscript{sem}.T | 5′-attggaatggagccaggttcacctctggcagttgcatt | 214–236 bp |
|         | KS\textsubscript{sem}.B | 5′-gtcctgcctgaatgctggacgccctggagga | 1452–1478 bp |
| HeLa cells | KS\textsubscript{glfp}.T | 5′-attcagggtgcagggagctggctggggtgggg | 100–124 bp |
|         | KS\textsubscript{glfp}.B | 5′-gtcctgcctgaatgcgtggagcgcattggg | 193–213 bp |
| Yeast  | KS\textsubscript{cem}.T | 5′-actttgcactgtaaatggggtggggtgggg | 75–96 bp |
|         | KS\textsubscript{cem}.B | 5′-ttatacttcattgatgccactggggtggggtgggg | 1432–1453 bp |

*The base pair numbers are according to the sequence in EST cDNA clone IMAGE-3446492.

**Human Mitochondrial β-Ketoacyl Synthase**

The Escherichia coli β-ketoacyl synthase DNA sequence was amplified by the PCR from plasmid pCMV-SPORT6 using the primers KS\textsubscript{sem}.T and KS\textsubscript{sem}.B. The resulting SpeI/ClaI fragment was incorporated into pUG35 (15) giving the multi-copy yeast expression plasmid pKS\textsubscript{sem}.GFP. The plasmid was transformed into the Saccharomyces cerevisiae mutant BY4743 (Euroscarf, Frankfurt/Main, Germany; MAT\textsubscript{a}, his3Δ1, lys2Δ1, ura3Δ0,cdc25Δ::kanMX4, ura3Δ0, and 10% glycerol). The enzyme was stable for at least 6 months when stored at 70 °C.

**Tissue Specificity of Expression of Human Mitochondrial β-Ketoacyl Synthase**—A 32P-labeled probe was synthesized by the random priming method using [γ-32P]ATP (3000 Ci/mmol), a PCR-amplified mitochondrial β-ketoacyl synthase template cDNA and NEBlot kit (New England Biolabs Ltd). A 32P-labeled probe (14) was hybridized to total RNA (1 μg) isolated from HeLa cells, HeLa cells stably expressing human β-ketoacyl synthase, and 1 μg of total RNA isolated from yeast. The probes were examined on a trichloroacetic acid precipitation.

**Expression and Purification of Human Mitochondrial β-Ketoacyl Synthase**—The recombinant pQE 90L vector was expressed in E. coli DH5\textsubscript{a} cells. Cells were grown at 37 °C in LB medium to a density equivalent to an \textit{OD}\textsubscript{600} of 0.5 and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside at room temperature overnight. Cells were harvested and using a microfluidizer (Microfluidics, Newton MA) were lysed in buffer (25 mM Tris-HCl, pH 7.8, 300 mM NaCl) containing protease inhibitors (leupeptin 5 μg/ml, trans-epoxysuccinyl-L-GB 10 μM, pepstatin 1 μg/ml, and antitryptsin 5 μg/ml). The cell debris was pelleted by centrifugation at 36,000 × g for 40 min at 4 °C, and the resulting supernatant was passed through a 0.45-μm filter and loaded at 20 °C onto a Hitrap Chelating HP column (5 ml bed volume, Amersham Biosciences). The column was washed with 50 mM imidazole in buffer A (25 mM Tris-HCl, pH 7.8, 300 mM NaCl, 10% glycerol) and then the tightly bound proteins were eluted with 250 mM imidazole in buffer A. The latter protein fraction was rechromatographed on the same column, essentially in the same manner, except that an additional wash with 100 mM imidazole in buffer A was included prior to elution of the tightly bound proteins. The purified protein was subjected to ultrafiltration, both to remove imidazole and to effect transfer to the storage buffer (25 mM Tris-HCl, pH 7.8, 300 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, and 10% glycerol). The enzyme was stable for at least 6 months when stored at −80 °C.

**β-Ketoacyl Synthase Activity Using Acetyl-CoA as Primer**—Recombinant mitochondrial β-ketoacyl synthase was assayed for condensing activity using [1-14C]acetyl-CoA as the primer and malonyl-ACP\textsubscript{mt}, as the chain extender. The malonyl-ACP\textsubscript{mt} was first generated in situ from malonyl-CoA and holomCP\textsubscript{mt} using human mitochondrial malonyl transference (10). The reaction mixture contained 83 mM potassium phosphate buffer, pH 6.8, 0.3 mM dithiothreitol, 1 mM EDTA, 200 μM malonyl-CoA, 60 μM holomCP\textsubscript{mt}, and 100 ng of human mitochondrial malonyl transference in a final volume of 30 μl and was incubated at 37 °C for 15 min. Then [1-14C]acetyl-CoA (54 Ci/mmol, 50 μM final concentration) was added. The mixture was aliquoted into two tubes and incubated for 20 min at 37 °C without or with 1 μg (725 nmol) of mitochondrial β-ketoacyl synthase. The reaction was terminated by trichloroacetic acid precipitation.

**Synthesis of Acyl-ACP\textsubscript{mt} Substrates**—The C2:0–C16:0 acyl-ACP\textsubscript{mt} thioesters were synthesized enzymatically using recombinant human phosphopantetheinyl transferase (15) and C-terminally His\textsubscript{6}-tagged apoACP\textsubscript{mt}. A typical reaction mixture, 5 ml in volume, containing 20 mM BisTris-HCl, pH 6.5, 10 mM MgCl\textsubscript{2}, 15 μM apoACP\textsubscript{mt} and 75 μM acyl-CoA, was incubated with 250 μM phosphopantetheinyl transferase at 37 °C overnight. The reaction mixture was then desalted into buffer B (20 mM BisTris-HCl, pH 6.5, 10% glycerol) using a Vivisap concentrator (5,000 Da cutoff, Vivascience) and repurified at 20 °C on a HiTrap Q HP anion exchange column. The bound proteins were eluted with a 250–500 mM NaCl gradient in buffer B, followed by a 5-mM gradient of 250–500 mM NaCl in buffer B. The anion exchange chromatography profile exhibited a partial separation between residual unmodified apoACP\textsubscript{mt} and the various acyl-ACP\textsubscript{mt} species, which eluted later. Fractions were analyzed by SDS-PAGE to assess protein purity and by mass spectrometry to identify those fractions that contained authentic acyl-ACP\textsubscript{mt} thioesters, essentially free of residual apoACP\textsubscript{mt}. Starting with ~1 mg of apoACP\textsubscript{mt}, the overall yields of acyl-ACP\textsubscript{mt} thioesters were typically ~60%, with the exception of C16-ACP\textsubscript{mt} for which the yield was 30%.

**Isolation of Apo and Holo Forms of Human Mitochondrial ACP**—The C-terminally His\textsubscript{6}-tagged apoACP\textsubscript{mt} was expressed in an Sf9/baculoviral host/vector system and purified as described earlier, and the holo-form was derived from it using human phosphopantetheinyl transferase (15).

**Mass Spectrometry**—Samples were desalted using a C\textsubscript{18} ZipTip (Millipore, Bedford, MS), mixed with the matrix (sinapinic acid, 10 mg/ml in 30% acetonitrile, 0.1% trifluoroacetic acid) in a 10:1 ratio (v/v), and 1 μl of the resulting mixture was spotted onto a stainless steel matrix-assisted laser desorption ionization time-of-flight biospectrometry work station (Bruker Daltonics, Billerica, MA) in a positive linear mode. The mass scale was externally calibrated utilizing cytochrome c. Both singly and doubly charged ions were used for a molecular mass determination.

**β-Ketoacyl-ACP Synthase Assay**—The β-ketoacyl synthase activities were assayed according to Garwin et al. (16). The incubation mixture (total volume 20 μl) contained 0.2 mM potassium phosphate buffer, pH 7.8.
Human Mitochondrial \(\beta\)-Ketoacyl Synthase

6.8, 1 mM EDTA, 0.3 mM dithiothreitol, 660 mM malonyl/acyetyl transferase (a recombinant form of the single domain derived from the rat cytosolic type I FAS (17)), 10 \(\mu\)M acyl-ACP\(_{\text{mit}}\), 20 \(\mu\)M [2-\(\text{C}^1\)]malonyl-CoA (52 C/mmol), 1–50 \(\mu\)M acyl-ACP\(_{\text{mit}}\), and 25 ng (27 nm) of mitochondrial \(\beta\)-ketoacyl synthase. The malonyl-ACP\(_{\text{mit}}\) was first generated from malonyl-CoA and ACP\(_{\text{mit}}\), using malonyl/acyetyl transferase, by incubation for 15 min at room temperature. Then the reaction was started by addition of acyl-ACP\(_{\text{mit}}\) and mitochondrial \(\beta\)-ketoacyl synthase, continued for 2 min at 37 °C, and finally terminated by the addition of 0.4 ml of reducing reagent (0.1 M \(\text{K}_2\text{HPO}_4\), 0.4 M KCl, 30% tetrahydrofuran, and 5 mM \(\text{NaN}_3\)). The contents of the assay tubes were mixed vigorously and incubated at 37 °C for 45 min. Toluene (0.4 ml) was added, the contents of the tubes were vortexed for 2 min, and a portion (0.4 ml) of the upper phase was taken for liquid scintillation spectrometry. Blank reactions were performed without acyl-ACP\(_{\text{mit}}\). Kinetic parameters were determined using EnzymeKinetics (Trinity Software); values represent the means ± S.D. for calculations using Lineweaver-Burk and non-linear regression methods.

Effects of Cerulenin on Mitochondrial \(\beta\)-Ketoacyl Synthase Activity—Mitochondrial \(\beta\)-ketoacyl synthase (0.25 \(\mu\)g) was preincubated for 15 min at room temperature with 0–1 mM cerulenin in a final volume of 20 \(\mu\)l. Portions of the reaction mixture were then assayed for residual activity in 0.2 M potassium phosphate buffer, pH 6.8, 1 mM EDTA, 0.3 mM dithiothreitol, 660 mM malonyl/acyetyl transferase, 10 \(\mu\)M acyl-ACP\(_{\text{mit}}\), 20 \(\mu\)M [2-\(\text{C}^1\)]malonyl-CoA (52 C/mmol), 10 \(\mu\)M C6-ACP\(_{\text{mit}}\), and 25 ng of \(\beta\)-ketoacyl synthase (final volume of 20 \(\mu\)l).

RESULTS

Identification of the Putative Human Mitochondrial \(\beta\)-Ketoacyl Synthase—A single candidate sequence for a freestanding, type \(\beta\) \(\beta\)-ketoacyl synthase was identified in both the human and mouse genomic sequence data bases (sequences 85% identical). Compared with \(\beta\)-ketoacyl synthase sequences from prokaryotes, the putative 459-residue mammalian mitochondrial counterparts have extensions of residues 443–449. This analysis provided a strong indication near the C terminus that contributes to the oxyanion hole (residue 273), two threonine function (residue 380), a glycine residue that allows entrance into the substrate-binding tunnel (residue 273), two threonine residues that hydrogen bond with the ACP phosphopantetheine moiety (residues 350 and 352), and a glycine-rich motif near the C terminus that contributes to the oxyanion hole (residues 443–449). This analysis provided a strong indication that mammalian nuclear genomes encode a single, type \(\beta\) \(\beta\)-ketoacyl synthase having an extended sequence at the N terminus. The coding sequence for the human enzyme is located on chromosome 3 (3p24.2) and that for the mouse counterpart on chromosome 14.

Verification That the Putative Mitochondrial \(\beta\)-Ketoacyl Synthase Contains an N-terminal Mitochondrial Targeting Sequence—A cDNA encoding the putative human \(\beta\)-ketoacyl synthase mitochondrial targeting sequence (residues 1–38) fused in-frame with the coding sequence for EGFP, a red-shifted variant of green fluorescent protein, was coexpressed in HeLa cells together with a fusion protein consisting of the mitochondrial targeting sequence of cytochrome c coupled to the Discosoma sp. red fluorescent protein. Confocal fluorescence microscopy revealed that the KS\(_{\text{mit1–38}}\)-EGFP colocalized with the red fluorescent mitochondrial marker protein (Fig. 2). In contrast, the EGFP protein lacking the putative mitochondrial targeting sequence of the \(\beta\)-ketoacyl synthase expressed throughout the cytoplasm. Thus, this experiment confirmed that the N-terminal region of the full-length \(\beta\)-ketoacyl synthase indeed is a mitochondrial targeting sequence.

Complementation of the Yeast cem1 Mutant Strain by the Human \(\beta\)-Ketoacyl Synthase—The in vivo function of the cDNA-encoded human mitochondrial \(\beta\)-ketoacyl synthase was established by complementation of the mitochondrial \(\beta\)-ketoacyl synthase defect of a S. cerevisiae cem1 mutant (2). This mutation is characterized by a nuclear-encoded respiratory-defective petite phenotype, resulting in the inability to grow on non-fermentable carbon sources such as glycerol or lactate. Consequently, the four spores originating from a sporulated BY4743 diplod consist of two lactate-positive and two lactate-negative segregants (Fig. 3B). In accordance with the use of kanMX4 as a disruption cassette for CEM1, the two lactate-negative spores are G418-resistant (Fig. 3B). In contrast, sporulation of BY4743 cells transformed with pKS\(_{\text{mit}}\)-GFP resulted in four respiratory-competent spores, even though two of them were again G418-resistant and hence contained the defective cem1Δ allele (Fig. 3A). Obviously, the \(\beta\)-ketoacyl synthase defect in these cells was compensated by the human \(\beta\)-ketoacyl synthase homolog expressed from pKS\(_{\text{mit}}\)-GFP. Because this plasmid contains the yeast URA3 gene as a selection marker, the uracil-protoporphy of all four spores proved the equal distribution of the multicopy plasmid among the meiotic segregants. The C-terminal fusion of GFP to pKS\(_{\text{mit}}\)-GFP-encoded human mitochondrial \(\beta\)-ketoacyl synthase apparently did not interfere with the \(\beta\)-ketoacyl synthase function. Furthermore, the successful complementation of the respiratory defect implied that the mitochondrial targeting sequence at the N terminus of the human \(\beta\)-ketoacyl synthase was recognized in the fungal host. This conclusion was supported by the observation that in situ fluorescence elicited by the C-terminal GFP marker was not distributed throughout the cytoplasm but was limited to specific regions of the cell likely corresponding to mitochondria (data not shown).

Tissue Distribution of Human Mitochondrial \(\beta\)-Ketoacyl Synthase—Analysis of a multiple tissue Northern blot with a radiolabeled cDNA probe revealed that mitochondrial \(\beta\)-ketoacyl synthase transcripts are most abundant in the heart and skeletal muscle, as well as the liver and kidney, tissues that contain high levels of active mitochondria (Fig. 4). Transcripts were present at relatively low levels in the placenta, brain, spleen, and lung and were barely detectable in the colon, thymus, and leukocytes. This relatively broad tissue expression contrasts with that of the type I cytosolic FAS, which is not expressed at significant levels in heart or skeletal muscle and is expressed at high levels in tissues such as liver that are involved in lipogenesis and energy homeostasis.

Expression and Purification of the Human Mitochondrial \(\beta\)-Ketoacyl Synthase—The mitochondrial \(\beta\)-ketoacyl synthase construct KS\(_{\text{mit1–38}}\), in which the first 37 residues constituting the mitochondrial targeting sequence were replaced by a His\(_{10}\)-tag, expressed well in E. coli, although appreciable amounts of the protein were sequestered in inclusion bodies when the cells were grown at temperatures above 20 °C. The protein could be
FIG. 1. Sequence alignments of putative mitochondrial, prokaryotic type II, and mammalian type I counterparts. The alignment was performed using ClustalW. Conserved residues known to be important for catalytic function are shown boxed in boldface. Numbering is for the human mitochondrial enzyme. Accession numbers for the sequences are, in order of presentation, NP_060367, XP_127578, AB073746, CAB58180, XP322142, NP_416826, NP_287229, and GO1880.
obtained in high purity by two successive metal-ion affinity chromatography steps, and its mobility on SDS-PAGE corresponded closely to that of a species with the expected molecular mass of 45.8 kDa (Fig. 5).

Substrate Specificity and Kinetic Properties of the Human Mitochondrial β-Ketoacyl Synthase—The type II FAS systems of prokaryotes and chloroplasts typically employ a specialized enzyme (β-ketoacyl synthase III) for catalysis of the first elongation step that uses acetyl-CoA, rather than acetyl-ACP, as the primer. Therefore, the possibility that the mitochondrial β-ketoacyl synthase could utilize acetyl-CoA directly as a substrate for elongation was evaluated using the malonyl thioester of human mitochondrial ACP (ACPmit) as the chain extender; however, no activity was observed even when high concentrations (0.725 μM) of enzyme were used.

For evaluation of catalytic activity using various acyl-ACPmit thioesters as primers, we devised a novel, simple, efficient procedure for synthesis of these substrates by exploiting the broad substrate specificity of the human phosphopantetheinyl transferase. This enzyme will transfer the phosphopantetheinyl moiety from CoA to a variety of apoACPs, essentially independent of whether the donor CoA substrate is offered as the free thiol, or as an acyl-CoA thioester. Thus, acyl-CoAs containing 2–16 C atoms were all efficiently converted to the corresponding acyl-ACPs by this enzyme. The human phosphopantetheinyl transferase has a remarkably broad tolerance not only for the acyl-3-phosphopantetheinyl donor but also for the carrier protein acceptor and can phosphopantetheinylate both peptidyl and acyl carrier proteins from prokaryotes and eukaryotes (15). Thus, the enzyme is ideally suited for routine synthesis of acyl-ACPs of any acyl chain length and ACP origin.

In the kinetic studies, care was taken to validate steady state conditions with each substrate. Typically, only 27 nM enzyme and incubation times of 2 min were used. When 40-fold higher concentrations of enzyme were used, a significant radiolabel from malonyl moieties was recovered in the toluene extract even when no priming substrate was provided, indicating that malonyl decarboxylase activity of the β-ketoacyl synthase most likely was generating acetyl moieties that were used as the primer. At steady state, using only 27 nM enzyme, no significant incorporation of radiolabel was observed in the absence of priming substrate indicating that formation of acetyl priming moieties by decarboxylation was negligible. Under these conditions, no more than 20% of the substrate was utilized during the course of the assay.

Acyl-ACPmit thioesters containing acyl moieties with 2–14 C atoms all were elongated by the mitochondrial β-ketoacyl synthase; only C-16-ACPmit was a very poor substrate (Fig. 6A). When compared in the acyl-ACPmit concentration range 5–10 μM, the activities ranked in the following order: C-12 > C-10 > C-6 > C-8 > C-4 > C-2 > C-14 > C-16. However, the $K_m$ values varied according to the acyl chain-length of the substrate. Thus, the enzyme exhibited $K_m$ values in the low micromolar range for C-4–C-12 ACP thioesters but had markedly lower affinity for C-2 and C-14 substrates. Although it was not possible to estimate actual kinetic parameters for C-2 because of an apparently very high $K_m$ value, the $V_{max}$ was >250 nmol min$^{-1}$ mg$^{-1}$ for this substrate and values for the other substrates were in the range 100–400 nmol min$^{-1}$ mg$^{-1}$, ex-

![Fig. 2. The N-terminal 38 residues of the putative β-ketoacyl synthase contain a mitochondrial targeting sequence. HeLa cells were cotransfected with two constructs, one encoding the red fluorescent protein (EGFP) or a chimera consisting of the first 38 residues of the β-ketoacyl synthase fused to the N terminus of the green fluorescent protein (KSmit1–38EGFP). Cells were fixed then analyzed for green and red fluorescence by confocal microscopy.](image1.png)

![Fig. 3. Complementation of the yeast cem1 mutation by the human mitochondrial β-ketoacyl synthase. pKSmit-GFP transformed (A) and untransformed (B) BY4743 cells were sporulated, and spore tetrads were dissected by micromanipulation. Individual spores from two representative tetrads were grown on the indicated media for 2–4 days at 30 °C. The media are described under “Experimental Procedures.”](image2.png)

![Fig. 4. Multiple tissue Northern blot for mitochondrial β-ketoacyl synthase. Each lane of the blot contains ~1 μg (normalized to give consistent signal for control B-actin gene across all lanes) of purified poly(A)$^+$ RNA.](image3.png)

![Fig. 5. Electrophoretic analysis of purified mitochondrial β-ketoacyl synthase. Purified enzyme was electrophoresed on 10% polyacrylamide gels and either stained with Coomassie Brilliant Blue (A) or electroblotted onto a polyvinylidene difluoride membrane for Western analysis using mouse anti-His6 and alkaline phosphatase-conjugated, goat anti-mouse IgG antibodies (B). The molecular masses of prestained markers, in kDa, are shown on the left of the figure.](image4.png)
except for C-12-ACP, which was >1000 nmol·min⁻¹ mg⁻¹.

Inhibition of the Human Mitochondrial β-Ketoacyl Synthase by Cerulenin—Cerulenin, an antibiotic produced by Cephalosporium caerulens, is a potent inhibitor of both prokaryotic type II and eukaryotic type I FAS systems (28) and acts by binding to the hydrophobic pocket formed at the β-ketoacyl synthase dimer interface and reacting with the active site cysteine through its C-2 carbon (20, 29). The human mitochondrial β-ketoacyl synthase was also inactivated by cerulenin (Fig. 7), although sensitivity appears low compared with the prokaryotic β-ketoacyl synthases I and II for which IC₅₀ values below 25 μM are typically reported (25).

**DISCUSSION**

This study establishes that the human nuclear genome encodes a single mitochondrially targeted β-ketoacyl synthase. This is the fourth nuclear-encoded component of a putative human mitochondrial FAS system to be characterized to date; the others are the ACP, malonyl transferase, and enoyl reductase. In common with the other three human mitochondrial FAS enzymes, as well as the fungal and plant mitochondrial β-ketoacyl synthases, the animal β-ketoacyl synthases appear more closely related to prokaryotic and plastid counterparts than to the cytosolic type I FAS of the same species. Thus, phylogenetic analysis reveals that all of the mitochondrial β-ketoacyl synthases associated with cytosolic FASs (cFAS) and modular polyketide synthases (mPKS) are shown as dashed lines, and the distantly related thiolases that catalyze the reverse reaction in fatty acid oxidation are shown as dotted lines. All other lineages represent type II β-ketoacyl synthases and the three types are distinguished by the notation KSI, KSII, or KSIII.

Present day prokaryotes and plant plastids employ three β-ketoacyl synthases with different specificities to fulfill their fatty acid requirement. They are β-ketoacyl synthase III, which catalyzes the initial condensation step using acetyl-CoA as primer, and β-ketoacyl synthases I and II, which catalyze the remaining elongation reactions using acyl-ACPs as primers. All three types of enzyme share a similar overall fold and differ primarily in the structure at the active site, reflecting their different specificities (31). The β-ketoacyl synthases I and II have similar specificities, except that β-ketoacyl synthase II is able to elongate 16:1–18:1. Because only one type II β-ketoacyl synthase was identified in the human genome, the substrate specificity of this enzyme was of particular interest. The human mitochondrial enzyme, which appears more closely related to prokaryotic β-ketoacyl synthases I and II than to β-ketoacyl synthase III enzymes (Fig. 7), is unable to utilize acetyl-CoA as the primer. However, in contrast to the prokaryotic β-ketoacyl synthases I and II, it is able to catalyze the initial condensation reaction using a 2-carbon primer, presented as acetyl-ACP. Otherwise, the specificity of the mitochondrial enzyme (Fig. 6) is quite similar to that of the prokaryotic β-ketoacyl synthases I and II (32) in that, with all three types, enzyme activity falls off significantly with substrates containing more than 12 C atoms. However, the specific activity of the human mitochondrial enzyme is 20-fold higher than that reported for the E. coli β-ketoacyl synthases I and II (32). It is unclear whether the disparity reflects a true difference in catalytic efficiency or can be attributed to suboptimal assay conditions used for the prokaryotic enzymes.

Recently, the mitochondrial β-ketoacyl synthase from A. thaliana has been identified, cloned, and characterized (18). This enzyme too exhibits a high sequence similarity with β-ketoacyl synthases I and II of prokaryotes and is able to restore growth of the E. coli CY244 strain, which lacks both β-ketoacyl synthases I and II. The authors concluded that the A. thaliana mitochondrial enzyme does not use either acetyl-CoA or acetyl-CoA as the primer, and catalyzes the initial condensation step using acetyl-CoA as the primer. However, in contrast to the prokaryotic β-ketoacyl synthases I and II, it is able to catalyze the initial condensation reaction using a 2-carbon primer, presented as acetyl-ACP. Otherwise, the specificity of the mitochondrial enzyme (Fig. 6) is quite similar to that of the prokaryotic β-ketoacyl synthases I and II (32) in that, with all three types, enzyme activity falls off significantly with substrates containing more than 12 C atoms. However, the specific activity of the human mitochondrial enzyme is 20-fold higher than that reported for the E. coli β-ketoacyl synthases I and II (32). It is unclear whether the disparity reflects a true difference in catalytic efficiency or can be attributed to suboptimal assay conditions used for the prokaryotic enzymes.

**FIG. 6. Substrate specificity of the human mitochondrial β-ketoacyl synthase.** A, substrate concentration dependence of the β-ketoacyl synthase reaction with different chain length acyl-ACP primers. B, kinetic parameters.

**FIG. 7. Inhibition of β-ketoacyl synthase by cerulenin.** Human mitochondrial β-ketoacyl synthase was preincubated with cerulenin and activity assayed using C6:0-ACP as substrate, as described under “Experimental Procedures.”

**FIG. 8. Phylogenetic tree of β-ketoacyl synthases.** Multiple sequence alignments were performed using ClustalW, and the unrooted phylogenetic analysis reveals that all of the mitochondrial β-ketoacyl synthases I and II, which catalyze the initial condensation step using acetyl-ACP as primer, and β-ketoacyl synthases I and II, which catalyze the remaining elongation reactions using acyl-ACPs as primers. All three types of enzyme share a similar overall fold and differ primarily in the structure at the active site, reflecting their different specificities (31). The β-ketoacyl synthases I and II have similar specificities, except that β-ketoacyl synthase II is able to elongate 16:1–18:1. Because only one type II β-ketoacyl synthase was identified in the human genome, the substrate specificity of this enzyme was of particular interest. The human mitochondrial enzyme, which appears more closely related to prokaryotic β-ketoacyl synthases I and II than to β-ketoacyl synthase III enzymes (Fig. 7), is unable to utilize acetyl-CoA as the primer. However, in contrast to the prokaryotic β-ketoacyl synthases I and II, it is able to catalyze the initial condensation reaction using a 2-carbon primer, presented as acetyl-ACP. Otherwise, the specificity of the mitochondrial enzyme (Fig. 6) is quite similar to that of the prokaryotic β-ketoacyl synthases I and II (32) in that, with all three types, enzyme activity falls off significantly with substrates containing more than 12 C atoms. However, the specific activity of the human mitochondrial enzyme is 20-fold higher than that reported for the E. coli β-ketoacyl synthases I and II (32). It is unclear whether the disparity reflects a true difference in catalytic efficiency or can be attributed to suboptimal assay conditions used for the prokaryotic enzymes.
ACP as primer but instead uses acetyl moieties derived by decarboxylation of malonyl-ACP. In this respect, the enzyme appears to differ from the human mitochondrial β-ketoacyl synthase, which clearly is able to utilize acetyl-ACP as primer.

The major products of the prokaryotic type II FAS pathway are long chain acyl-ACP’s containing 16 and 18 C atoms, but one of the intermediates, octanoyl-ACP, is siphoned off for the production of lipooyl-ACP. Experiments with plant mitochondria indicate that the major products of this mitochondrial type II FAS system also are octanoyl and long chain length acyl-ACPs (5). Furthermore, the profile of fatty acids synthesized by soluble extracts of *E. coli* CY244 cells supplemented with the *A. thaliana* mitochondrial β-ketoacyl synthase also showed a bimodal distribution with maxima at C-8 and C-14–16. The hypothesis that the mitochondrial FAS system might be responsible for producing both octanoyl and long chain length acyl-ACPs is supported by mutational disruption of the mitochondrial FAS system of fungi. For example, disruption of the nuclear-encoded gene for mitochondrial ACP in *Neurospora crassa* results in an accumulation of lysophospholipids in mitochondrial membranes and an accompanying respiratory-deficient phenotype. Disruption of the nuclear-encoded gene for either the mitochondrial β-ketoacyl synthase or ACP in *S. cerevisiae* also produces a respiratory-deficient phenotype and, in the case of the ACP-defective strain, it has been established that cellular lipoic acid concentration is reduced to <10% of that of the wild-type strain (4, 33). Furthermore, addition of lipoic acid to the growth medium could not compensate for the block in endogenous lipoic acid synthesis, suggesting that in this species lipoic acid may not readily be taken up into mitochondria (4). Our finding that the human mitochondrial β-ketoacyl synthase is able to restore normal growth to the *cem1* mutant strain implies that the substrate specificity and kinetic properties of the human enzyme are entirely compatible with those of the endogenous *S. cerevisiae* enzyme and allow the synthesis and release of octanoyl-ACP from the elongation cycle for conversion to lipooyl-ACP. An interesting feature of the kinetic properties of the human mitochondrial β-ketoacyl synthase is the higher *Kₘ* for octanoyl-ACP (11 μM), compared with the values for hexanoyl- and decanoyl-ACP (2 μM). Possibly, this property may result in a higher pool size for the C-8 intermediate that could facilitate diversion into the lipoate pathway. For many years it has been generally assumed that in mammals the requirement for lipoic acid is met by dietary intake. In this pathway, free lipoate, taken up via vitamin transporters (34, 35) is activated to lipoyl-AMP by a lipoytating enzyme, and then the lipoyl moiety is transferred to a lysine residue in the acceptor protein by a lipoyl-AMP:Nε-lysine lipoyltransferase; both enzymes have been cloned and characterized from mammalian sources (36). However, recent studies have revealed that mammalian mitochondrial also contain a lipoic acid synthase, which converts octanoyl-ACP to lipoyl-ACP (37). Although a lipooyl-ACP-dependent transferase required for the lipoylation of mitochondrial proteins (performed by the lIP and LIP2 gene products of prokaryotes and plant mitochondria, respectively (38, 39)) has yet to be identified in mammalian mitochondria, this finding raised the possibility that endogenously synthesized lipoic acid may be utilized for the lipoylation of mitochondrial proteins, as it is in fungal and plant mitochondria. In mammals, all of the known lipoate-containing proteins, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine cleavage enzyme, are located in the mitochondria (40) and the presence of a lipoate-producing system in this organelle may ensure an adequate supply of this cofactor, essential for mitochondrial function, independent of dietary availability.

Collectively then, the complementation of the *cem1* knockout by the human mitochondrial β-ketoacyl synthase, the similar substrate specificities, and close evolutionary relationship of type II human mitochondrial and prokaryotic β-ketoacyl synthases suggest that the type II enzymes fulfill similar roles in these different environments, namely the supply of octanoyl-ACP for lipoic acid production and the production of long chain length fatty acyl moieties for membrane phospholipid biosynthesis. Nevertheless, until unequivocal experimental evidence is obtained validating this hypothesis, the possibility that the FAS pathway might generate other products important for mitochondrial function, such as myristate for protein modification, should not be discounted. In this regard, the finding that ACP isolated from *N. crassa* mitochondria appeared to have a β-hydroxymyristoyl moiety attached to the phosphopantetheine (41) remains something of an enigma. Evaluation of the effects of down-regulation of expression of mitochondrial FAS components on mitochondrial function may resolve these questions.

In recent years the type II fatty acid-synthesizing systems of microorganisms have been identified as promising targets for the development of new therapeutic agents for the treatment of a variety of infectious diseases, based primarily on the premise that such agents likely would have little or no effect on the type I FAS system present in the cytosol of the animal host cells (31, 42, 43). Demonstration of the presence of a type II FAS system in animal mitochondria indicates that caution should be exercised in the screening of such compounds, and assurance should be sought that they have no serious side effects on mitochondrial function.

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**REFERENCES**

1. Mikolajczyk, S., and Brody, S. (1990) *Eur. J. Biochem.* 187, 431–437

2. Harington, A., Herbert, C. J., Tung, B., Getz, G. S., and Slonimski, P. P. (1993) *Mol. Microbiol.* 9, 545–555

3. Schneider, R., Brors, B., Burger, F., Camrath, S., and Weiss, H. (1997) *Curr. Genet.* 32, 384–388

4. Brody, S., Oh, C., Hoja, U., and Schweizer, E. (1997) *FEBS Lett.* 408, 217–220

5. Gueguen, V., Machere, D., Jaspion, M., Douce, R., and Bourguignon, J. (2000) *J. Biol. Chem.* 275, 5021–5025

6. Torkko, J. M., Koivuranta, K. T., Miinalainen, I. J., Yagi, A. I., Schmitz, W., Kastaniotis, A. J., Airenne, T. T., Gurvitz, A., and Hiltunen, K. J. (2001) *Mol. Cell. Biol.* 21, 6243–6253

7. Schneider, R., Brors, B., Massow, M., and Weiss, H. (1997) *FEBS Lett.* 407, 249–252

8. Runswick, M. J., Fearnley, I. M., Skehel, J. M., and Walker, J. E. (1991) *FEBS Lett.* 286, 121–124

9. Triepels, R., Smitink, J., Loeffen, J., Smets, R., Buskens, C., Tijdens, F., and van den Heuvel, L. (1999) *J. Inherit. Metab. Dis.* 22, 163–173

10. Zhang, L., Joshi, A. K., and Smith, S. (2003) *J. Biol. Chem.* 278, 40067–40074

11. Miinalainen, I. J., Chen, Z. J., Torkko, J. M., Piriia, P. L., Sormunen, R. T., Bergmann, U., Qin, Y. M., and Hiltunen, K. J. (2003) *J. Biol. Chem.* 278, 20154–20161

12. Rangan, A. K., and Smith, S. (1993) *J. Biol. Chem.* 268, 22508–22513

13. Niedenthal, R. K., Riles, L., Johnston, M., and Hegemann, J. H. (1996) *Yeast* 12, 773–786

14. Ausubel, M. F., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York

15. Joshi, A. K., Zhang, L., Rangan, V. S., and Smith, S. (2003) *J. Biol. Chem.* 278, 31142–31149

16. Garwin, J. L., Klages, A. L., and Cronan, J. E., Jr. (1980) *J. Biol. Chem.* 255, 11949–11956

17. Rangan, V. S., Serre, L., Witkowski, H. E., Bari, A., and Smith, S. (1997) *Protein Eng.* 10, 561–566

18. Yamasuo, R., von Wettstein-Knowles, P., and Wada, H. (2004) *J. Biol. Chem.* 279, 6242–6251

19. Mochel, M., Dehesh, K., Edwards, P., and Lindqvist, Y. (2001) *J. Biol. Chem.* 276, 403–404

20. Moche, M., Schneider, G., Edwards, P., Dehesh, K., and Lindqvist, Y. (1999) *J. Biol. Chem.* 274, 6001–6004

21. Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., and Lindqvist, Y. (1998) *EMBO J.* 17, 1183–1191

22. Olsen, J. G., Kadziola, A., von Wettstein-Knowles, P., Sigggaard-Andersen, M., Lindquist, Y., and Larsen, S. (1999) *FEBS Lett.* 400, 46–52

23. Olsen, J. G. (2001) *The Molecular Structure and Function of β-Ketoacyl Synthase I*. Ph.D. Thesis, University of Copenhagen, Copenhagen

24. Olsen, J. G., Kadziola, A., von Wettstein-Knowles, P., Sigggaard-Andersen, M.,
and Larsen, S. (2001) *Structure (Camb.)* 9, 233–243
25. Price, A. C., Choi, K. H., Heath, R. J., Li, Z., White, S. W., and Rock, C. O. (2001) *J. Biol. Chem.* 276, 6551–6559
26. Heath, R. J., and Rock, C. O. (2002) *Nat. Prod. Rep.* 19, 581–596
27. Price, A. C., Rock, C. O., and White, S. W. (2003) *J. Bacteriol.* 185, 4136–4143
28. Tomoda, H., Kawaguchi, A., Omura, S., and Okuda, S. (1984) *J. Biochem.* 95, 1705–1712
29. Funabashi, H., Kawaguchi, A., Tomoda, H., Omura, S., Okuda, S., and Iwasaki, S. (1989) *J. Biochem.* 105, 751–755
30. Page, R. D. M. (1996) *Comput. Appl. Biosci.* 12, 357–358
31. Heath, R. J., White, S. W., and Rock, C. O. (2001) *Prog. Lipid Res.* 40, 467–497
32. Edwards, P., Nelsen, J. S., Metz, J. G., and Dehesh, K. (1997) *FEBS Lett.* 402, 62–66
33. Schneider, R., Massow, M., Lisowsky, T., and Weiss, H. (1995) *Curr. Genet.* 29, 10–17
34. Wang, H., Huang, W., Fei, Y. J., Xia, H., Yang-Feng, T. L., Leibach, F. H., Devoe, L. D., Ganapathy, V., and Prasad, P. D. (1999) *J. Biol. Chem.* 274, 14875–14883
35. Prasad, P. D., Wang, H., Kekuda, R., Fujita, T., Fei, Y. J., Devoe, L. D., Leibach, F. H., and Ganapathy, V. (1998) *J. Biol. Chem.* 273, 7501–7506
36. Fujisawa, K., Takeuchi, S., Okamura-Ikeda, K., and Motokawa, Y. (2001) *J. Biol. Chem.* 276, 28819–28823
37. Morikawa, T., Yasuno, R., and Wada, H. (2001) *FEBS Lett.* 498, 16–21
38. Jordan, S. W., and Cronan, J. E., Jr. (2003) *J. Bacteriol.* 185, 1582–1589
39. Wada, M., Yasuno, R., Jordan, S. W., Cronan, J. E., Jr., and Wada, H. (2001) *Plant Cell Physiol.* 42, 650–656
40. Perham, R. N. (2000) *Annu. Rev. Biochem.* 69, 961–1004
41. Brody, S., and Mikolajczyk, S. (1988) *Eur. J. Biochem.* 173, 353–359
42. Kremer, L., Douglas, J. D., Baulard, A. R., Morehouse, C., Guy, M. R., Alland, D., Dover, L. G., Lakey, J. H., Jacobs, W. R., Jr., Brennan, P. J., Minnikin, D. E., and Besra, G. S. (2000) *J. Biol. Chem.* 275, 16857–16864
43. Wrenger, C., and Muller, S. (2004) *Mol. Microbiol.* 53, 105–113