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Molecular analysis of methylmalonyl-CoA mutase deficiency: identification of three missense mutations in *mut*⁰ patients*

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Abstract Genetic defects in the methylmalonyl-CoA mutase (MCM) gene cause methylmalonic acidemia (MMA). Only three mutations have been reported among Oriental patients to date. We studied fibroblast cell lines established from three Japanese patients with MCM deficiency. Enzymatic study showed that these patients had the *mut*⁰ type of MMA. Nucleotide sequencing of MCM cDNAs identified three missense mutations: a T to A change at nucleotide position 2082, which results in an amino acid substitution of Glu669 for valine (V669E); a T to A change at position 1179 with the corresponding amino acid substitution of Asp368 for valine (V368D); and a G to A change at position 1182 with the corresponding amino acid substitution of His369 for arginine (R369H). Each of the three missense mutations abolished MCM activity according to a transient expression study. Alignment of these mutations with a recently reported homology model of human MCM allowed us to speculate on the effect of these nonconservative amino acid substitutions on MCM activity: V368D and R369H affected residues in the β/α -(TIM-) barrel domain, on one of the two α -helices that form the dimer interface, while V669E altered a residue in the adenosylcobalamin-binding domain in the C terminus.

Key words Methylmalonic acidemia · Methylmalonyl-CoA mutase · Adenosylcobalamin · Organic aciduria · Mutation

Introduction

Methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) deficiency is an autosomal recessive disorder characterized clinically by neonatal or infantile metabolic ketoacidosis (Fenton and Rosenberg 1995). It is responsible for *mut* types of methylmalonic aciduria (MMA), whereas *cbl* types of MMA are due to inherited abnormalities in the metabolism of adenosylcobalamin, a cofactor for MCM. The *mut* types of MMA are further classified into two subtypes: *mut*⁻ types exhibit decreased MCM activity with an altered K_m for adenosylcobalamin; *mut*⁰ types show no residual activity even in the presence of excess cofactor (Fenton and Rosenberg 1995). MCM is a homodimer of identical subunits with an apparent molecular weight of 77.5 kDa and is located in the mitochondrial matrix (Fenton et al. 1982). The MCM gene has been mapped to human chromosome 6p12-21.1, consists of 13 exons, spreads over more than 30 kb, and produces mRNA with a length of 2.7 kb (Ledley et al. 1988; Jansen et al. 1989; Nham et al. 1990).

Molecular analysis of MCM deficiency among Caucasian and African-American patients has revealed at least 20 mutations to date (Ledley and Rosenblatt 1997; Adjalla et al. 1998). Each of the mutations was found only in one family, except for G717X, which has been identified in three patients of African-American origin evaluated at one hospital (Crane et al. 1992), suggesting considerable genetic heterogeneity of MCM deficiency in Caucasian populations. Only three mutations have been reported in Oriental patients. We previously reported a nonsense mutation (g425t, which results in E117X) and a two-base deletion frame shift (769delCA) in Japanese patients (Ogasawara et al. 1994b). A splicing error mutation (INV2 + 5G > A) was also described by Toyo-Oka et al. (1995). The E117X substitution accounted for 7 of 32 mutant MCM alleles (22%), while each of the other two mutations were found in a single family. The presence of a prevalent E117X mutation and the absence of a missense mutation suggested that the spectrum of MCM mutations in Oriental patients may be

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distinct from that of Caucasians. However, the majority of MCM mutations in Oriental populations remain to be elucidated. We report here on the identification of three missense mutations responsible for *mut*⁰ MMA. Alignment of these mutations with a recently reported homology model of human MCM allows us to speculate on the effect of each substitution on MCM activity (Thomä and Leadlay 1996).

Materials and methods

Patients

We studied three Japanese patients with MCM deficiency who showed initial symptoms, such as vomiting, poor feeding, and/or respiratory distress, during the neonatal period (days 1–8). Although they survived the acute phase with intensive medical treatment, moderate to severe mental retardation was observed in all patients.

Enzymatic analysis of fibroblast cultures

Fibroblast cultures (Nos. 69, 133, and 422) were obtained from the three patients. The diagnosis was enzymatically confirmed by our previously described method employing high-performance liquid chromatography (Kikuchi et al. 1989). A propionate incorporation study was also carried out to distinguish between the *mut*⁻ and *mut*⁰ types (Willard et al. 1976).

Extraction of RNA and Northern blotting

mRNA was isolated from cultured skin fibroblasts according to the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987) followed by oligo-dT column (Pharmacia LKB Biotechnology, Uppsala, Sweden) chromatography. One microgram of mRNA was electrophoresed through a 1.0% agarose gel in the presence of formaldehyde and transferred to a nylon membrane (Sambrook et al. 1989). The membrane was hybridized with ³²P-labeled MCM cDNA, and subsequently with ³²P-labeled β -actin cDNA.

Reverse transcription/polymerase chain reaction (PCR) of MCM mRNA and nucleotide sequencing of MCM cDNA

One microgram of the isolated poly(A⁺) RNA was used to synthesize first-strand cDNA, and the entire coding region of MCM cDNA was amplified by PCR as described previously except that each amplification primer had an additional *Bam*HI or *Stu*I recognition sequence at the 5' end (Ogasawara et al. 1994b). PCR products were digested with *Bam*HI and *Stu*I (Takara, Kyoto, Japan) and subcloned into the *Bam*HI–*Sma*I site of pGEM–Blue plasmid (Promega, Madison WI, USA). Isolated plasmid from multiple inde-

pendent clones was subjected to sequencing analysis by the dideoxy chain termination method.

Direct sequencing of MCM cDNA and genomic MCM locus

To confirm the presence of mutations detected by the plasmid-sequencing analysis and to identify allelic status of the mutations, PCR copies of MCM cDNA and genomic DNA were sequenced directly (Winship 1989). Primer sequences in introns were based on data on the human genomic MCM structure (Ogasawara et al., unpublished data). The direct sequencing was performed by the following protocol. Amplified fragments of MCM cDNA and genomic MCM DNA were electrophoresed on a 1% agarose gel, visualized with ethidium bromide. The desired bands were excised from the gel and the DNA was recovered by a glass powder method. The purified templates (0.5 pmol) were mixed with primers (100 pmol) in 40 mM Tris HCl (pH 7.5), 20 mM MgCl₂, 50 mM NaCl, and 10% dimethyl sulfoxide. The mixture was boiled for 3 min and immediately immersed in liquid nitrogen. The template–primer mixture was then placed on ice and subjected to sequencing reaction using Sequenase (USB, Cleveland OH, USA) according to the manufacturer's protocol except that 10% dimethyl sulfoxide was included throughout the reaction.

Detection of g425t mutation by *Mbo* II restriction enzyme digestion

The E117X mutation, which has been found in Japanese patients with a relatively high incidence, was screened by a previously described method by PCR amplification of a DNA fragment in exon 2/intron 2 followed by digestion with *Mbo*II (New England Biolabs, Beverly, MA, USA).

Transient expression study

MCM-deficient fibroblast cell culture 325 was transformed by replication origin-defective simian virus 40 (ori⁻ SV40) and used for transient expression analysis of various MCM mutations (Gulzman et al. 1980).

MCM mutations were introduced into MCM cDNA by a Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Normal or mutant MCM cDNA was subcloned into a mammalian expression vector, pUC-CAGGS (Niwa et al. 1991), which carries chicken β -actin promoter and cytomegalovirus enhancer. The sequence of MCM cDNA in the recombinant expression vector was confirmed by nucleotide-sequencing analysis. Culture 325 with ori⁻ SV40 seeded in a 175 cm² flask was transfected with each of the recombinant vectors (20 μ g) and pSV- β -gal (5 μ g) using Lipofectamine reagent (Gibco BRL, Gaithersburg MD, USA). The cells were harvested 72 h after the transfection and assayed for MCM activity and β -galactosidase activity.

Results

MCM activity was undetectable (less than 100pmol of succinyl-CoA formed per minute per milligram protein, while the control value in normal fibroblasts was 2019 ± 434 pmol of succinyl-CoA formed per minute per milligram protein) in all three fibroblast cultures derived from the patients with or without the presence of excess adenosylcobalamin ($5\mu\text{M}$) by the high-performance liquid chromatographic method. The more sensitive propionate incorporation study showed that propionate incorporation was not stimulated by a high concentration of hydroxycobalamin ($1\mu\text{g/ml}$) in the cultures, indicating that these cells exhibited a *mut*⁰ phenotype.

Screening of genomic DNA for the E117X mutation, which was associated with markedly decreased mRNA (Ogasawara et al. 1994b), revealed that both cultures 133 and 422 were heterozygous for the mutation. Northern blot analysis detected MCM mRNA in the three cultures (Fig. 1, lanes 2, 3, and 4). We then analyzed MCM mRNA from the three cultures by reverse transcription/PCR amplification of MCM cDNA followed by nucleotide sequencing. The obtained nucleotide sequences were compared with human MCM cDNA sequence in the GenBank database (accession Nos. M37510 and J04774), since the original report of MCM cDNA contained several cloning or sequencing errors (Jansen et al. 1989). The most significant difference was a deletion of 24 nucleotides (nt 1382–1405) in the previous report. The nucleotides were in-frame, adding an extra eight amino acids (NDVYDAAL), and did not alter the downstream amino acid sequences. Our sequencing analy-

sis of MCM cDNA from two normal Japanese individuals indicated that the 24 nucleotides were indeed present in normal cDNA, and the subunit of MCM precursor protein consists of 750 amino acids. Other differences were single- or double-nucleotide changes.

Sequencing of MCM cDNA obtained from culture 69 revealed a single-base change of T to A at nucleotide position 2082 in MCM cDNA, which resulted in an amino acid substitution of Glu669 for Val669 (V669E) in exon 12 (Fig. 2a). Direct sequencing of amplified MCM cDNA identified the same single-base substitution. Exon 12 of the MCM gene was then amplified with intronic primers (sense primer, 5'-GGTATTGCTAGATTGCTCTCC-3'; antisense primer, 5'-TCAAGATTCCCAATCACAGTAC-3') and directly sequenced. The sequencing analysis showed the presence of both T and A at this position, indicating that the cell line was a compound heterozygote of the V669E mutation and another as yet unidentified mutation associated with markedly reduced expression.

Sequencing of MCM cDNA obtained from culture 133 identified a single-base change of T to A at nucleotide position 1179, which resulted in an amino acid substitution of Asp368 for Val368 (V368D) in exon 6 (Fig. 2b). Direct sequencing of genomic DNA indicated that culture 133 is a compound heterozygote of V368D and E117X.

Sequencing of MCM cDNA obtained from culture 422 revealed a single-base change of G to A at position 1182, which resulted in an amino acid substitution of His369 for Arg369 (R369H) in exon 6 (Fig. 2c). Direct sequencing of genomic DNA showed that culture 422 is a compound heterozygote of R369H and E117X.

Transient expression studies were carried out to determine whether the identified missense mutations were causes of MMA in these patients. We used fibroblast culture 359 for the transfection with each recombinant expression vector instead of COS cells, because COS cells exhibited a significantly high level of basal MCM activity. Culture 359 was first transformed with ori⁻SV40 to obtain an enhanced growth rate and an unlimited life span. The transformed culture did not show MCM activity (Table 1). Normal and mutated MCM cDNA was introduced into the culture and transiently expressed under the control of

Fig. 1 Northern blot analysis. Lane 1, control fibroblast; lane 2, fibroblast culture 69; lane 3, fibroblast culture 133; lane 4, fibroblast culture 422; lane 5, fibroblast culture 325. The membrane was hybridized with **a** an MCM cDNA probe and **b** subsequently with a β -actin cDNA probe

Table 1 Transient expression analysis of MCM cDNA harboring missense mutations identified in the study. Each transfection experiment was performed in triplicate. The efficiency of transfection was monitored by assaying β -galactosidase activity in the cell extract

Expression vector used for transformation	MCM activity ^a
Mock	ND ^b
pUC-CAGGS/normal MCM cDNA (sense)	1508 ± 442
pUC-CAGGS/normal MCM cDNA (antisense)	ND
pUC-CAGGS/MCM cDNA with V368D	ND
pUC-CAGGS/MCM cDNA with R369H	ND
pUC-CAGGS/MCM cDNA with V669E	ND
Normal fibroblast cultures ($n = 8$)	2019 ± 434

^aExpressed as picomoles of succinyl-CoA formed per minute per milligram protein

^bNot detected (less than 100pmol of succinyl-CoA formed per minute per milligram protein)

Fig. 2 **a** Nucleotide sequencing of MCM cDNA derived from culture 69. The autoradiogram shows a base substitution of T to A, which resulted in an amino acid substitution of Glu669 for valine. **b** Nucleotide sequencing of MCM cDNA derived from culture 133. The autoradiogram shows a base substitution of T to A, which resulted in an amino acid substitution of Asp368 for valine. **c** Nucleotide sequencing of MCM cDNA derived from culture 422. A substitution of G to A, which resulted in an amino acid substitution of His369 for arginine was identified

chicken β -actin promoter and cytomegalovirus enhancer. Normal MCM cDNA in pUC-CAGGS increased the MCM specific activity from undetectable to 1508 ± 442 pmol of succinyl-CoA formed per minute per milligram protein, which was comparable to the value in normal fibroblasts (Table 1). In contrast, increase of MCM activity was not observed by the transfection of any of the expression vectors carrying the three different point mutations, V669E, V368D, or R369H (Table 1).

Discussion

Recently, the crystal structure of MCM from *Propionibacterium shermanii* at 2 Å resolution was reported, and the cobalamin-binding and coenzyme

substrate-binding domains were identified (Mancia et al. 1996). Furthermore a three-dimensional homology model of human MCM generated from the structure of the α subunit of *P. shermanii* MCM provided valuable information for interpreting the effect of various mutations on MCM function (Thomä and Leadlay 1996). The three mutations identified in this study substitute amino acid residues that are conserved between human MCM and the α subunit of *P. shermanii* MCM. Both V368D and R369H affect amino acid residues in helix Ia7 of the β/α -(TIM-) barrel domain on one of the two α helices that form the dimer interface. An arginine residue at 369 is salt bridged to Glu415 provided by the other subunit. Therefore, these two mutations might affect dimerization of MCM subunits, possibly destabilizing mutant MCM proteins.

R369H has been independently described in a report by Adjalla et al. (1998) as "unpublished results" by Kogekar, Janata and Fenton. It was reported that the mutation was associated with low mRNA phenotype. However, our Northern blot analysis of cell culture 422, which is a compound heterozygote of R369H and E117X, showed approximately the normal amount of MCM mRNA (Fig. 1, lane 4). Since E117X homozygotes exhibited MCM mRNA at levels less than 1% of normal controls (Ogasawara et al. 1994b), it seems unlikely that R369H is associated with decreased amounts of MCM mRNA.

V669E affected an amino acid residue in the adenosylcobalamin-binding domain in the C terminus of MCM, where clustering of MCM mutations has been noted (Crane and Ledley 1994). The core of the B_{12} binding domain is formed by five parallel β strands (II β 1–II β 5) that alternate with α helices (II α 1–II α 5), and a valine residue at 669 is located in strand II β 3 (Thomä and Leadlay 1996). Strands II β 3 and II β 4 form one side of a deeply buried pocket in which the dimethylbenzimidazole portion of the cobalamin is accommodated, while the other side of the pocket is flanked by side-chains from helices II α 1 and II α 5. The V669E mutation results in a replacement of a conserved hydrophobic residue with an acidic hydrophilic residue, and is likely to affect the key function of the enzyme. Also, there is a possibility that the mutation affects the stability of mutant MCM protein.

Our initial attempt of *in vitro* expression analysis of each mutation was performed in COS cells using an eukaryotic expression vector, pEUK-C1 (Clontech). However, a significantly high level of intrinsic MCM activity in COS cells and a relatively weak gene expression driven by the SV40 late promoter hampered precise measurement of extraneous MCM activity. To establish a cell culture suitable for the *in vitro* expression study of MCM, we transformed MCM-deficient fibroblast culture 359 with ori⁻ SV40. Since MCM mRNA in culture 325 was not detected by Northern blot analysis (Fig. 1, lane 5) and no mutase protein was detected by Western blot analysis using anti-MCM antibody (unpublished results), it is unlikely to have complementation with extrinsic mutant MCM protein expressed in the cells. Molecular analysis of culture 325 indicated that it is a compound heterozygote of E117X and another as yet unidentified mutation. The transformed and cloned culture

originating from culture 325 did not show MCM activity. The establishment of the expression system allowed us to measure precisely the MCM activity produced by each mutant MCM cDNA. None of the three mutations exhibited residual MCM activity in our experiments. This observation is in line with the possible detrimental effect of each amino acid substitution on an important functional domain in the MCM protein, and is compatible with the severe clinical pictures of the three patients.

Our previous study showed the relatively high incidence of the E117X mutation among Japanese patients (Ogasawara et al. 1994b). In contrast, the 769 Δ ca, V669E, V368D, and R369H mutations were identified only once in 18 patients with MCM deficiency (data not shown). These results suggested that MCM deficiency may also be genetically heterogeneous in Japanese populations. Since MCM deficiency is one of the most prevalent organic acidemias in Japan, it is important to further characterize MCM mutations to establish DNA diagnosis of MMA in the population. Also, the interpretation of various mutations with defined clinical pictures and biochemical properties in relation to three-dimensional homology modeling of human MCM would provide further insights into the genotype-phenotype correlation of MCM deficiency and the structure-function relationship in the MCM protein.

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