Cloning, Expression, Characterization, and Interaction of Two Components of a Human Mitochondrial Fatty Acid Synthase

MALONYLTRANSFERASE AND ACYL CARRIER PROTEIN

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The possibility that human cells contain, in addition to the cytosolic type I fatty acid synthase complex, a mitochondrial type II malonyl-CoA-dependent system for the biosynthesis of fatty acids has been examined by cloning, expressing, and characterizing two putative components. Candidate coding sequences for a malonyl-CoAacetyl carrier protein transacylase (malonyltransferase) and its acyl carrier protein substrate, identified by BLAST searches of the human sequence data base, were located on nuclear chromosomes 22 and 16, respectively. The encoded proteins localized exclusively in mitochondria only when the putative N-terminal mitochondrial targeting sequences were present as revealed by confocal microscopy of HeLa cells infected with appropriate green fluorescent protein fusion constructs. The mature, processed forms of the mitochondrial proteins were expressed in S9 cells and purified, the acyl carrier protein was converted to the holoform in vitro using purified human phosphopantetheinyltransferase, and the functional interaction of the two proteins was studied. Compared with the dual specificity malonyl/ acetyltransferase component of the cytosolic type I fatty acid synthase, the type II mitochondrial counterpart exhibits a relatively narrow substrate specificity for both the acyl donor and acyl carrier protein acceptor. Thus, it forms a covalent acyl-enzyme complex only when incubated with malonyl-CoA and transfers exclusively malonyl moieties to the mitochondrial holocarboxyacyl carrier protein. The type II acyl carrier protein from Bacillus subtilis, but not the acyl carrier protein derived from the human cytosolic type I fatty acid synthase, can also function as an acceptor for the mitochondrial transferase. These data provide compelling evidence that human mitochondria contain a malonyl-CoAacetyl carrier protein-dependent fatty acid synthase system, distinct from the type I cytosolic fatty acid synthase, that resembles the type II system present in prokaryotes and plastids. The final products of this system, yet to be identified, may play an important role in mitochondrial function.

The existence of a mitochondrial system for the biosynthesis of fatty acids in animals was first reported 40 years ago and postulated to function by a reversal of the process of fatty acid β-oxidation (1–3). Indeed one laboratory reported that short and medium chain-length fatty acids could be produced using the partially purified mitochondrial β-oxidation enzymes, β-ke- toacyl thiolase, β-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase (4), and another found that inhibitory antibodies to pig heart 3-keotoacyl-CoA thiolase inhibited, in a parallel fashion, the fatty acid elongation system of pig heart mitochondria (5). The picture was complicated by reports that liver and heart mitochondria have two distinct fatty acid-synthesizing systems, one acetyl-CoA-dependent, possibly involving some of the β-oxidation enzymes, the other malonyl-CoA-dependent and apparently resembling the cytosolic FAS system. Most studies were in agreement that the acetyl-CoA-dependent system was an elongation pathway in which one or more C2 units, derived from acetyl-CoA, were added to preexisting saturated and unsaturated fatty acids of a broad range of chain length (C12–C16). In this pathway, at least one of the β-oxidation enzymes appears to be substituted by a specific biosynthetic enzyme; the FAD-dependent acetyl-CoA dehydrogenase being replaced by a more thermodynamically favorable enzyme, enoyl-CoA reductase (6).

The possible presence of a malonyl-CoA-dependent system capable of synthesizing saturated fatty acids C14–C16 de novo in mitochondria was hotly debated with some investigators suggesting that contamination with cytosolic FAS and/or components of a microsomal malonyl-CoA-dependent elongation system was very likely responsible for the widely varying activity of this pathway reported by different laboratories (7–10). Perhaps because of these early conflicting reports and inherent difficulties in working with this system, the animal mitochondrial FAS has received little attention over the last 25 years, and almost all of the recent advances in our knowledge have come from studies with fungi and plants.

The first notable advance was the discovery that mitochondria of both Neurospora crassa and Saccharomyces cerevisiae contain a small molecular mass phosphopantetheinylated protein that resembles closely the acyl carrier proteins (ACP) characteristically associated with the type II FAS systems of prokaryotes and plants (11, 12). Unexpectedly this mitochondrial system for the biosynthesis of fatty acids in animals was first reported 40 years ago and postulated to function by a reversal of the process of fatty acid β-oxidation (1–3). Indeed one laboratory reported that short and medium chain-length fatty acids could be produced using the partially purified mitochondrial β-oxidation enzymes, β-ke-toacyl thiolase, β-hydroxyacyl-CoA dehydratase, and enoyl-CoA reductase (4), and another found that inhibitory antibodies to pig heart 3-keotoacyl-CoA thiolase inhibited, in a parallel fashion, the fatty acid elongation system of pig heart mitochondria (5). The picture was complicated by reports that liver and heart mitochondria have two distinct fatty acid-synthesizing systems, one acetyl-CoA-dependent, possibly involving some of the β-oxidation enzymes, the other malonyl-CoA-dependent and apparently resembling the cytosolic FAS system. Most studies were in agreement that the acetyl-CoA-dependent system was an elongation pathway in which one or more C2 units, derived from acetyl-CoA, were added to preexisting saturated and unsaturated fatty acids of a broad range of chain length (C12–C16). In this pathway, at least one of the β-oxidation enzymes appears to be substituted by a specific biosynthetic enzyme; the FAD-dependent acetyl-CoA dehydrogenase being replaced by a more thermodynamically favorable enzyme, enoyl-CoA reductase (6).

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mitochondrial ACP was found to be associated with the NADH-ubiquinone reductase (proton-pumping respiratory complex 1) in N. crassa. This is not the case in S. cerevisiae, however, since this organism lacks complex 1 and respires using an alternative, non-proton-pumping, ubiquinone oxidoreductase. Disruption of the nuclear-encoded gene for ACPmit in both N. crassa and S. cerevisiae produced respiratory-deficient phenotypes (12, 13). In S. crassa, complex 1 was improperly assembled, and lysophospholipids accumulated in mitochondrial membranes, and in S. cerevisiae, mitochondrial ACP was reduced to less than 10% of that of the wild-type strain. Studies on plants have also revealed that mitochondria are capable of synthesizing both long chain acyl-ACPs and octanoyl-ACP, the direct precursor of that of the wild-type strain. Studies on plants have also revealed that mitochondria are capable of synthesizing both long chain acyl-ACPs and octanoyl-ACP, the direct precursor of lipic acid (14, 15). Meanwhile genes encoding several putative mitochondrial proteins, β-ketoacyl synthase (CEM1), β-ketoacyl reductase (OAR1), malonyl-CoA:ACP transacylase (MCT1), and enoyl reductase (YBR026c), that resemble type II FAS enzymes were identified in S. cerevisiae (16–18). Disruption of each of these genes produced a respiratory deficient phenotype.

In summary, fungal and plant mitochondria appear to contain an ACP-dependent lipogenic system that likely is responsible for the production of fatty acids that play essential roles in mitochondrial function. An ACP-like protein was found in animal mitochondria several years ago and shown to be a subunit of respiratory complex 1 (19, 20). However, the role of this ACP is not known, and no other components of a putative animal mitochondrial FAS system have been identified or characterized. In an attempt to clarify the role of this system, we have initiated a program to identify and characterize the components of a putative type II mitochondrial FAS in humans. In this communication we report the identification, cloning, and expression of a mitochondrial malonyltransferase and the characterization of its interaction with mitochondrial ACP.

**EXPERIMENTAL PROCEDURES**

**Prediction of Mitochondrially Imported Proteins**—The software programs PSORT (PSORT.nibb.ac.jp), iPSORT (hypothesiscreator.net/iPSORT), and MitoProt II (www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) were used to predict mitochondrially targeted proteins (21, 22).

**Cloning of the Human Mitochondrial Malonyltransferase**—Based on the available sequence information (GenBank™ accession number GI 85743683), PCR primers were designed to amplify the putative human mitochondrial malonyltransferase using expressed sequence tag cDNA clone ATCC 4811508 as the template DNA. The PCR procedure used was essentially as described earlier (23). Three sets of primers were designed to facilitate expression of the full-length mitochondrial MT (MTmit1), the putative N-terminally processed form lacking the first 21 residues (MTmit22), and an N-terminally truncated form lacking the first 59 residues that is of similar length to the MT of Escherichia coli (MTmit60). Thus, the primer sets MTbac.T/B in primer names indicates sense/antisense primer, respectively, and the numbers 1, 22, 60, and 67 correspond to the residue at which the encoded protein begins. Uppercase letters indicate the primer sequence matches cDNA sequence, while bases in lowercase are not sequence matches cDNA sequence, while bases in lowercase are not.

**Expression of the Human Mitochondrial Malonyltransferase in E. coli**—DNA fragments encoding MTmit22 and MTmit60 were cloned into the pET-29c(+)- vector, and expression was attempted in BL21-CodonPlus cells (Stratagene, La Jolla, CA). Cells were grown at 37°C in LB medium to an OD 600 of 0.5–0.6 and induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside for 5 h. However, in both cases, induction of protein expression resulted in cessation of cell growth within 1 h. The level of protein expression was very low, and almost all of the recombinant protein was recovered in inclusion bodies. Lowering the isopropyl-1-thio-β-d-galactopyranoside concentration, lowering the growth temperature, or using alternative host strains did not improve expression of soluble mitochondrial MT.

**Expression of the Human Mitochondrial Malonyltransferase**—The amplified constructs encoding MTmit1, MTmit22, and MTmit60 using QIAGEN Quick PCR purification Kit (Qiagen Inc., Valencia, CA), digested at XhoI and NotI sites that had been engineered into the amplification primes T and B, respectively, and cloned into the modified baculoviral transfer vector pFast Bac 1 (Invitrogen). To facilitate purification of the encoded proteins by metal ion affinity chromatography, an in-frame coding sequence for a C-terminal His6 tag was engineered into the cloned cDNAs. Authenticity of the cloned PCR fragments was confirmed by DNA sequencing, recombinant baculoviral stocks were generated, and the encoded protein was expressed in S/F insect cells using the Bac-to-Bac baculoviral expression system (Invitrogen) according to the manufacturer’s instructions. The amino acid sequences of the encoded proteins are shown in Supplemental Fig. 1. A mammalian intron was added to the C terminus of both His6 andFlag affinity tags at the C terminus was engineered by digesting the MTmit1,FB plasmid DNA with the restriction enzymes NheI and NotI and ligating the cognate the c. Hs_flag.T/B linker (Table I) in-frame with the coding sequence

**Subcellular Fractionation of S/F Cells Expressing the Full-length and Putative N-terminally Processed Forms of Human Mitochondrial Malonyltransferase**—S/F cells were infected with recombinant baculovirus encoding either the MTmit1, or MTmit22 proteins carrying only the His6 affinity tag, harvested by centrifugation at 600 × g for 5 min, washed with PBS, and resuspended at 4°C in 5 volumes of mitochondrial isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.35, 1 mM EDTA) containing protease inhibitors (5 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml aprotinin, and 1 mM pepstatin, 5 μg/ml antitrypsin). The cellular suspension was disrupted using a Teflon/glass homogenizer and centrifuged at 800 × g for 10 min at 4°C to remove cell debris. The supernatant was then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected and further centrifuged at 100,000 × g for 30 min at 4°C. The 100,000 × g supernatant (cytosol) was collected, and the pellet (microsomal fraction) was resuspended in isolation buffer to the same volume as that of the cytosolic fraction. The 10,000 × g pellet (mitochondrial fraction) was washed twice with isolation buffer and resuspended in isolation buffer in the same volume as cytosol.

**Purification of the Human Mitochondrial Malonyltransferase**—S/F cells were infected with baculoviruses encoding either MTmit22 or MTmit60. For purification of the cytosolic form, insect cells were homogenized at 4°C in buffer A (50 mM HEPES, pH 8, 300 mM NaCl, 10% glycerol) containing protease inhibitors and centrifuged at 140,000 × g for 60 min at 4°C. The supernatant was filtered (0.45 μm) and loaded
FIG. 1. N-terminal sequence elements direct the putative FASmit proteins into mitochondria. Constructs encoding FASmit-GFP fusion proteins, with and without the putative N-terminal mitochondrial targeting sequences, were expressed in HeLa cells, and the subcellular distribution of protein expression was compared with that of mitochondrial markers by confocal microscopy. Anti-cytochrome c antibodies followed by fluorescent secondary antibodies were used as the mitochondrial marker for experiments on MT targeting and pDsRed2-Mito, a mammalian expression vector encoding a fusion of Discosoma sp. red fluorescent protein with the mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase, was utilized for experiments on ACP targeting. A, localization of the expressed ACPmit-GFP, fusion proteins with (ACPmit-GFP) and without (ACPmit-GFP) the putative N-terminal targeting sequence, and the GFP alone control. B, localization of the expressed mitochondrial MT fusion proteins with (MTmit-GFP) and without (MTmit-GFP) the putative N-terminal targeting sequence and the GFP alone control.

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. The bound proteins were eluted with 250 mM imidazole in buffer A. For further purification of the doubly tagged MTmit fractions containing the enzyme were pooled and applied to a column containing 2 mL of anti-FLAG M2 agarose (Sigma), which was equilibrated with buffer A. The column was washed with 20 mL of buffer A, and the bound proteins were eluted with 1.5 mg/mL FLAG peptide in buffer A.

Cloning, Expression, Purification, and Phosphopantetheinylation of the Human Mitochondrial ACP—The putative ACPmit sequence (20) was identified and cloned from a human expressed sequence tag using the PCR. The coding sequence for the putative processed form lacking the mitochondrial targeting sequence (ACPmit) was cloned into a baculoviral vector that also encoded a C-terminal His6 tag, and the recombinant protein was expressed in insect cells harboring plasmids encoding C-terminally His6-tagged versions of ACP-A (M15 pQE60[acpA], Ref. 25) and PCP (M15 pQE70[TycC3PCP], Ref. 26) were obtained from Dr. Mohamed A. Marahiel at the Institut für Biochemie, Fachbereich Chemie, Philipps-Universität Marburg, Marburg, Germany. Cells were grown at 37 °C with 30 μM 3-choro-4-hydroxybenzoate (A27200) and 10 μM H9262-isoleucine for 3 days. The soluble components were incubated at 37 °C overnight. The holo-ACP-A or holo-PCP were separated from phosphopantetheinyltransferase by nickel ion affinity chromatography. Confirmation that at least 90% of the apoforms had been converted to the corresponding holoforms was obtained by incubation of a portion of the product with [1-14C]acetyl-CoA and human phosphopantetheinyltransferase and assaying the incorporation of radiolabel into the ACP (only residual apoform is radiolabeled by [1-14C]acetyl-phosphopantetheine transfer (24)).

Expression and Purification of the Malonyl/Acetyltransferase and Acyl Carrier Protein Domains of Mammalian FASs—A cDNA fragment encoding the ACP domain of the human cytosolic FAS (amino acid residues 2117–2205) was cloned into the expression vector pQE80L (Qiagen) and expressed as a soluble protein in E. coli. The recombinant ACP was purified to homogeneity by a combination of nickel ion affinity chromatography and gel filtration. Details are described elsewhere (24).

Verification That the Putative N-terminal Mitochondrial Recognition Elements Direct the Mitochondrial FAS Proteins into Mitochondria—A 1170-bp fragment encoding MTmit was amplified using PCR primer set MTmitGFP/T8 (Table I) and directionally cloned into the KpnI/BamHI sites of pEGFP-N3 vector (Clontech) to facilitate expression as the N-terminal partner of a MTmit/Green fluorescent protein (GFP) fusion protein. A second fusion protein construct (primer set MTmitGFP/T5), lacking the coding sequence for the N-terminal 21 amino acids (putative targeting sequence), was similarly engineered as a control. Authenticity of the cloned fragments was confirmed by DNA sequencing. Expression vectors encoding the fusion proteins were transfected into HeLa cells
using FuGENE 6 reagent (Roche Applied Science), and the cells were cultured for 60 h. Cells were then fixed with paraformaldehyde in PBS, treated with 50 mM NH₄Cl (in PBS), permeabilized with 0.1% Triton X-100 (in PBS), washed with PBS, and treated with bovine serum albumin blocking solution. The cells were then treated with the primary antibody (purified mouse anti-cytchrome c monoclonal antibody), washed with PBS, and treated with the fluorescent secondary antibody (Cy3-conjugated affinity-purified goat anti-mouse IgG, Jackson Immunoresearch Labs., West Grove, PA). Finally the washed cells were mounted in glycerol/PBS and examined for green and red fluorescence in a Sony confocal fluorescence microscope.

The ACPₘᵢᵗ-GFP fusion constructs were generated in a similar manner. The two ACP-specific PCR-amplified fragments (ACP-gfp/T/B and ACP-gfp/T/B) primer set products for constructs with and without the putative mitochondrial targeting sequence, respectively; Table I) were cloned into the EcoRI/KpnI sites of pEGFP-N3 vector, and authenticity of the cloned fragments was confirmed by DNA sequencing. Transfection into HeLa cells was carried out using FuGENE 6 reagent as above except that ACPₘᵢᵗ-GFP fusion constructs were cotransfected with plasmid pHsRed2-Mito (Clontech) for fluorescent labeling of mitochondria. Cells were cultured for 60 h, fixed with paraformaldehyde as above, and directly analyzed for green and red fluorescence by confocal microscopy.

Assay of Human Mitochondrial Malonyltransferase Activity—Typical assay systems contained 83 mM potassium phosphate buffer, pH 6.8, 20 μM 2-14C-malonyl-CoA, 10–20 μM holo-ACP, and 50 ng of MTₘᵢᵗ. Incubations were carried out at 20 °C for 2 min. Proteins were precipitated with acid, washed, and assayed for radioactivity. Blank reactions were performed without enzyme. Kinetic parameters were determined using EnzymeKinetics (Trinity Software); values represent the means ± S.D. for calculations using six different methods.

RESULTS

Identification of Sequences of the Putative Mitochondrial ACP and Malonyltransferase Proteins—BLAST searches of the eukaryotic sequence data bases, using as probe sequences malonyltransferases from either prokaryotic, type II, or eukaryotic type I FAS systems, identified likely malonyltransferase sequences with N-terminal extensions in several species (see Supplemental Fig. 1A). Sequences for putative malonyltransferases from human, rat, mouse, Drosophila, and Neurospora were predicted by several computational methods (PSORT, iPSORT, and MitoProt II) to have a high probability of containing N-terminal mitochondrial targeting sequences (see Supplemental Fig. 1A). In addition, all of these sequences contained three positionally conserved features that are characteristic of malonyl and malonylacetyltransferases: a Gly-Xaa-Ser-Xaa-Gly serine esterase active site motif, a conserved Arg residue that interacts with the 3-carboxylate of the malonyl substrate, and a conserved His residue that is required for activation of the Ser nucleophile (27–30). On the basis of this analysis, we identified expression sequence tag cDNA clones encoding the putative human mitochondrial MT protein for further study. A similar approach identified ACP sequences in animals, fungi, and plants that have a high probability of containing putative mitochondrial targeting sequences (see Supplemental Fig. 1B). The coding sequence for the putative mitochondrial MT is located on human chromosome 22 (22q13.31), and that for the mitochondrial ACP is located on chromosomes 16 (16p12.3) in the human genome sequence data base. The coding sequences for both proteins are interrupted by three introns.

Verification That the Putative N-terminal Mitochondrial Recognition Elements Direct the Mitochondrial FAS Proteins into Mitochondria—Vectors encoding mitochondrial ACP and MT sequences fused to the N terminus of GFP were engineered and expressed in HeLa cells. Confocal fluorescence microscopic analysis revealed that both the ACPₘᵢᵗ-GFP and MTₘᵢᵗ-GFP chimeras colocalized with mitochondrial markers when the putative N-terminal mitochondrial targeting sequence was present (Fig. 1, A and B, left column, ACPₘᵢᵗ-GFP and MTₘᵢᵗ-GFP, respectively). In contrast, GFP expressed throughout the cytoplasm when either the entire mitochondrial ACP or MT coding sequence or the putative mitochondrial targeting sequence was omitted from the cDNA constructs (Fig. 1, A and B, center and right columns, GFP, ACPₘᵢᵗ-GFP, and MTₘᵢᵗ-GFP, respectively). These results confirmed that the sequences identified do indeed encode bona fide mitochondrially targeted proteins.

Expression of Mitochondrial Malonyltransferase in S9 Cells: Subcellular Localization of Recombinant Protein—Because the mitochondrial MT could not be expressed as a soluble protein in E. coli (see “Experimental Procedures” for details), we explored the possibility of using the insect S9/baculoviral host/vector system. Cells infected with recombinant baculoviruses encoding either the full-length (MTₘᵢᵗ₁) or N-terminally truncated (MTₘᵢᵗ₂) forms of the His₆-tagged enzyme were homogenized, and mitochondrial, microsomal, and cytosolic fractions were prepared and analyzed by SDS-PAGE and Western blotting using anti-His antibodies as the primary antibodies. The total volume of each fraction was identical as was the volume of the portion applied to the 10% polyacrylamide-SDS gel. Lanes 1, 2, and 3, mitochondrial, microsomal, and cytosolic fractions, respectively; lane M, prestained standards with molecular masses in kDa shown on the left. The distribution of immunoreactive material in the mitochondrial, microsomal, and cytosolic fractions, estimated by densitometry, was 46, 40, and 14%, respectively, for MTₘᵢᵗ₁, and 87, 12.6, and 0.4%, respectively, for MTₘᵢᵗ₂.

![Fig. 2. Subcellular location of recombinant human malonyltransferase expressed in insect S9 cells.](image-url) Cells infected with baculoviruses encoding either the full-length (MTₘᵢᵗ₁) or N-terminally truncated (MTₘᵢᵗ₂) forms of the His₆-tagged enzyme were homogenized, and mitochondrial, microsomal, and cytosolic fractions were prepared and analyzed by SDS-PAGE and Western blotting using anti-His antibodies as the primary antibodies. The total volume of each fraction was identical as was the volume of the portion applied to the 10% polyacrylamide-SDS gel. Lanes 1, 2, and 3, mitochondrial, microsomal, and cytosolic fractions, respectively; lane M, prestained standards with molecular masses in kDa shown on the left. The distribution of immunoreactive material in the mitochondrial, microsomal, and cytosolic fractions, estimated by densitometry, was 46, 40, and 14%, respectively, for MTₘᵢᵗ₁, and 87, 12.6, and 0.4%, respectively, for MTₘᵢᵗ₂.
The electrophoretic mobility of this species was identical to that of the faster moving species found associated with the mitochondrial fraction in cells infected with baculovirus encoding the full-length protein. These results indicated that most of the full-length MTmit1 species was taken up and partially processed by insect cell mitochondria but that a small portion became associated with the microsomal fraction and remained unprocessed. More surprising was the observation that much of the recombinant protein produced by direct expression of the putative processed form (MTmit22) was associated with the mitochondrial and microsomal fractions. The proportion of MTmit22 recovered in the cytosolic fraction could not be increased by inclusion of non-ionic detergent in the homogenization medium.

**Purification of Mitochondrial Malonyltransferase**—Initial attempts to purify the His-tagged MTmit22 protein from the cytosolic fraction met with limited success. Although a partially purified preparation that exhibited malonyltransferase activity could be obtained by nickel ion affinity chromatography, the preparation was refractory to further purification by ion exchange chromatography or ammonium sulfate precipitation. A more extensively N-terminally truncated form, MTmit60, that corresponds closely in length to the E. coli engineered at the C terminus of MTmit22, and the doubly tagged MTmit22 was used for subsequent functional characterization of the enzyme.

**Expression, Purification, and Phosphopantetheinylation of the Human Mitochondrial ACP**—The His-tagged ACPmit expressed extremely poorly in E. coli but could be expressed at high levels in insect Sf9 cells and readily purified from the cytosol with yields of −0.5 mg/g wet weight of cells. The protein expressed as a mixture of apo- and holoforms but could be completely converted to the holoform by treatment with human phosphopantetheinyltransferase (24). The preparation is homogeneous as judged by SDS-PAGE and staining with Coomassie Brilliant Blue (Fig. 4).

**Acyl Donor Substrate Specificity of Mitochondrial Malonyltransferase**—Whereas the formation of a covalent acyl-enzyme intermediate was readily demonstrated using [2-14C]malonyl-CoA as the acyl donor, incubation with [1-14C]acyetyl-CoA did not result in radiolabeling of the enzyme (Fig. 3), suggesting that the mitochondrial MT was specific for malonyl moieties. This possibility was confirmed by examination of the reaction kinetics under steady state conditions (Fig. 5). At a fixed concentration of ACPmit22 (20 μM) an apparent K<sub>m</sub> of 3.5 μM was derived for malonyl-CoA, but no activity was observed with acetyl-CoA as acyl donor (Fig. 5A). Thus, the mitochondrial MT closely resembles typical type II MTs, as exemplified by that of E. coli (32), that exhibit marked specificity for malonyl moieties and is distinctly different from the transferase domains of cytosolic type I FASs that are equally active in the transfer of malonyl and acetyl moieties (29, 33, 34).

**Acyl Acceptor Substrate Specificity of Mitochondrial Malonyltransferase**—The ability of MTmit22 to transfer malonyl moieties to a variety of carrier proteins was examined under steady state conditions at a fixed concentration (20 μM) of malonyl-CoA. The natural substrate, the human ACPmit68, and the ACP-A from B. subtilis functioned equally well as acceptor substrates (Fig. 5B). However, the ACP domain derived from the human cytosolic FAS and the PCP domain derived from the non-ribosomal surfactin peptide synthase from B. brevis were extremely poor acceptors. By comparison, the malonyl/acyetyltransferase domain from cytosolic type I FASs exhibited a broader specificity and was active with ACPs from both type I (ACP<sub>mit</sub>) and type II (ACP<sub>mit</sub> and B. subtilis ACP-A) FAS systems (Fig. 5C).
DISCUSSION

This study represents the first successful isolation and functional characterization of components of an animal mitochondrial FAS system and reveals that the ACP species present in human mitochondria that has been shown to be associated with respiratory complex 1 interacts functionally in a very specific manner with a mitochondrial malonyltransferase. Both the ACP and malonyltransferase are encoded by nuclear genes, and their importation into mitochondria is absolutely dependent on the presence of an N-terminal targeting sequence as demonstrated by our confocal fluorescence microscopy studies with HeLa cells (Fig. 1). Nevertheless, when the putative N-terminally processed form of the malonyltransferase was directly overexpressed in insect Sf9 cells, most of the recombinant protein associated nonspecifically with the mitochondrial and microsomal fractions, and, in the course of purification, the malonyltransferase recovered from the cytosolic fraction also showed a tendency to adhere nonspecifically to various chromatographic media, significantly limiting recovery. In contrast, direct overexpression of the N-terminally processed form of the mitochondrial ACP yielded a predominantly cytosolic protein that was readily purified to homogeneity (Fig. 4). Although inspection of the amino acid sequence of the mitochondrial malonyltransferase does not reveal any clear evidence that it may be a membrane protein, in its natural environment it must interact functionally with the mitochondrial ACP, which is a subunit of the respiratory complex 1, itself an amphipathic protein with arms that extend into both the matrix and membrane compartments (35). Whether the observed properties of the mitochondrial malonyltransferase have any significance in terms of its physical location within the mitochondria is unclear at present.

The human mitochondrial transferase shows an absolute specificity for malonyl moieties and appears to recognize only ACPs of the type II group (Fig. 5). In contrast, the transferase domain of the cytosolic type I animal FAS has a dual donor-substrate specificity, transferring both acetyl and malonyl moieties with equal efficiency (29), and in vitro is capable of utilizing either type I or type II ACPs as acceptor substrate (Fig. 5). Thus, the human mitochondrial malonyltransferase exhibits functional properties that are closer to those of a prokaryotic or plastid type II enzyme than to the transferase component of the type I cytosolic FAS of the same species. This kinship with prokaryotic malonyltransferases is also clearly evident at the amino acid sequence level. Phylogenetic analysis of malonyltransferase sequences reveals that all mitochondrial forms, including those of fungi, protozoa, insects, and mammals, orig-
Fig. 6. Phylogenetic tree of malonyltransferases. Mitochondrial MTs are distinguished by dark highlighting, type I proteins are distinguished by light highlighting, and prokaryotic type II proteins lack highlighting. Subscripts indicate the following: mit, mitochondrial; fas, domain of cytosolic fatty acid synthase; pks, domain of a modular polyketide synthase. The sequence alignment and homology comparison was performed with MacVector 7.0, and the phylogenetic tree was generated by PAUP, version 4.0b10 (36).

inate from a single ancestral branch (Fig. 6). The functional and structural similarities between mitochondrial and prokaryotic malonyltransferases are consistent with the generally accepted hypothesis that mitochondria originated as free-living prokaryotes. Similar conclusions have been drawn from phylogenetic analysis of mitochondrial ACP sequences (16).

After 3 decades of uncertainty, we now have very strong evidence that there is a malonyl-CoA/ACP-dependent FAS system functional inside mammalian mitochondria in addition to the acetyl-CoA-dependent system. However, the source of malonyl-CoA as the substrate for the mitochondrial FAS system is unknown. Both the α- and β-isofoms of acetyl-CoA carboxylase that have been described in animals generate malonyl-CoA in the cytosol, although the β-isofom is associated with the outer mitochondrial membrane (37–39). The α-form generates malonyl-CoA for utilization by the cytosolic FAS, whereas the β-form is believed to provide malonyl-CoA primarily for the regulation of carnitine palmitoyltransferase I, which controls entry of fatty acids into the mitochondria for oxidation (40). Malonyl-CoA is also produced by the β-oxidation of odd chain-length dicarboxylic fatty acids, but this process is thought to be exclusively peroxisomal (41). There are no reports indicating that a malonyl-CoA-translocating system might be present on the outer mitochondrial membrane. An alternative way of generating malonyl-CoA intramitochondrially could be via the mitochondrial propionyl-CoA carboxylase, which has some activity toward acetyl-CoA (42); in this regard, we cannot formally exclude the possibility that methylmalonyl-CoA, formed by carboxylation of propionyl-CoA, is the actual chain extender substrate used by the mitochondrial FAS, although branched chain fatty acids are not normally found in mitochondrial lipids. Finally another possible mechanism for generating malonyl-CoA intramitochondrially could be via a malonyl-CoA synthetase (“malonate kinase”). Malonyl-CoA synthetases have been fairly well characterized in prokaryotes (43) and recently have been implicated in plant mitochondria in the formation of malonyl-CoA that is used for fatty acid synthesis (14). The presence of an active malonyl-CoA decarboxylase in mammalian mitochondria has always seemed paradoxical since the major pool of malonyl-CoA is thought to be extramitochondrial (44). However, in view of the evidence presented in this report, the possibility should now be considered that the mitochondrial malonyl-CoA decarboxylase may play a role in regulating the intramitochondrial malonyl-CoA pool that is utilized by the mitochondrial FAS system.

The exact role of the malonyl-CoA-dependent pathway in mitochondrial function is yet to be ascertained. Northern analysis indicates that both the human mitochondrial ACP (20) and the phosphopantetheinyltransferase responsible for its post-translational modification (24) are expressed in a broad range of tissues, and are particularly abundantly in heart and skeletal muscle, tissues that have a high content of very active mitochondria. One possible role may be in the biosynthesis of lipoic acid as has been proposed for the mitochondrial FAS system in fungi (16). Only very recently has it been demonstrated that the key enzyme of lipoic acid biosynthesis, lipoic acid synthase, an iron-sulfur protein, is present in mammalian cells and is, in fact, a mitochondrial enzyme (45). The mouse lipoic acid synthase can complement an E. coli lipA mutant defective in lipoic acid synthesis. A putative lipoic acid synthase can be found in the human sequence data base that is 92% identical in sequence to the authentic mouse lipoic acid synthase. It seems likely therefore that mammalian mitochondria synthesize lipoic acid essentially by the same mechanism as do plant mitochondria (14, 15) and E. coli (46) using a lipoic acid synthase to introduce two sulfur atoms into an octanoyl moiety attached in thioester linkage to a mitochondrial ACP. A second possible function of the animal mitochondrial FAS system could be in the biosynthesis of longer chain-length fatty acids that could be utilized for the remodeling of mitochondrial phospholipids. However, the possibility that the mitochondrial FAS system provides some other, as yet unidentified, fatty acid-like moiety for a specialized function cannot be ruled out. At the time of writing this report, a publication appeared documenting the discovery of a 2-enoyl thioster reductase in human mitochondria that could also be a player in a mitochondrial FAS system (47). We also have identified several DNA sequences that may encode other components of a mitochondrial type II FAS system, and we are presently engaged in their expression and characterization. These studies should provide the information and tools required to establish the role of the type II FAS in mitochondrial function.
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