Molecular Cloning of CoA Synthase
THE MISSING LINK IN CoA BIOSYNTHESIS*¶§

Molecular cloning of CoA synthase in cells and participates in the metabolism of fatty acids, carbohydrates, and amino acids (1–3). CoA is also involved in the regulation of several key reactions in intermediary metabolism. It is estimated that about 4% of all cellular enzymes utilize CoA or its thioster derivatives as substrates. The biosynthesis of CoA in mammalian cells occurs in five steps, which utilize pantothenate (vitamin B5), ATP, and cysteine (4). In the first step, pantothenic acid is phosphorylated to 4’-phosphopantethenic acid in a reaction mediated by pantothenate kinase. This is a rate-limiting step in CoA biosynthesis, and the activity of pantothenate kinase is strongly inhibited by coenzyme A and all of its acyl esters (5–7). The product of the first reaction is then converted to 4’-phosphopantethenoyl cysteine, which is subsequently decarboxylated to 4’-phosphopantotheine. The 4’-phosphopantethenoyl cysteine synthase and phosphopantethenoyl cysteine decarboxylase catalyze these two reactions, respectively. Another rate-limiting step in this biosynthetic pathway involves the conversion of 4’-PP to dPCoA by 4’-phosphopantotheine adenyltransferase. Dephospho-CoA kinase phosphorylates the 3’-hydroxyl group of the ribose ring of dPCoA in the final stage of CoA biosynthesis.

The tissue level of CoA is regulated by various extracellular stimuli, including hormones, nutrients, and cellular metabolites. It has been shown that insulin, glucose, fatty acids, pyruvate, and ketone bodies inhibit CoA biosynthesis, while glucocorticoids and glucagon, as well as drugs such as clofibrate, increase tissue concentration of CoA (8–12). Altered homeostasis of CoA has been observed in diverse disease states, such as diabetes, starvation, alcoholism, Reye syndrome, medium-chain acyl CoA dehydrogenase deficiency, vitamin B12 deficiency, hypertension, and certain types of tumors (13–19).

Enzymatic activities responsible for each step of CoA biosynthesis have been purified from various mammalian sources and characterized. However, these studies have not been extended into protein sequence analysis and cDNA cloning of enzymes involved in the pathway of CoA biogenesis. Recently, bioinformatic studies led to the identification and characterization of a gene and a cDNA coding for mammalian pantothenate kinase (20, 21). Here, we report molecular cloning of CoA synthase, which encodes a protein of 563 amino acids. Sequence alignments, mutational analysis, and biochemical characterization indicated that CoA synthase possesses two enzymatic domains, which mediate the last two steps in CoA biosynthesis: conversion of 4’-PP into dPCoA and subsequently into CoA.

EXPERIMENTAL PROCEDURES

Cell Cultures and Antibodies—HEK293 cell line was purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Anti-Myc (9E-10) monoclonal antibody was purchased from Santa Cruz. A His-tag fusion protein, containing the C-terminal domain of CoA synthase (His-dCoAK) was used to raise specific polyclonal antibodies. Immunoreactive sera were affinity-purified on an Actigel matrix containing His-dPCoA fusion protein.

Yeast Two-hybrid Screen—The DupLEX-A yeast two-hybrid system was used in this study (OrgGENE Technologies). The pE202/S6Kα "bait" construct was created by standard cloning techniques and used to screen a mouse embryo cDNA library. Autoactivation assay, testing for nuclear localization of the bait fusion protein, selection of positive

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clones, and mating assay were performed as recommended by the manufacturer.

Northern and Western Blot Analysis—A mouse tissue mRNA blot (a generous gift from Dr. V. Buchman) was hybridized with an 850-bp cDNA fragment of CoAsy. The probe was labeled using the Readiprime™II random prime labeling kit from Amersham Biosciences. Chicken/H9252-actin cDNA probe was used as a control.

Homogenates of adult rat tissues and cell lines were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, and a mixture of protease inhibitors (Roche Molecular Diagnostics). A portion of total lysate (40 μg/H9262) from each tissue and cell line was separated by SDS-PAGE and immunobotted with affinity-purified anti-CoAsy antibodies.

Plasmid Construction and Expression Studies—Full-length CoAsy was amplified by PCR and cloned into pcDNA3.1 vector (Invitrogen) in frame with the N-terminal Myc-tag epitope. The dPCoAK domain was cloned into pET23d plasmid (Novagen) in-frame with His-tag sequences, located at the N terminus. Expression and affinity purification of His-dPCoAK was carried out in BL21 DE3 cells and on NTA-agarose, respectively. Transient transfection of HEK293 cells was performed using Polyfect under conditions recommended by the manufacturer (Qiagen). Immunoprecipitation assays and Western blot analysis were carried out as described previously (22).

QuikChange site-directed mutagenesis kit (Stratagene) was used to generate a point mutation in the PPAT domain (His 203 to Ala). All constructs were verified by DNA sequencing.

Analysis of CoA Synthase Activities—The PPAT activity of CoAsy was measured as described below. The immunoprecipitates, containing Myc-CoAsy were mixed with 0.2 mM 4'-PP, 0.25 mM ATP, 0.5 μCi of [γ-32P]ATP, in BB1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1.5 mM DTT). The reaction mixture was incubated at 25 °C for 30 min. Reaction products were separated by descending paper chromatography using Whatman 3MM paper and developing system containing isobutyric acid:0.5N ammonium hydroxide (100:60) and 1 mM EDTA (23). A phosphoimager system (Bio-Rad) was used to identify the position of radiolabeled products.

Dephospho-CoA kinase activity of immune complexes or recombinant His-dPCoAK was assayed at 25 °C for 30 min in buffer containing 150 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1.5 mM DTT, 0.2 mM dPCoA, 0.25 mM ATP, 0.5 μCi of [γ-32P]ATP. The reaction products were separated and analyzed in the same way as described above for the PPAT assay. Both PPAT and dPCoAK activities were also measured spectrophotometrically using enzymatic systems as described previously (24).

RESULTS

Using the full-length coding sequence of ribosomal S6 kinase αII (S6KαII) as bait in a yeast two-hybrid screen of a mouse embryo cDNA library, we isolated 16 positive clones, which were confirmed by mating assay. Restriction analysis and DNA sequencing revealed that 13 clones contained the same insert.

One of these cDNA clones, designated Ukr1, was fully sequenced and found to have an open reading frame that translated into a protein of 563 amino acids (Fig. 1). The deduced amino acid sequence has a calculated molecular weight of 62,023. Sequence alignments indicated that the central region of Ukr1 shows high homology to phosphopantetheine adenyl-
transfersases, which belong to the superfamily of cytidylyltransferases (Supplementary data, Fig. 1A). The C-terminal region is highly homologous to the catalytic domain of dephospho-CoA kinases (Supplementary data, Fig. 1B). The N terminus of Ukr1 does not exhibit signatures of any known domains or motifs. The presence of PPAT and dPCoAK domains strongly indicated that Ukr1 cDNA might encode a protein involved in the biosynthesis of CoA. This was an interesting observation, as there is a previous report describing purification of an enzyme from pig liver possessing both PPAT and dPCoAK activities (25). This bi-functional enzyme with a molecular mass of ~57 kDa was termed CoA synthase, as it had the potential to mediate the final stages of CoA biosynthesis. As the protein was never sequenced and the gene was not identified, the hypothesis of a bifunctional CoA synthase was never formally proven. Based on the observation that Ukr1 contained domains predicted to have both PPAT and dPCoAK activities combined with the fact that the Ukr1 gene product has a predicted molecular mass of 62 kDa, we hypothesized that Ukr1 cDNA encoded CoA synthase.

Bioinformatic analysis revealed that homologues of mouse Ukr1/CoAsy are present in Homo sapiens, Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, and Saccharomyces cerevisiae and exhibit 87, 33, 28, 29, and 23% homology at the protein level, respectively (Fig. 1A). To our knowledge, none of the gene products of these data base entries have been biochemically characterized nor shown to possess PPAT and/or dPCoAK activities. It is important to note that in prokaryotes and lower eukaryotes PPAT and dPCoAK activities reside on different proteins (Fig. 1B). The merger of both enzymatic activities in higher eukaryotes suggests the existence of a novel, more recently evolved mechanism in the regulation of CoA biosynthesis. Moreover, the N-terminal extension is only present in eukaryotes and might contain some regulatory sequences. Interestingly, the C. elegans homologue contains a highly hydrophobic insert in the middle of dPCoAK domain and a hydrophobic C-terminal extension.

The expression level of CoAsy in tissues and cell lines was examined by Northern and Western blot analysis. Probing of total RNA from mouse tissues with a radioactively labeled transcript of chicken colon, lung, intestine, and spleen showed the lowest levels of expression. Levels were found in kidney and liver, whereas two minor bands at around 1.7 and 2 kb (Fig. 2A). The fragment of CoAsy revealed a major transcript of 2.3 kb and total RNA from mouse tissues with a radioactively labeled probe was detected by descending paper chromatography. The C-terminal region of CoAsy containing the dPCoAK domain was expressed in bacteria as His-tag fusion protein (His-dPCoAK). Recombinant protein was purified by affinity chromatography on NTA-agarose and used to generate polyclonal antibodies. Affinity-purified antibodies specifically recognized His-dPCoAK and the full-length Myc-CoAsy in Western blotting. Immunoblot analysis of CoAsy expression in mouse tissues (Fig. 2B) and various cell lines (C). The antigen-antibody complexes were detected using enhanced chemiluminescence and fluoroimaging system (Bio-Rad).

To analyze expression of CoAsy at the protein level, the C-terminal region of CoAsy containing the dPCoAK domain was expressed in bacteria as His-tag fusion protein (His-dPCoAK). Recombinant protein was purified by affinity chromatography on NTA-agarose and used to generate polyclonal antibodies. Affinity-purified antibodies specifically recognized His-dPCoAK and the full-length Myc-CoAsy in Western blotting and immunoprecipitation experiments (Supplementary data, Fig. 2). When rat tissue lysates were immunoblotted with anti-CoAsy antibody, a major immunoreactive band was observed at around 60 kDa, which was slightly lower than recombinant Myc-CoAsy expressed in HEK293 cells. CoAsy expression is high in kidney and liver, whereas ovaries and lung express low levels of the protein (Fig. 2B). There is a good correlation between CoAsy tissue expression patterns at the RNA and protein levels. Immunoblotting also revealed that CoAsy was present in a wide range of cell lines including 3T3-L1 adipocytes and J774.4 macrophages (Fig. 2C). Therefore, we conclude that CoAsy is a widely distributed enzyme, but particularly significant levels of expression are observed in tissues exhibiting elevated lipid metabolism such as heart, liver, and fat.

To determine whether the CoAsy cDNA encodes a protein possessing the predicted enzymatic activities, Myc-tagged CoAsy was expressed in HEK293 cells and recombinant protein immunoprecipitated with anti-Myc antibody. Immune complexes were divided and assayed for PPAT and dPCoAK activities as described under "Experimental Procedures." When dPCoA was used as a substrate, the appearance of radioactively labeled CoA was detected by descending paper chromatography (Fig. 3A). Bacterial preparations of His-dPCoAK domain were also tested in this assay and found to possess dPCoAK activity (Fig. 3A). To test the presence of PPAT activ-
Vitamin B₅ is a key component in the biosynthesis of CoA. It is readily available from diverse dietary sources, a fact that is underscored by the difficulty encountered in attempting to induce pantothenate deficiency. Vitamin B₅ deficiency has not been linked with any particular disease, but results in generalized malaise clinically. It has been demonstrated that tissue CoA levels are not significantly altered in pantothenate deficiency, suggesting that cells are equipped to preserve their pantothenate content, possibly by a recycling mechanism for utilizing pantothenate obtained from the degradation of pantothenate-containing molecules.

Biosynthesis of CoA is a universal pathway, conserved from prokaryotes to mammals. Multistep biosynthesis of CoA and the enzymes involved in this process are shown in Fig. 3B. This study reports the molecular cloning and biochemical characterization of mammalian CoA synthase, previously a gap in the genetics of the mammalian CoA biosynthetic pathway. The presence of PPAT and dPCoAK domains in the predicted amino acid sequence of murine CoAsy strongly suggested its involvement in the biosynthesis of CoA. Expression studies in HEK293 cells provided evidence that recombinant CoAsy possesses both PPAT and dPCoAK activities. These findings were further supported by mutational studies and biochemical analysis of bacterially expressed dPCoAK domain. Interestingly, D. melanogaster and C. elegans have homologues of mammalian CoAsy, while in lower eukaryotes, such as S. cerevisiae and bacteria, the PPAT and dPCoAK activities reside on different proteins. These differences suggest the existence of distinct modes of regulation of CoA biosynthesis. Indeed, the CoA-synthesizing complex in S. cerevisiae was found to have a molecular mass around 400 kDa, while in higher eukaryotes, the existence of such a complex has not been reported (23). The compartmentalization of the CoA biosynthetic pathway is poorly understood in mammals. Since mitochondria and peroxisomes contain the greatest concentrations of CoA, it has been proposed that the last enzymes in the pathway are located inside these compartments (28). Others report that mitochondria can transport CoA into the matrix, implying that all CoA synthesizing enzymes are cytosolic proteins (29). Analysis of subcellular localization of CoAsy supports the latter hypothesis, as CoAsy is located predominately in the cytoplasm.

Metabolic labeling experiments have revealed that both pantothenate and 4'-PP accumulate in the cell, suggesting that pantothenate kinase and PPAT catalyze rate-limiting steps in the pathway (30). Therefore, it is apparent that the PPAT activity of CoAsy might be regulated in cellular responses to extracellular stimuli or environmental changes. The availability of the molecular reagents that have evolved from this study will allow us to study regulatory mechanisms governing the final stages of CoA biosynthesis.

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