Molecular mechanisms leading to three different phenotypes in the cblD defect of intracellular cobalamin metabolism

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The cblD defect of intracellular vitamin B12 metabolism can lead to isolated methylmalonic aciduria (cblD-MMA) or homocystinuria (cblD-HC), or combined methylmalonic aciduria and homocystinuria (cblD-MMA/HC). We studied the mechanism whereby MMADHC mutations can lead to three phenotypes. The effect of various expression vectors containing MMADHC modified to contain an enhanced mitochondrial leader sequence or mutations changing possible downstream sites of reinitiation of translation or mutations introducing stop codons on rescue of adenosyl- and methylcobalamin (MeCbl) formation was studied. The constructs were transfected into cell lines derived from various cblD patient’s fibroblasts. Expression of 10 mutant alleles from 15 cblD patients confirmed that the nature and location of the mutations correlate with the biochemical phenotype. In cblD-MMA/HC cells, improving mitochondrial targeting of MMADHC clearly increased the formation of adenosylcobalamin (AdoCbl) with a concomitant decrease in MeCbl formation. In cblD-MMA cells, this effect was dependent on the mutation and showed a negative correlation with endogenous MMADHC mRNA levels. These findings support the hypothesis that a single protein exists with two different functional domains that interact with either cytosolic or mitochondrial targets. Also a delicate balance exists between cytosolic MeCbl and mitochondrial AdoCbl synthesis, supporting the role of cblD protein as a branch point in intracellular cobalamin trafficking. Furthermore, our data indicate that the sequence after Met116 is sufficient for MeCbl synthesis, whereas the additional sequence between Met62 and Met116 is required for AdoCbl synthesis. Accordingly, western blot studies reveal proteins of the size expected from the stop codon position with subsequent reinitiation of translation.

INTRODUCTION

In mammals, vitamin B12 (cobalamin) is essential for normal development and survival and must be provided in the diet from animal products or from supplements. It is converted intracellularly to two active coenzyme forms, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). AdoCbl is the coenzyme for mitochondrial methylmalonyl-CoA mutase (MCM) which converts L-methylmalonyl-CoA to succinyl-CoA and is involved in catabolism of odd-chain fatty acids and some amino acids. Deficiency of MCM (MIM 609058) leads to accumulation of methylmalonic acid in body fluids (1) and results in a severe clinical phenotype. MeCbl is the coenzyme for cytosolic methionine synthase (MS) which converts homocysteine to methionine and is essential for normal one-carbon metabolism (2). Deficiency of MS (MIM 156570) causes increased levels of homocysteine in body fluids and a severe neurological phenotype.

Vitamin B12 has to be converted to its active cofactors in a series of intracellular reactions defined as eight different

We previously reported that the cblD defect is associated with three distinct biochemical phenotypes: deficient synthesis of either both cobalamin coenzymes or only MeCbl or AdoCbl (5). Using microcell-mediated chromosome transfer and refined genetic mapping, we identified the gene responsible for cblD as MMADHC (methylmalonic aciduria, cblD type) (3). Individuals with cblD-MMA/HC and cblD disorders cause combined methylmalonic aciduria and homocystinuria (MMA/HC), cblA, cblB, cblD-MMA and mut cause isolated MMA and cblD-HC, cblE, cblG result in isolated HC.

We have speculated that in patients with cblD-MMA, there is reinitiation of translation whereby Met62 acts as second start codon. In patients with cblD-HC, we showed that the nonsense mutations in the C-terminal part affect MeCbl formation but not the functional domain for the synthesis of AdoCbl (3).

### RESULTS

#### Mutations detected in cblD patients

Table 1 summarizes the phenotype, and nucleotide and protein changes in the 10 patients previously reported and 7 new ones (3,6). Of a total of 15 mutations, two, c.133dupG and c.229dupG, have not been reported.

#### Expression studies of mutant alleles

The effect of mutant alleles on MeCbl and AdoCbl synthesis was studied in an immortalized combined cblD-MMA/HC cell line (D007) confirming the functional significance of various mutations (Supplementary Material, Fig. S1). We show that mutant alleles associated with the cblD-HC phenotype (c.737A>G, p.D246G; c.776T>C, p.L259P) were unable to rescue MeCbl synthesis. In contrast, MeCbl synthesis was clearly rescued by mutant alleles associated with the cblD-MMA phenotype (c.57_64del, p.S20X; c.60insAT, p.L21IfsX2; c.133dupG, p.A45GfsX15; c.160C>T, p.R54X; c.229dupG, p.N77EfsX5). Regarding AdoCbl synthesis, mutant alleles associated with cblD-HC clearly rescued AdoCbl synthesis. The missense allele c.545C>A (p.T182N) found in a patient with cblD-HC compound heterozygous for the cblD-HC allele c.746A>G rescued neither MeCbl nor AdoCbl synthesis. The

### Table 1. Phenotype and MMADHC mutations detected in 17 patients from 16 families with cblD defect

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<th>ID</th>
<th>Allele 1 Nucleotide change</th>
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<th>Location</th>
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*aMMA, isolated methylmalonic aciduria; HC, isolated homocystinuria; MMA/HC, combined methylmalonic aciduria and homocystinuria.
bAppears homozygous at cDNA level; clearly heterozygous at genomic level.
cNot detectable at cDNA level suggesting low steady-state levels of mRNA; clearly heterozygous at genomic level.
dSiblings.
nonsense allele c.683C>G (p.S228X), identified in the homozygous state in a combined defect patient, was used as a negative control.

Rescue of AdoCbl synthesis in cblD-MMA/HC and cblD-MMA cells

In previous studies, we showed that AdoCbl synthesis was poorly corrected by transfection with the wild-type allele, except when using a vector in which a V5-polypeptide was attached to the C-terminus of the wild-type protein (3). We hypothesized that the low efficiency of rescue of AdoCbl synthesis by the wild-type construct is caused by inefficient mitochondrial targeting. Analysis of the amino acid sequence of MMADHC by the Mitoprot II software (7) predicted a cleavable, N-terminal mitochondrial leader sequence (MLS) of 11 amino acids (MANVLCNRARL).

To investigate the efficiency of the endogenous MLS, we prepared a construct (cblD_MLS_ALDH2) in which this putative MLS of MMADHC by the Mitoprot construct is caused by inefficient mitochondrial targeting. Analysis of the amino acid sequence of MMADHC by the Mitoprot II software (7) predicted a cleavable, N-terminal mitochondrial leader sequence (MLS) of 11 amino acids (MANVLCNRARL).

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Attempts to increase the level of rescue of AdoCbl synthesis with the cblD wild-type allele in a cblD-MMA cell line homozygous for p.S20X (D003 or D010) by using another type of vector (pcDNA3) for transient transfection were not successful. When using stable transfection in a retroviral system (pBABE), again only a small but statistically significant ($P \leq 0.001$) increase in AdoCbl from 1.3 to 3.4% was seen (Supplementary Material, Fig. S2).

**MMADHC mRNA expression**

Real-time polymerase chain reaction (PCR) showed considerable differences in the levels of MMADHC mRNA in cblD cell lines (Fig. 3A). Clearly reduced levels were obtained in a patient with the combined cblD-MMA/HC phenotype and homozygous for a premature termination codon (PTC) in exon 5 (D006, c.419dupA, p.Y140X), and to a lesser extent in a patient with an isolated cblD-MMA phenotype and homozygous for a premature termination codon (PTC) in exon 4 (D004, c.160C>T, p.R54X/c.307_324dup, p.L103_S108dup). However, mRNA levels were not significantly reduced in other patients with the cblD-MMA phenotype and PTCs in exons 3 (D003 and D010 [data not shown]), homozygous for c.57_64del, p.S20X; D012, homozygous for c.133dupG, p.A45GfsX15); in exons 3 and 5 (D008, c.60insAT, p.L211fsX2/c.455dupC, p.C153MfsX10) and in exon 4 (D017, homozygous for c.228dupG, p.N77EfsX5). Normal mRNA levels were also found in patients with the cblD-MMA/HC phenotype and homozygous for PTCs in exons 3 and 5 (D008, c.419dupA, p.S20X; D012, homozygous for c.133dupG, p.A45GfsX15); in exons 3 and 5 (D008, c.60insAT, p.L211fsX2/c.455dupC, p.C153MfsX10) and in exon 4 (D017, homozygous for c.228dupG, p.N77EfsX5). mRNA levels were normalized to mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (data not shown). Similar results were obtained when the mRNA levels were normalized to the mRNA of the mitochondrial protein MRPL19 (data not shown).

A negative correlation was observed between the mean levels of expression of MMADHC mRNA and the level of rescue of AdoCbl synthesis in the different cblD-MMA cell lines. Thus, the correlation coefficients between mRNA synthesis and AdoCbl rescue were $R = 0.89$ and $R = 0.83$ for transfection with the wild-type and cblD_MLS_ALDH2 construct, respectively. No such correlation was observed in cblD-MMA/HC cell lines.

**MMADHC protein expression**

Endogenous production of the MMADHC protein was studied by immunoprecipitation in crude cell extracts. This showed a single band of 32.8 kDa corresponding to the full-length cblD protein in control cells. In cells of patient D003 homozygous for p.Ser20X, smaller bands of 26.5 and 20.5 kDa corresponding to MMADHC proteins starting at Met62 and Met116, respectively, were detected (Fig 3B). In cells of patient D017, homozygous for p.N77EfsX5, only the 20.5 kDa band corresponding to MMADHC starting at Met116 was present. No MMADHC protein was detected in cell lines D006 and D007 that are homozygous for p.Y140X and p.F204_A232del, respectively.
Effect of mutations associated with cblD-MMA on the level of rescue of AdoCbl synthesis

The negative correlation observed between the level of the rescue of AdoCbl synthesis and the level of mRNA among the cblD-MMA cell lines suggests that the endogenous mutant protein in these cells may affect the ability of the wild-type protein to produce AdoCbl. We therefore studied the effect of five cblD-MMA-associated mutations on rescue of AdoCbl synthesis by co-transfecting a combined cblD-MMA/HC cell line (D007, p.F204_A232del/p.F204_A232del) after co-transfection of a wild-type construct (cblD_MLS_ALDH2) in which the endogenous putative MLS of MMADHC is replaced by the MLS of aldehyde dehydrogenase 2 (ALDH2) together with wild-type (wt) or cblD-MMA-associated mutant alleles (p.S20X, p.L21IfsX2, p.A45GfsX15, p.R54X, p.N77EfsX5). Co-transfection together with empty vector (vector only) and with a cblD-MMA/HC-associated mutant allele (p.S228X) were used as controls. All constructs were prepared using pTracer vector and transfections were made by electroporation. Columns represent the mean and vertical lines the range of results from at least four replicate experiments with single determinations.

Effect of alternative start codons on synthesis of MeCbl and AdoCbl

All six patients with the cblD-MMA phenotype have at least one allele carrying a mutation that leads to premature termination of translation before the second or third methionine located at positions 62 and 116, respectively (Table 1). Four of these are homozygous and the other two are compound heterozygous and carry in addition (i) a deletion that leads to a frameshift and premature termination at amino acid residue 162 (p.C153FksX10 in D008), and (ii) an in-frame duplication of the amino acids 103–108 (p.L103_S108dup in D004). Both mutations are not detectable at the cDNA level but clearly heterozygous at the genomic level, suggesting that the steady-state level of mRNA from these alleles is very low. Since MeCbl synthesis is completely normal in these patients, we have speculated that there is reinitiation of translation at a downstream AUG codon, e.g. at Met62 and/or at Met116. Both codons are located within a sequence compatible with the Kozak consensus (3,9).

To test this hypothesis, we prepared constructs with selected changes in the MMADHC wild-type cDNA, transfected them into a cblD-MMA/HC cell line (D007, p.F204_A232del/p.F204_A232del) and investigated the rescue of cobalamin co-enzyme synthesis. As shown in Figure 5, N-terminal truncation to either Met62 (Fig. 5, construct 5) or Met116 (Fig. 5, construct 7), or introduction of a stop codon at position 89 (Fig. 5, construct 9), does not lead to lack of MeCbl synthesis. In contrast, introduction of a stop codon at position 118 (Fig. 5, construct 10) completely abolishes MeCbl synthesis. These findings confirm that translation can be initiated either at Met62 or at Met116, but not downstream of Met116.

Concerning AdoCbl synthesis, as expected there was no AdoCbl formation with truncation N-terminally to Met62 or Met116 as these constructs lack a MLS (Fig. 5). However, introduction of the MLS of ALDH2 N-terminally at Met62 (Fig. 5, construct 6) resulted in significant rescue of AdoCbl synthesis, whereas no rescue was observed when the MLS was added N-terminally to Met116 (Fig. 5, construct 8). This indicates that the sequence between Met62 and Met116 and downstream is essential for mitochondrial AdoCbl synthesis. Thus, the protein truncated N-terminally to Met116 has lost the dual function being unable to support AdoCbl synthesis but being fully active in MeCbl synthesis.

The removal of possible alternative translation start sites by mutating Met62 and Met116 to glutamine residues (10) (Fig. 5, constructs 3 and 4) did not significantly affect the...
synthesis of MeCbl ($P = 0.294$) or AdoCbl ($P = 0.640$). This suggests that initiation of translation at alternative start sites plays at most a minor role in the wild-type.

**DISCUSSION**

Our studies provide further evidence for the idea that the MMADHC protein contains various domains responsible for (i) targeting the protein towards the mitochondria; (ii) MeCbl synthesis and (iii) AdoCbl synthesis. Accordingly, the nature and location of mutations within the protein determine one of three biochemical phenotypes, i.e. combined MMA/HC, isolated MMA or isolated HC.

To date, we have identified a total of 17 patients, 5 with a combined defect and 6 each with isolated MMA or isolated HC (Table 1). In line with our idea, each patient with cblD-MMA carries on at least one mutant allele causing a premature stop toward the N-terminal part of the protein. Importantly, one patient (D017) is homozygous for a duplication leading to a stop codon at position 81 (p.N77EfsX5) which is downstream from the second (Met62) but upstream from the third (Met116) initiation codon supporting the idea that reinitiation of translation can also occur at Met116. All six patients with cblD-HC carry at least one missense mutation causing amino acid substitutions toward the C-terminal part of the protein. Interestingly, these three mutations are located within a short stretch of 14 amino acids (p.D246G, p.Y249C, p.L259P). Using expression studies, we have proven the functional significance of these mutations.

This small region is one of the five putative sites of interaction between MMADHC and MMACHC according to recent phage-display experiments (11). Thus, our data support the hypothesis that mutations in the C-terminal part of MMADHC could lead to a defect in the MeCbl pathway by preventing binding with MMACHC.

Further supporting our concept, three additional reported patients with cblD-MMA are homozygous for mutations leading to a premature stop codon N-terminal of Met62 (6). All five patients with the combined defect carry mutations that are predicted to lead to premature stop codons C-terminal of Met116.

Central to the various features of this special protein are reinitiation of translation at downstream start codons and both cytosolic and mitochondrial localization. Our studies conclusively show that a truncated protein lacking the first 115 amino acids is fully capable of MeCbl synthesis. In support of reinitiation of translation and in line with naturally occurring mutations in patients, we have shown that truncation N-terminally to Met62 or Met116, or introduction of a stop codon after Met116, still allow formation of MeCbl (Fig. 5). In line with our concept, western blot studies showed formation of protein of the sizes expected from the position of stop codons and subsequent reinitiation of translation (Fig. 3B).

Our studies show that AdoCbl synthesis requires targeting of the cblD protein to the mitochondria (positions 1–61) and an intact sequence downstream from Met62 (Fig. 5). More precise identification of the essential sequence for AdoCbl synthesis requires further studies. Dual localization of proteins has been shown to be caused by various mechanisms, including alternative splicing, initiation codon scan-through, multiple in-frame start codons, incomplete secretion, reverse translocation, spontaneous folding and others (12–18). Furthermore, proteins that are localized in both mitochondria and the cytosol have been shown to be more likely to have a weak MLS (19). Thus, the nature of the MLS can shift the balance between mitochondrial and cytosolic localization.
alleles also show variable rescue of AdoCbl synthesis (Fig. 4). The cblD_MLS_ALDH2 and various cblD-MMA mutant interact with full-length protein or with binding partners located in the short polypeptides that are produced in cblD-MMA patients thereby reducing AdoCbl synthesis. Another possibility is that Met116 could affect formation of functional complexes in cblD-MMA cells due to reinitiation of translation at Met62 or that if MMADHC is part of a complex necessary for the formation of AdoCbl, a shorter MMADHC protein produced in cblD-MMA cells due to reinitiation of translation at Met62 or Met116 could affect formation of functional complexes thereby reducing AdoCbl synthesis. Another possibility is that the short polypeptides that are produced in cblD-MMA patients (from Met1 to the stop codon resulting from the mutation) could interact with full-length protein or with binding partners located in the cytosol or at the mitochondrial membrane. In support of these findings, co-transfection of cblD-MMA/HC cells with the cblD_MLS_ALDH2 and various cblD-MMA mutant alleles also show variable rescue of AdoCbl synthesis (Fig. 4). Again the level of rescue appears to correlate with the level of endogenous mRNA (Fig. 3A).

Essentially normal amounts of mRNA were observed in patients D005 (p.R250X/p.R250X) and D009 (p.S228X/p.S228X). This can be explained by the well-established rule that PTCs residing in the last or penultimate exon (that is, they are not followed by more than one exon–exon junction) generally do not elicit NMD (23). As expected, severely reduced mRNA levels were found in the cblD-MMA/HC patient 006 homozygous for a PTC in exon 5 (p.Y140X/p.Y140X). Regarding the truncating mutations found in the cblD-MMA cell lines before the second (D003 and D010, D008, D0012, D004) or third (D017) start codon would all fulfill the rules of a PTC eliciting NMD, but instead have (near) normal levels of mRNA (Fig. 3). We can only speculate about the immunity of these cblD-MMA cell lines to NMD. A possible explanation would be that translation reinitiation downstream of a PTC inhibits NMD (24), indicating that PTC recognition by translating ribosomes is not sufficient for NMD, and that NMD depends on events that are subsequent to translation termination and are precluded by reinitiation (23).

Interaction of MMADHC with MMACHC may play a role in the regulation of the balance between AdoCbl and MeCbl synthesis. Thus, we observed that co-transfection of the cbIC gene with cblD wild-type cDNA in cblD-MMA/HC or cblD-MMA cell lines doubled the level of AdoCbl synthesis with less effect on MeCbl synthesis, compared with cblD wild-type alone (unpublished results). This is supported by the finding that MMADHC acts as a binding partner for MMACHC both in vitro and in vivo (11).

In summary, our findings suggest that under physiological conditions, there is a delicate balance of intracellular localization of MMADHC and that this balance is influenced by the efficacy of the MLS, supporting the concept that the cblD protein acts as a branch point in intracellular cobalamin trafficking. Our data indicate that the sequence after Met116 is sufficient for MeCbl synthesis, whereas the sequence from Met62 downstream is required for AdoCbl synthesis. We propose the existence of one single protein with two different functional domains that interact with either cytosolic or mitochondrial targets. Further studies on interactions with possible partner proteins and determination of the 3-D structure of MMADHC and sub-cellular localization will help further elucidate the role of this intriguing protein in intracellular cobalamin metabolism.

MATERIALS AND METHODS

Cell culture

Fibroblasts were obtained for diagnostic purposes with informed consent from the patients or their parents and referring clinicians approved the use of the cells for our investigation of the origin of the disease. Fibroblasts were routinely grown in Earl’s minimal essential medium (Amimed, Basel, Switzerland) supplemented with 10% fetal bovine serum (Amimed) as described earlier (5). Fibroblasts were transformed by transfection with pRNS1 (25) using electroporation as previously described (3).

Mutation analysis

Genomic DNA and total RNA were extracted from cultured patient fibroblasts using the QIAamp DNA Mini Kit and RNeasy Kit (Qiagen, Hombrechtikon, Switzerland), respectively. For mutation analysis, genomic DNA and cDNA synthesized from total RNA by reverse transcription (RT–PCR) were amplified by PCR using specific primers as described earlier (3). To confirm mutations identified in RT–PCR products, the corresponding exons were amplified by PCR from genomic DNA using flanking intronic primers and were sequenced by the ABI BigDye method (Applied Biosystems, Rotkreuz, Switzerland). All mutations were checked for their correct designation using ‘mutalyzer’ (www.mutalyzer.nl) according to the guidelines of the Human Genome Variation Society.

Preparation of constructs for transfection

Constructs containing the MMADHC wild-type and mutant cDNA were prepared in mammalian expression vectors as
described earlier (26). Shortly, cDNA was produced from total RNA by reverse transcription using the Titan One Tube RT–PCR Kit (Roche, Basel, Switzerland), cloned into pCR-Blunt-II-TOPO (Invitrogen, Basel, Switzerland), sub-cloned into pTracer-CMV2 (Invitrogen) at the EcoRI site and propagated in Escherichia coli DH5α competent cells (Invitrogen). Plasmids for transfections were isolated using the QIAfilter Plasmid Maxi Kit (Qiagen). Introduction of the sequence of base pairs corresponding to the MLS of ALDH2 into E. coli and propagated in pCR-Blunt-II-TOPO (Invitrogen, Basel, Switzerland), sub-cloned into TaqMan Universal PCR Master Mix (Applied Biosystems) at different positions and production of truncated and mutant MMADHC cDNAs was achieved using site-directed mutagenesis and appropriate oligonucleotides (Supplementary Material, Table S1). All PCR reactions were carried out using Hot Gyro Polymerase (Solis Biodyne, Tartu, Estonia). PCR products were cloned into pTracer-CMV2, as described above. All constructs were sequenced to verify that they contained the indicated changes.

Transfection studies

 Constructs containing wild-type and different mutant cDNA sequences in pTracer-CMV2 were transfected transiently into transformed patient and control fibroblasts by electroporation as described earlier (3). Rescue of cellular function was tested by measuring the synthesis of MeCbl and/or AdoCbl from $^{57}$Co cyanocobalamin, as described previously (5).

Real-time PCR

cDNA was synthesized from total RNA using the TaqMan Reverse Transcription Reagent (Applied Biosystems). Analysis of the abundance of the MMADHC mRNA was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s recommendations, except that the total reaction volume was reduced to 10 μl. The TaqMan probes (Applied Biosystems) used were Hs00739517_g1 (MMADHC) and as controls Hs00608519_m1 (MRPL19) and Hs99999905_m1 (GAPDH).

Western blot studies

Fibroblasts from control and CblD patients were lysed with radio immuno precipitation assay buffer (Invitrogen), immunoprecipitated with proteinG-sepharose (Invitrogen) and a polyclonal mouse anti-human MMADHC antibody (Biogenes, Berlin, Germany). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on a 12% gel, transferred to polyvinylidene difluoride membranes, blocked at room temperature for 1 h with 1% bovine serum albumin in phosphate buffered saline containing 0.1% Tween 20 and incubated overnight with a monoclonal mouse anti-human MMADHC antibody (AMSBio, Lugano, Switzerland). Visualization of protein bands was performed using a donkey anti-rabbit antibody conjugated with horseradish peroxidase (Jackson ImmuNoResearch, Newmarket, UK) and SuperSignal reagent according to the manufacturer’s instructions (Perbio, Lausanne, Switzerland). As protein molecular size marker, we used Kaleidoscope Precision Plus (BioRad, Reinach, Switzerland).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement

None declared.

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REFERENCES


