Cloning and expression of rat pancreatic β -cell malonyl-CoA decarboxylase

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To gain insight into the function and regulation of malonyl-CoA decarboxylase (MCD) we have cloned rat MCD cDNA from a differentiated insulin-secreting pancreatic β -cell-line cDNA library. The full-length cDNA sequence shows 69% identity with the cDNA cloned previously from the goose uropygial gland, and predicts a 492 amino acid protein of 54.7 kDa. The open reading frame contains an N-terminal mitochondrial targeting sequence and the C-terminal part of the enzyme ends with a peroxisomal (Ser-Lys-Leu) targeting motif. Since the sequence does not reveal hydrophobic domains, MCD is most likely expressed in the mitochondrial matrix and inside the peroxisomes. A second methionine residue, located 3' of the mitochondrial presequence, might be the first amino acid of a putative cytosolic MCD, since the nucleotide sequence around it fits fairly well with a consensus Kozak site for translation initiation. However,

primer extension detects the presence of only one transcript initiating upstream of the first ATG, indicating that the major, if not exclusive, transcript expressed in the pancreatic β -cell encodes MCD with its mitochondrial presequence. The sequence also shows multiple possible sites of phosphorylation by casein kinase II and protein kinase C. mRNA tissue-distribution analysis indicates a transcript of 2.2 kb, and that the MCD gene is expressed over a wide range of rat tissues. The distribution of the enzyme shows a broad range of activities from very low in the brain to elevated in the liver and heart. The results provide the foundations for further studies of the role of MCD in lipid metabolism and metabolic signalling in various tissues.

Key words: fatty acid oxidation, insulin secretion, malonyl-CoA, mitochondrial presequence, peroxisomes.

INTRODUCTION

Malonyl-CoA has three established functions in animal cells; it is the carbon precursor used by fatty acid synthase to form palmitate, it is required for fatty-acid elongation in the endoplasmic reticulum and it controls the mitochondrial oxidation of fatty acids through its interaction with carnitine palmitoyl-transferase I (CPT-I) [1,2]. This metabolic intermediate may be considered as an intracellular signal of nutrient abundance, which regulates fuel partitioning in various tissues [3,4]. The malonyl-CoA–CPT-I interaction is a central element of a 'fuel cross-talk' signalling network in several organs including the heart [5], skeletal muscle [6] and liver [7], and may be implicated in the short and/or long term control of insulin secretion in the pancreatic β -cell [8,9]. The intracellular concentration of malonyl-CoA in lipogenic tissues, such as liver and adipose tissue is thought to result from its rate of formation by acetyl-CoA carboxylase and its utilization by fatty acid synthase (FAS). However, other tissues like the heart [10] and pancreatic islet [11] express FAS at a very low level, yet show rapid (within minutes) variations in malonyl-CoA under a number of experimental conditions which increase or decrease its concentration. The fate of malonyl-CoA in the β -cell and the heart is unclear and it is attractive to believe that the intracellular concentration of this metabolic signalling molecule is controlled by an additional enzyme, at least in non-lipogenic tissues expressing low FAS.

A prime candidate for such function is malonyl-CoA decarboxylase (MCD). Little is known about the role and regulation of this enzyme in mammalian cells. Early studies

showed that the enzyme is located mainly in the mitochondrial matrix in various tissues including liver, mammary gland and brain [12–15]. Its precise role in this intracellular compartment is uncertain. It has been hypothesized that MCD protects key mitochondrial matrix enzymes, such as pyruvate carboxylase, from inhibition by malonyl-CoA generated by propionyl-CoA carboxylase, which can use acetyl-CoA as a false substrate at a low rate [15]. MCD deficiency in children is a rare metabolic disorder which results in metabolic acidosis, hypoglycemia and abnormal excretion of malonate in the urine [15–19]. Whether some MCD in mammalian cells is expressed in the cytosol and/or, like CPT-I [1], in the outer mitochondrial membrane to regulate cytosolic malonyl-CoA concentration and fuel partitioning, is not known. Nonetheless, MCD accumulates in the cytoplasm of the uropygial gland of the goose that synthesizes special wax-type lipids [20]. The function of MCD in this particular organ is to ensure that methylmalonyl-CoA is the only substrate for FAS, so that methyl-branched fatty acids are generated in the absence of malonyl-CoA [20]. The cDNAs from the goose cytosolic (uropygial gland) and mitochondrial (liver) enzymes have been cloned [21,22]. The data indicated that the two MCD enzymes are encoded by a single gene and that the use of two promoters allowed synthesis of a longer form containing a mitochondrial targeting presequence in the liver [22].

As a first step to a better understanding of the control of intracellular malonyl-CoA levels and the role of MCD in mammalian cells and, in particular, in the β -cell, where malonyl-CoA may act as a signalling molecule [4], we have cloned the rat MCD cDNA from an insulin-secreting pancreatic β -cell line

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Abbreviations used: MCD, malonyl-CoA decarboxylase; INS-I, insulin-secreting pancreatic β -cell line; CPT-I, carnitine palmitoyltransferase I; FAS, fatty acid synthase; RT, reverse transcriptase.

(INS-1). In this study we report the sequence of rat MCD and its expression in a number of tissues. The role of the mitochondrial form of MCD is discussed in relation to the control of intracellular malonyl-CoA concentration.

EXPERIMENTAL

Probe synthesis, library screening and DNA sequencing

Reverse transcriptase (RT)-PCR was made on total mRNA from INS-1 with the Superscript RT and *Taq* polymerase (GIBCO), according to the supplier's instructions. The annealing temperature was 55 °C. For PCR, degenerated oligonucleotides were made from the goose MCD sequence [21]. The sense primer, positioned at base 547 of the goose sequence, was 5'-GANTS-TGARGCTGTGCAYCCTGT3'; the antisense primer, starting at base 1102, was 5'-TACARRTACCAGGCRCACARYCTC-AT3'. The 580 bp fragment obtained was subcloned into pBluescript (Stratagene), sequenced, digested with restriction enzyme and purified and was used as a probe.

An INS-1 cDNA library [23], constructed in Lambda ZAP Express (Stratagene), was screened using the purified 580 bp PCR fragment labelled with [α -³²P]dCTP by random priming. After two rounds of screening, four clones were excised *in vivo* and assayed for MCD activity in human kidney 293 cells 24 h after DNA transfection (5 μ g/10⁶ cells) using the calcium phosphate precipitate technique [24]. The clone bearing the highest enzymic activity was sequenced in both directions using the deletion technique (Erase-a-Base; Promega) and the dye-primer sequencing strategy (Autoread Sequencing Kit and the ALF DNA sequencer; Pharmacia). The size of this cDNA was 2020 bp. An additional full-size positive clone of 2062 bp was sequenced to confirm the sequence obtained from the clone with the highest MCD activity in 293 cells after transfection. DNA transfection of the β -galactosidase gene, under the control of the cytomegalovirus promoter, served as a negative control for MCD activity and as positive control for the efficiency of transfection (about 20%). INS-1 [25] and human kidney 293 cells [26] were cultured as described previously.

mRNA measurements

Northern-blot analysis was carried out using standard techniques for the gel preparation and transfer [24]. Briefly, 20 μ g of total RNA per lane were separated on a 1% (w/v) agarose gel in the presence of formaldehyde, transferred by capillary attraction on to a Zeta probe QT membrane (Bio-Rad), fixed with UV light, prehybridized, hybridized in the presence of the labelled 580 bp MCD cDNA fragment described above and washed according to the supplier's protocol. The signals were revealed with a Fuji PhosphorImager. A control hybridization with a 18 S rRNA precursor probe (0.77 kb *EcoRI*-*Bam*HI fragment, position 1-1780, of mouse 18 S rRNA cDNA subcloned in pUC830) was carried out on the same membrane to confirm the ethidium-bromide staining of the RNA. RT-PCR was performed using total RNA obtained from rat tissues by the guanidinium isothiocyanate acidic phenol technique [27]. Reverse transcription was done with 5 μ g RNA as described above. cDNAs from sorted pancreatic α - and β -cells (kindly provided by Dr Frans Schuit, Vrije University, Brussels, Belgium) were prepared as described previously [28]. PCR reactions were performed with one third of the RT reaction, using the same degenerate primers and conditions described above. The conditions were compared with PCR made on the same material with rat β -actin oligonucleotides primers [28]. Reactions were monitored after 15, 25 and 35 cycles

and analysed on 1% (w/v) agarose gels stained with ethidium bromide.

Assay of MCD activity

MCD activity was measured according to a protocol described by Kudo et al. [5]. Briefly, MCD activity was measured in the supernatants (140000 *g* for 10 min) of extracts of transfected human kidney 293 cells after cell sonication, and various Wistar rat tissues obtained after tissue homogenization (Polytron) in ice-cold buffer (10 mM Hepes, pH 7.4/75 mM KCl/20 mM sucrose/1 mM EGTA/0.5 mM dithiothreitol containing protease inhibitors [5]). Extracts (approx. 50 μ g of protein) were incubated for 10 min in the presence of 1 mM malonyl-CoA. The reactions were then stopped with perchloric acid and the precipitated proteins were sedimented by centrifugation. The conversion of the acetyl-CoA formed by MCD to [¹⁴C]citrate was obtained by a coupled enzymic reactions, where [¹⁴C]aspartate (203 mCi/mmol; Amersham) was first transformed to [¹⁴C]oxaloacetate with glutamic-oxaloacetic transaminase and then the [¹⁴C]oxaloacetate reacted with acetyl-CoA to form [¹⁴C]citrate in the presence of citrate synthase. In the final step of the process, unreacted [¹⁴C]aspartate and [¹⁴C]citrate were separated by stirring the solution into a 1:2 (w/v) suspension of Dowex 50W-8X 9 (100-200 mesh) and centrifugation at 400 *g* for 10 min. The unreacted [¹⁴C]aspartate was contained within the pellet; the radioactivity in supernatant, which contained the [¹⁴C]citrate, was estimated by scintillation spectroscopy. MCD activity, expressed as nmol of acetyl-CoA formed/min per mg protein, was quantified by comparison with acetyl-CoA standards.

RESULTS

The cloning of the rat INS-1 cDNA was performed using a two-step approach with degenerated oligonucleotides. One of the selected pairs (see Experimental) tested on INS-1 mRNA generated a single product of 580 bp, as predicted by comparison with the goose cDNA. Sequencing of this fragment indicated 67% identity with the goose nucleotide sequence and no match was found with any other sequence available on the BLAST server [29]. The chosen fragment was then used as a probe to screen an INS-1 cDNA library. Among the four clones excised *in vivo*, one of 2020 bp had high MCD activity (6.9 nmol acetyl-CoA/min per mg of protein) after transfection into human kidney 293 cells. For reasons which were not clear, another clone of 2062 bp, although full length, had much lower activity (0.4 nmol acetyl-CoA/min per mg of protein). Human kidney 293 cell did not express the enzyme at a detectable level, either under non-transfected conditions or following transfection with the β -galactosidase gene. Sequencing of both clones revealed an open reading frame of 1476 bases corresponding to an amino acid sequence of 492 residues and a predicted protein of 54.7 kDa (Figure 1). A poly(A)-tail with a polyadenylation signal at the end of the 3' non-coding region was also present. Previous studies with MCD enzyme(s) purified from various mammalian tissues gave a molecular mass of approx. 170 kDa [14], suggesting that the enzyme might multimerize *in vivo*. The deduced rat protein sequence of MCD showed 69% identity with the goose sequence from amino acid 39, a methionine residue, to the end of the sequence (Figures 1 and 2). The nucleotide sequence surrounding that encoding this methionine residue (AGCGCCATGG) reasonably fits a Kozak consensus site (GCCA/GCCATGG) [30], suggesting that it may be a site of translation initiation. A sequence of 38 amino acids, in the same nucleotide reading frame, was present on the N-terminal side of this methionine residue. The first four amino acids of this

goose MCD 1 MRGLRRGLSLRISPRLGWAVPRSLRRLRRLAAGPWVQSASGVSVERGCAS
rat MCD 1 MRGL-----SPSL--RA--RRLL--PLVYPPRPSPGPRPLSGRLTASA

goose MCD 51 MEELRLRSLVPLLEDTETKELKAPDPRRSRLRRLRRLVLRAGSRRLTGA
rat MCD 39 MEELRLRSLVPLLEDTETKELKAPDPRRSRLRRLRRLVLRAGSRRLTGA

goose MCD 101 CLARDFGADGQVAVFSAKVLQRAERLRRLQAGLLQAEDRRVYVLTTRYNA
rat MCD 89 CLARDFGQVHDGQVAVFSAKVLQRAERLRRLQAGLLQAEDRRVYVLTTRYNA

goose MCD 151 LFQRLRLDGGGRFLVQLRDLVEGLAARAVDGPVHKMSGVLKNLSEW
rat MCD 139 LFHRLISKLDGGGRFLVQLRDLVEGLAARAVDGPVHKMSGVLKNLSEW

goose MCD 201 FSDGFLNLERVTVSPCEVLQKISDVAHVPRNWNMDLRRRVGPYRRCYF
rat MCD 169 FSDGFLNLERVTVSPCEVLQKISDVAHVPRNWNMDLRRRVGPYRRCYF

goose MCD 251 FSHCACTCEPLILRLVALTDISSSIOSIVKDVESLTDKRNTRAAVEY
rat MCD 239 FSHCACTCEPLILRLVALTDISNNIOSIVKECCPFSITEKRNTRAAVEY

goose MCD 301 SISCDAQQGGVGLGNHLIKRVKELQKDLPIIAFSSLSPIPGFTKWLVL
rat MCD 289 SISLTQQQGGVGLGTFPIKRVVKEKQKFFPIILGAFSSLSPIPGFTKWLVL

goose MCD 351 GLSSSLDKELKSNLEFFESRRQEISITEDSTTSLKLLKLTNSWEVKSEK
rat MCD 339 GLGNLVGKELKSNLEFFESRRQEIATVETDPVHSLGLSSGSEKSEKSEK

goose MCD 401 LVRRLHSLPLMRICAWYLYGKEKRGYALNPVANFHLQNGAELWRINWMDST
rat MCD 389 LQAQLQGLPLMRICAWYLYGKEKRGYALNPVANFHLQNGAYWRINWMDST

goose MCD 451 SPRGLIASCGWVNYRYPLEDTASNSAAVLGKHKHKASEQVLSVVSQFQ
rat MCD 438 SPRGLISCGLVNRYRILEDTGPNVISLGLSKGKASEQVLSVVAQFS

goose MCD 501 NSKL
rat MCD 488 NSKL

Identical residues are shown by white-on-black type

Tissue distribution of MCD mRNA was investigated by Northern-blot analysis and complemented by a semi-quantitative RT-PCR study (Figure 3). An mRNA of about 2.2 kb was present in all rat tissues examined, and was expressed at relatively high levels in liver, kidney, heart and adipose tissues. The diffuse signal obtained was because the MCD transcript co-migrated with the 18 S rRNA. To confirm this ubiquitous distribution,

The ATG, which may serve as a translation start site for a putative cytosolic MCD, is framed. The mitochondrial presequence is underlined at the beginning of the sequence and the positive arginine residues are denoted with black bullets. The Ser-Lys-Leu (SKL) peroxisomal-targeting sequence at the C-terminus is underlined. Two possible polyadenylation signals (AACAAA or ATTAA) are present in the 5' non-coding region. The putative phosphorylation site residues are enclosed in squares for protein kinase C and in circles for casein kinase II.

sequence (Met-Arg-Gly-Leu), which starts with a methionine residue, were identical with that of the goose sequence; however, the remaining part of the sequence, from amino acids 5–38, showed relatively little similarity with the goose sequence. This portion of the rat MCD protein had the features of a mitochondrial targeting sequence [31,32], since it was rich in positively charged (eight arginine residues) and hydroxylated amino acids, and lacked acidic amino acids. Therefore it is possibly a cleavable.

Figure 3 Northern-blot and RT-PCR analysis of MCD mRNA in various tissues

(A) Northern-blot analysis of rat tissues with a rat MCD probe and an 18 S rRNA cDNA fragment, used as a control to determine RNA loading on to the gel. (B) Ethidium bromide stained agarose gels of RT-PCR experiments carried out with MCD (top and bottom) and β -actin (middle) primers using total RNA isolated from various rat tissues and sorted pancreatic islet cells.

RT-PCR was performed on the rat tissues and on sorted pancreatic α - and β -cells. After 25 cycles of PCR, the relative signals among tissues were similar to those obtained at 35 cycles (Figure 3B), except that the band intensities were weaker. A β -actin control PCR on the same samples allowed comparisons to be made. A control PCR of non-reverse transcribed material gave no signal (results not shown), thus excluding genomic DNA contamination of the RNA preparations. Thus the RT-PCR results confirmed the Northern-blot analysis and, furthermore, indicated that MCD mRNA was expressed in the α - and β -cells of pancreatic islet tissue to an extent similar to that found in INS-1.

MCD activity was measured in tissue extracts and the results, presented in Figure 4, show a broad range of activities from high levels in liver, heart and INS-1 to low activity in the brain and spleen. MCD activity was undetectable in the duodenum, non-transfected human kidney 293 cells and 293 cells transfected with Rous sarcoma virus- β -galactosidase. The tissue distribution of MCD mRNA (Figure 3) did not correlate with enzymic activity measurements (Figure 4). This discrepancy may be explained by differences among tissues in the translational control of the MCD gene. Another possibility to consider is that MCD is an allosteric enzyme regulated by covalent modification(s), and that

Figure 4 MCD activity in various tissues

MCD activity was determined in total cell extracts of various rat tissues, INS-1 and in human kidney 293 cells, either non-transfected or transfected with MCD or β -galactosidase (β -gal) cDNAs. nd, not detectable. The results are the means $\pm$ S.E. of four independent experiments.

the enzyme is differentially phosphorylated in various tissues. Consistent with this view, the rat MCD sequence shows many potential phosphorylation sites and alkaline phosphatase treatment of heart tissue extracts before measuring the activity resulted in higher MCD activity [37].

DISCUSSION

The deduced MCD amino acid sequence, from amino acid 39 (following the mitochondrial presequence) to the stop codon, showed 69% identity with that of the goose uropygial gland enzyme, indicating that this gene is well conserved between birds and mammals. As shown by its N-terminal sequence, rat INS-1 MCD is possibly a mitochondrial matrix protein. It contained a typical presequence which allows the precursor to enter the mitochondrial matrix where it is removed by proteolysis, likely at position 31 which contained a PRLCSG cleavage signal. Interestingly, MCD also possessed a peroxisomal targeting sequence. This second, potential site would not conflict with the general view of MCD as a mitochondrial enzyme. Thus experiments to discover the subcellular distribution of the enzyme to date [13,14] did not distinguish between peroxisomal and mitochondrial fractions, as this requires high resolution fractionation procedures [38]. This dual localization is of interest as both organelles are implicated in fatty-acid metabolism and contain malonyl-CoA-sensitive carnitine acyl-transferases [1,39,40]. However, the cloned mitochondrial CPT-I enzymes, unlike MCD, do not possess a peroxisomal targeting sequence [1].

The role of mammalian MCD in the mitochondrial matrix is unclear. Previous studies have suggested a role in detoxification by preventing malonyl-CoA accumulation catalysed by propionyl-CoA carboxylase, which can use acetyl-CoA as a pseudo-substrate [15]. Since malonyl-CoA is rapidly formed and degraded in tissues such as the heart or the pancreatic β -cell, which express very low FAS activity [5,11], the enzyme which

removes malonyl-CoA by using it as a substrate for lipogenesis. Thus it cannot be excluded that mitochondrial MCD regulates cytosolic malonyl-CoA concentration. The problem which arises is that malonyl-CoA is impermeable to mitochondria and a malonyl-carnitine shuttle has not yet been described. Indirect support for the idea that mammalian MCD regulates cytosolic malonyl-CoA and lipid partitioning can be found in that MCD, like CPT-I, appears to be expressed in the two cellular compartments where fatty acid oxidation occurs, i.e. mitochondria and peroxisomes.

With respect to the role of MCD in the pancreatic β -cell, if malonyl-CoA is a metabolic coupling factor in the endocrine pancreas [4], MCD might act as an 'off' signal for insulin release. However, it should be stated that the role of malonyl-CoA in the acute regulation of insulin secretion by glucose is presently debated, since gene-transfer experiments with acetyl-CoA carboxylase [41] and goose MCD [42] in INS-1 have respectively provided supportive and negative evidence for this hypothesis. However, the latter study, which aimed at over-expressing MCD in the cytosol, was carried out with recombinant adenoviruses in which the goose MCD cDNA was devoid of its mitochondrial presequence but still contained the Ser-Lys-Leu peroxisomal targeting motif. Thus it cannot be discounted that the enzyme was overexpressed mainly in peroxisomes and not in the cytoplasm of INS-1.

Much evidence has accumulated in recent years, indicating that malonyl-CoA is a cytosolic signal of glucose abundance, which regulates fuel partitioning and metabolic signal transduction in many tissues, particularly in liver, adipose, heart and pancreatic islet tissues [1,4,6,10,43]. Furthermore, malonyl-CoA-CPT-I interaction has been considered as an important site of intervention in diabetes and obesity [1,3]. If, in further studies, MCD proves to be implicated in the regulation of lipid metabolism and cytosolic malonyl-CoA concentration in mammalian cells, the enzyme may also be an excellent choice for pharmacological intervention in these diseases as well as for cardiac ischaemia, where excessive fat oxidation impairs glucose metabolism and heart function [5].

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