Common Missense Mutation G1528C in Long-Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency

Characterization and Expression of the Mutant Protein, Mutation Analysis on Genomic DNA and Chromosomal Localization of the Mitochondrial Trifunctional Protein α Subunit Gene

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Abstract

Mitochondrial trifunctional protein (MTP) is a recently identified enzyme involved in mitochondrial β-oxidation, harboring long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and long-chain 3-ketothiolase activity. A deficiency of this protein is associated with impaired oxidation of long-chain fatty acids which can lead to sudden infant death. Furthermore, it is clear that this inborn error of fatty acid oxidation is very frequent, second to medium chain acyl-CoA dehydrogenase deficiency. In most patients only the LCHAD activity of MTP is deficient with near normal activity of the two other enzyme activities of the complex. We recently described the occurrence of a frequent G1528C mutation in the cDNA coding for the α subunit of MTP. Using *S. cerevisiae* for expression of wild type and mutant protein we show that the G1528C mutation is directly responsible for the loss of LCHAD activity. Furthermore, we describe a newly developed method allowing identification of the G1528C mutation in genomic DNA. The finding of an 87% allele frequency of the G1528C mutation in 34 LCHAD deficient patients makes this a valuable test for prenatal diagnosis. Finally, we show that the gene encoding the α subunit of MTP is located on chromosome 2p24.1-23.3. (*J. Clin. Invest.* 1996. 98:1028–1033.) Key words: fatty acid • 3-hydroxyacyl-CoA dehydrogenase • hereditary disease • chromosome mapping • gene expression

Introduction

The mitochondrial β-oxidation of fatty acids is a complex catabolic pathway in which at least 10 different enzymes are involved. One of the latest enzymes added to this list is the mitochondrial trifunctional protein (MTP)[1] catalysing the last three steps of the β-oxidation of long-chain fatty acids (1, 2). This enzyme complex is composed of 4 α and 4 β subunits. The α subunit harbors the 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase activities, whereas the 3-ketoacyl-CoA thiolase activity is located on the β subunit. Both subunits are encoded by different mRNA's with an open reading frame of 2289 and 1422 bp respectively (3).

Long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency in man was first described in 1989 (4) and is now described in more than 15 patients (see 5 for review) although many remain to be reported (since our first report in 1989 we have identified 71 patients with a deficient long-chain 3-hydroxyacyl-CoA dehydrogenase activity [60 LCHAD-deficient and 11 MTP-deficient], unpublished observations). The clinical manifestations associated with a defect in this enzyme complex are very variable and include hypoglycaemia, cardiomyopathy and sudden death (see references 5 and 6 for discussion). Biochemically, two forms of LCHAD deficiency can be distinguished. In the first form described in two patients only (7, 8) all three activities of the MTP are deficient (MTP-deficiency). More frequent is the second form in which there is an isolated deficiency of the dehydrogenase activity (LCHAD-deficiency) with normal hydratase activity and a moderately decreased thiolase activity (59% of control) (9). In this last group of patients we and others found a G1528C mutation in the mRNA encoding for the α subunit of MTP (9, 10). This point mutation results in the substitution of glutamate to glutamine at aminoacid position 510 in the dehydrogenase region of the protein. The frequency of this mutation was found to be high: 24 out of 26 expressed only the C1528 mutant allele while the remaining two patients were found to be heterozygous for this allele (9).

In this paper we report the successful development of a PCR-RFLP method to identify this mutation in genomic DNA. This method allows unambiguous ascertainment of homo- and heterozygosity for the G1528C mutation obviating the need to analyze cDNA from both parents. This makes studies to determine the frequency of the G1528C mutation in the normal population now possible. Furthermore, definitive proof is presented showing that the G1528C mutation, indeed, causes loss of enzyme activity without changing the conformation and assembly of the MTP complex.

Finally, in situ hybridization studies located the gene for the α subunit of the MTP on chromosome 2p24.1-23.3.

Methods

Cell culture conditions, enzyme activity measurements, and gelfiltration experiments were performed exactly as described before (8).

Index patient. The patient with LCHAD-deficiency studied in this paper has been described previously (12).

RNA isolation and cDNA synthesis. Total RNA was isolated from cultured skin fibroblasts using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (13) and used to prepare cDNA (9).

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1. *Abbreviations used in this paper:* AFLP, acute fatty liver of pregnancy; GBP, Gastrin binding protein; HELLP, haemolysis elevated liver enzymes and low platelets; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; MTP, mitochondrial trifunctional protein.

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DNA isolation. Genomic DNA was isolated using diatoms matrix as described by Boom et al. (14).

Polymerase chain reaction. The cDNA (5–10 μl) or genomic DNA (1 μg) was amplified in a mixture (25 μl) containing 10 mM Tris-HCl (pH = 8.4 at 25°C), 1.4 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml BSA, 20-mer oligonucleotide primers as indicated (12.5 pmol each) and 2.5 U Taq polymerase (Promega Corp., Madison, WI). DNA amplification was performed in a PTC-100 thermocycler from M.J. Research Inc. programmed as follows: 120 s at 96°C initial to cycling, 30 cycles of 30 s at 94°C, 30 s at 55°C and 60 s per kb at 72°C followed by 5 min at 72°C.

Polymerase chain reaction of long fragments. A 10-kb PCR fragment was used as a probe for in situ hybridization. This fragment was amplified from human genomic DNA using a Expand Long Template PCR System (Boehringer Mannheim, Mannheim, Germany) with the following primerset: sense 5′-GCC TTT ATC CTG CAC CTC TG-3′ and antisense 5′-AAT AGC CAG ATG CCT GCA AGG C-3′.

Sequencing. PCR fragments were sequenced both in the sense and anti-sense direction using the dideoxy chain termination method of Sanger et al. (15), and nucleotide sequence analysis was performed with an Applied Biosystems 373A DNA sequencer.

RFLP analysis. For rapid screening of the G1528C mutation identified in the cDNA of the α subunit of the patient, cDNA was amplified by PCR using sense primer: 5′-GCC TTT ATC CTG CAC CTC TG-3′ and anti-sense primer: 5′-ACT GAC TGA GCG AGG CAT GA-3′. A different primer set was used with genomic DNA as template: sense primer 5′-CCC TTG CCA GGT GAT TGG C-3′ and antisense primer 5′-GTA TAG AAG CCA GGT CCA TCC TGC CAA G-3′. Subsequently, PCR products were directly digested with endonuclease PstI and were analyzed by agarose gel (2% wt/vol) electrophoresis containing ethidium bromide.

Construction of expression plasmids. Three expression plasmids were constructed for expression of wild type α-MTP, β-MTP, and G1528C mutated α-MTP in *S. cerevisiae*. The complete open reading frame of the gene coding for the α subunit of MTP was amplified in a PCR reaction using the following primer set: sense: 5′-TTT TCC CGG GAT GGT GGC CTG CCG GGC GAT TGG C-3′ and antisense: 5′-AAA ATC TAG ATC ACT GGT AGA ACT TCT TGT TAG GGC TG-3′. The open reading frame of the β subunit was amplified using the following primers: sense: 5′-TCG CGG ACG TCA GCC AAG ATT-3′ and antisense: 5′-GCA CAG AAA CTT CAG GTC AC-3′.

The yeast expression vector pIJl3 was constructed by insertion of the multiple cloning site of pUC19 into pSY1 (LEU2 gene, 2 μm origin of replication, *GAL7* promotor region and the PGK transcription termination signal sequence). The PCR product of the cDNA of the α-subunit was cloned in this yeast expression vector (pIJ3) downstream of the *GAL7* promotor (pYαMTP-G1528), allowing induction of transcription by addition of galactose to the culture medium. pYαMTP-C1528 was constructed by replacing the BfrI–EcoRI fragment of pYαMTP-G1528 for the BfrI–EcoRI fragment obtained from the index patient via PCR amplification. Plasmid pYES2 (Invitrogen) was used to create pYβMTP by cloning the PCR product of the β subunit downstream of the *GAL1* promotor. All plasmids were sequenced to rule out PCR mistakes.

Yeast culture and expression. Wild-type yeast (INVSC1: MATα, his3-Δ1, leu2, trp1-289, ura3-52 obtained from Invitrogen) and the transformed strains were grown on a medium containing 20 grams/liter lactate, 0.67 grams/liter Yeast Nitrogen Base without amino acids (Difco Laboratories Inc., Detroit, MI) and appropriate aminoacids to OD_{600} = 0.3–0.4. Overnight induction was initiated by addition of 2 grams/liter galactose. The cells were harvested and spheroplasts were prepared according to standard procedures (16). These spheroplasts were homogenized by sonication in a buffer containing 10 mM Mops (pH = 7.4), 160 mM NaCl, 0.1% wt/vol Triton X-100 and 1 mM PMSF. The homogenates were centrifuged at 10,000 *g* for 30 s and the supernatant was used for enzyme activity measurements.

Fluorescence in situ hybridization. FISH was performed on metaphase chromosomes from peripheral lymphocytes according to standard procedures. Probes were biotin-labeled by nick-translation and detected with Avidin-FITC. The signal was amplified with biotinylated goat anti-avidin and a second Avidin-FITC detection. The slides were examined under a Zeiss Axioplan epifluorescence microscope. For digital imaging microscopy the Cytovision Probe system (Applied Imaging) was used.

Results

Expression of wild-type and mutant MTP. In our previous study we reported on the frequent occurrence of a G1528C mutation in the cDNA encoding the α subunit of the MTP among a series of 26 long-chain 3-hydroxyacyl-CoA dehydrogenase deficient patients (9). This mutation changes the codon for glutamate at position 510 into glutamine in the dehydrogenase coding region of the α subunit. To establish whether this mutation results in an inactive enzyme complex, expression studies of the wild-type and mutant enzyme were done.

Initial experiments using *E. coli* for expression of α-MTP were unsuccessful since the protein was found to be enzymatically inactive despite its correct expression (not shown). Other expression systems such as COS cells could not be used because of the interference of endogenously present α- and β-MTP. Since *S. cerevisiae* lacks a mitochondrial β-oxidation system we decided to use this unicellular eukaryote for expression studies. Homogenates of transformed cells (derived from three independent transformants) as well as untransformed cells were prepared and the 3-hydroxyacyl-CoA dehydrogenase activity was measured using acetoacetyl-CoA (C4) and 3-ketopalmitoyl-CoA (C16) as substrates (Fig. 1).

Fig. 1 shows that cells grown on lactate or galactose contain a considerable 3-hydroxyacyl-CoA dehydrogenase activity

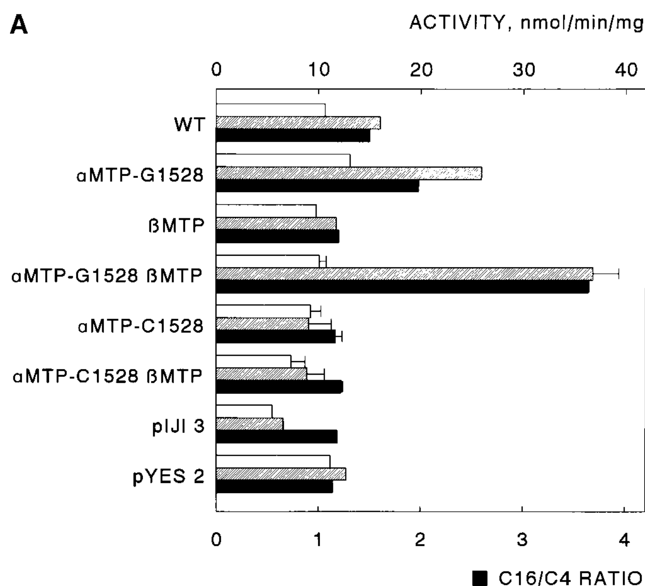

Figure 1. Expression of MTP in *S. cerevisiae*. Wild-type yeast (WT) cells were transformed with different expression plasmids (pIJl3, pYES2) containing the open reading frame of the wild type α-MTP gene (pYαMTP-G1528), mutant α-MTP gene (pYαMTP-C1528) and the open reading frame of the wild type β-MTP gene (pYβMTP). Subsequently, the 3-hydroxyacyl-CoA dehydrogenase activity was measured in homogenates prepared from spheroplasts using acetoacetyl-CoA (C4, *open bar*) and 3-ketopalmitoyl-CoA (C16, *hatched bar*) as substrates as described in Methods. *Solid bar*, C16/C4 ratio.

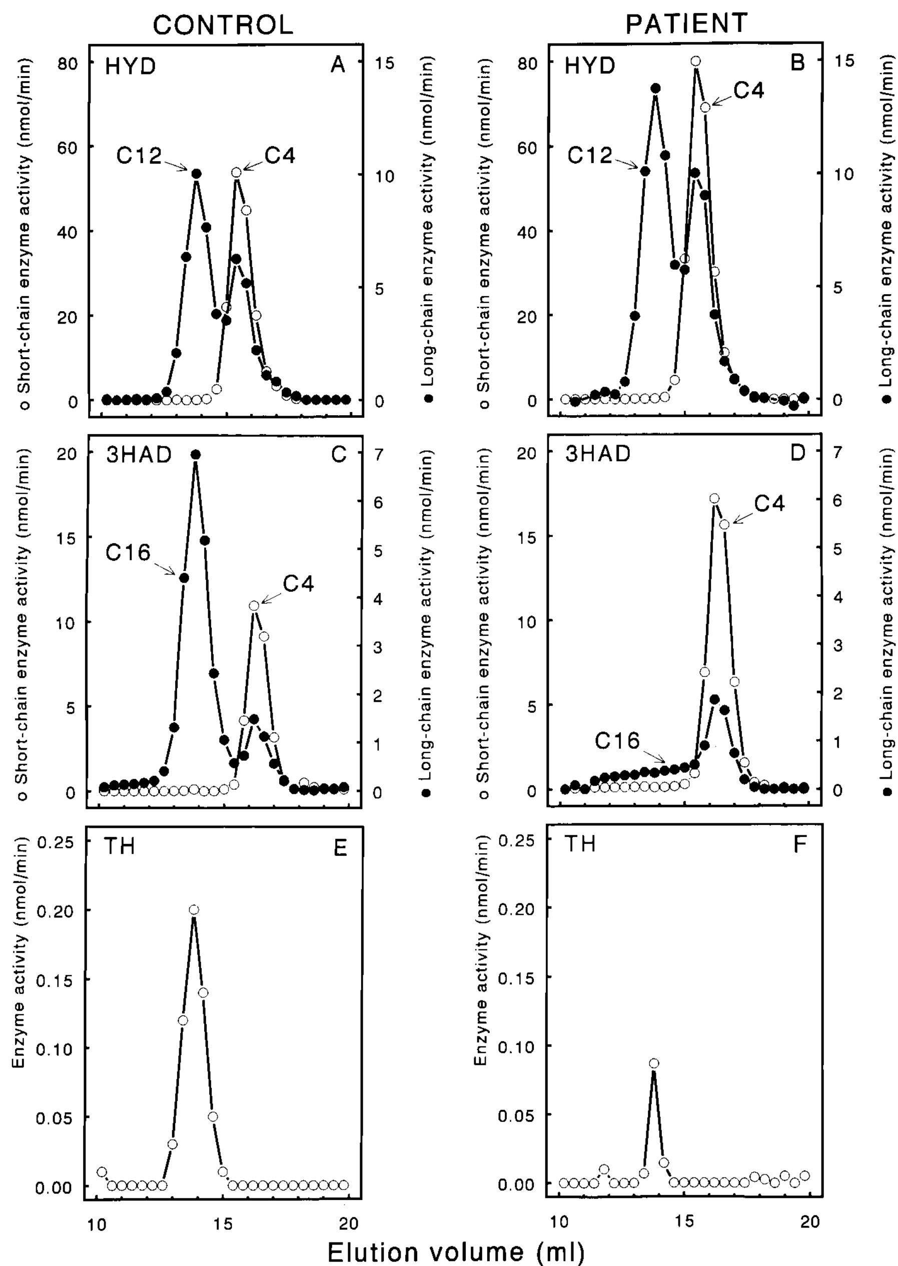

Figure 2. Gelfiltration of extracts of cultured skin fibroblast homogenates from a control subject and a LCHAD-deficient patient. Fibroblasts from a control subject and a LCHAD-deficient patient homozygous for the G1528C mutation were extracted as described in Methods and the extract was applied to a gelfiltration column. In the fractions eluting from the column the following enzyme activities were measured using different substrates: enoyl-CoA hydratase (HYD) with crotonyl-CoA (C4) and 2-dodecenoyl-CoA (C12); 3-hydroxyacyl-CoA dehydrogenase (3HAD) with acetoacetyl-CoA (C4) and 3-ketopalmitoyl-CoA (C16); 3-ketoacyl-CoA thiolase (TH) with 3-ketopalmitoyl-CoA (C16). For experimental details see Wanders et al. (8). *A*, *C*, and *E*, control fibroblasts. *B*, *D*, and *F*, LCHAD-deficient fibroblasts.

both with C4 and C16 as substrates. This activity most likely results from the peroxisomal multifunctional protein (17) containing 3-hydroxyacyl-CoA dehydrogenase activity. Indeed, growth of *S. cerevisiae* on oleate, a strong inducer of peroxisomes and the peroxisomal multifunctional protein (17), leads to a strong increase in activity as measured with the C4 and C16 substrates (not shown). Since MTP displays no activity with the C4 substrate (1), successful expression of MTP in yeast can be monitored by measuring activity with both the C4 and C16 substrates. Indeed, proper expression of MTP should lead to increased enzyme activity when measured with the C16 substrate whereas activity with the C4 substrate should remain unchanged thus leading to increased C16/C4-activity ratio's. Fig. 1 shows a somewhat higher C16/C4-activity ratio and C16 enzyme activity when *S. cerevisiae* was transformed with a plasmid containing full-length α-MTP cDNA (pYαMTP-G1528). However, the C16 enzyme activity was much higher if the cells were co-transformed with a plasmid containing the full-length β-MTP cDNA (pYβMTP). Immunoblot analysis showed a comparable level of expressed α-MTP protein in the two transformants. This suggests that the stimulation in enzyme activity is not due to increased α-MTP levels but due to the formation of a more active enzyme complex when the β subunit is co-expressed too.

To investigate the effect of the G1528C mutation on the 3-hydroxyacyl-CoA dehydrogenase activity of MTP, we transformed wild type yeast with plasmids containing wild-type β-MTP cDNA (pYβMTP) and mutant α-MTP cDNA (pYα-MTP-C1528). As seen in Fig. 1, the 3-hydroxyacyl-CoA dehydrogenase activities in these cells measured with the two substrates were the same as in wild type cells or in cells transformed with the two expression vectors only. In these cells the amount of α-MTP protein produced, as judged by immunoblotting, was the same as in cells transformed with pYαMTP-

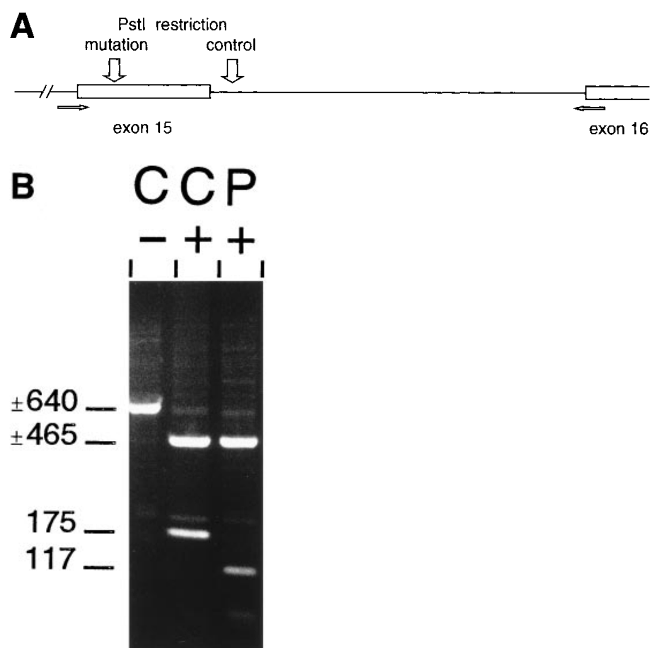

Figure 3. PCR-RFLP analysis of the G1528C mutation in the α-subunit of MTP (A) Schematic representation of a part of the gene of the α-subunit of MTP. Primers are located on the intron exon boundaries (horizontal arrows), the PstI restriction sites are indicated by vertical arrows. (B) Ethidium bromide stained agarose gel showing the PCR-RFLP analysis using genomic DNA of a control subject (C) and LCHAD-deficient patient (P). PCR products were either directly loaded (−), or digested with PstI (+) before electrophoresis.

G1528 (control α-MTP). These results show that the G1528C mutation found in the majority of LCHAD-deficient patients causes loss of 3-hydroxyacyl-CoA dehydrogenase activity of the MTP.

Characterization of the mutant protein. To investigate whether the disease causing mutation (G1528C) influences the conformation or assembly (α4β4) of the mutant enzyme complex, we performed gelfiltration experiments. The molecular weight of the mutant and wild type enzyme complex was determined by gelfiltration (Fig. 2) using fibroblast homogenates of control (*left*) and patients' fibroblasts (*right*).

Clear separation of the mitochondrial trifunctional protein and the monofunctional β-oxidation enzymes is obtained when control fibroblasts are used: the peak fraction of MTP elutes at 13.8 ml while crotonase and short-chain 3-hydroxyacyl-CoA dehydrogenase are eluting at 15.4 and 16.2 ml, respectively (see reference 8 for detailed discussion).

Gelfiltration experiments using fibroblasts from an LCHAD-deficient patient homozygous for the G1528C mutation (right hand panel) showed identical elution patterns for enoyl-CoA hydratase and 3-ketoacyl-CoA thiolase activity (compare panels *A* and *E* with *B* and *F* in Fig. 2). Although the profile for the thiolase activity is the same, the absolute activity in these fractions was lower as compared to control cells which agrees with the reduced thiolase activity in whole cell homogenates (59% of control, see (9). The long-chain enoyl-CoA hydratase and long-chain 3-ketoacyl-CoA thiolase activities coelute in exactly the same fractions (13.8 ml) as with control fibroblasts whereas long-chain 3-hydroxyacyl-CoA dehydrogenase activity was very low in these fractions. According to the identical elution profiles of long-chain enoyl-CoA hydratase and long-chain 3-ketoacyl-CoA thiolase activity which are components of MTP, we conclude that the molecular weights of the mutant MTP and the wild type MTP are about the same.

PCR-RFLP using genomic DNA. As reported earlier the common G1528C mutation can easily be identified in cDNA by PCR-RFLP with PstI (9). Initial experiments using the same primerset for PCR-RFLP on genomic DNA and cDNA gave aspecific amplification products, suggestive for a pseudogene (data not shown). We therefore started to analyze the genomic structure of the gene (and presumed pseudogene) for the α subunit. Meanwhile, the paper by Zhang and Baldwin (11) appeared reporting the genomic organization of a gene which was presumed to code for gastrin binding protein. The cDNA was found to show high similarity with that of the α subunit of MTP as described by Kamijo et al. (3). Interestingly, in the same paper Zhang and Baldwin (11) described the existence of a pseudogene.

On the basis of the DNA sequence published by Zhang and Baldwin (11) we designed two primers on the intron-exon boundaries flanking the mutation (intron 14 and 15). Using this primer set we obtained a specific PCR product after amplification with an estimated size of 640 bp. Direct sequencing of this PCR product confirmed that the amplified fragment was identical to the corresponding cDNA fragment also showing the exon-intron boundary as reported by Zhang and Baldwin (11). Furthermore we found a G→C mutation in the patient's DNA when compared to the control sequence, corresponding to nucleotide 1528 in the cDNA sequence. A second PstI restriction-site was predicted on the basis of this sequence (24 bp in intron 15). This second PstI restriction-site down stream of the mutation can be used as an internal control to check the efficiency of the restriction. PstI digestion of the amplified fragment (Fig. 3 *A*) revealed a different restriction pattern in the control (±465 and 175 bp) and the patient (±465, 117 and 58 bp), respectively (Fig. 3 *B*, 58-bp fragment not visible on photo). Indeed predicted fragments of 117 and 58 bp in length were obtained in the patient while these fragments were not present in the control. These results are consistent with homozygosity for the G1528C mutation in the patient in agreement with results obtained from cDNA analysis of the patient's father and mother. Using the method described above for analysis of genomic DNA we examined 34 LCHAD-deficient patients. All patients were found to carry the C1528 mutation. The majority of these patients (25/34) were homozygous for the G1528C mutation whereas nine patients were found to be heterozygous. Thus 59 of the 68 alleles investigated were found to carry the G1528C mutation (overall allele frequency: 87%).

Chromosomal localization of the α-MTP gene. On the basis of the genomic sequence we designed a second set of primers to amplify a large fragment suitable for in situ hybridization experiments. The primers were designed so that the amplified fragment of 10 kb contained 0.2 kb exon sequence only. A specific PCR product of 10 kb was obtained (not shown) which was used to localize the α-MTP gene. This fluorescence situ hybridization experiment (Fig. 4) showed labeling on the short arm of chromosome 2. The relative position of the signal on the chromosome axis was determined on 26 different chromosomes and revealed a localization at band 2p24.1–23.3 (95% confidence interval).

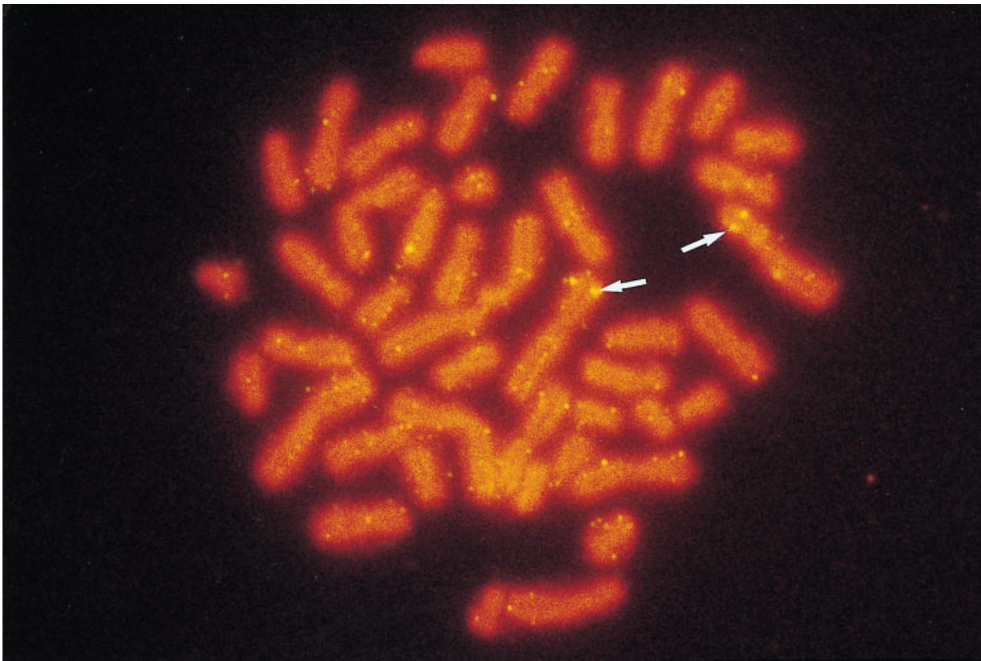

Figure 4. Metaphase cell after fluorescence in situ hybridization with a probe for the α-subunit of MTP. A PCR product (10 kb) amplified from genomic DNA is used as a probe for FISH. The sequence of this fragment ranges from nucleotide 887 to 1098 of the open reading frame of the α-subunit of MTP and include intron 9, 10, and 11. After labeling with biotin this fragment was used as a probe. Two specific signals (indicated by arrows) were present on both chromosomes 2p24.1-23.3.

Discussion

In this study we describe a method allowing identification of the G1528C mutation in the α subunit of the mitochondrial trifunctional protein using genomic DNA rather than cDNA. The genomic organization of the gene encoding the α subunit of the mitochondrial trifunctional protein gene was resolved recently by Zhang and Baldwin (11) who described the genomic organization of gastrin binding protein (GBP). As already recognized by the authors themselves, it appears that gastrin binding protein and the α subunit of MTP are one and the same. The cDNA sequence of the α subunit was recently found to be encoded by 20 exons (10) which is similar in structure to the genomic organization published by Zhang and Baldwin (11) giving further strength to the concept that GBP and α-MTP are identical. The GBP was initially purified and identified as a component of the gastrin/CCK-B receptor. This receptor is localized on the cell surface (18) where it controls the gastrin dependent acid secretion from parietal cells. The cloned "GBP"-gene is identical to the mitochondrial trifunctional protein cDNA, also carrying a mitochondrial targeting signal (3). A functional relation between the two proteins is therefore not very likely.

Using the genomic sequence published in detail by Zhang and Baldwin (11) for the selection of primers for PCR-RFLP analysis, we were able to amplify a fragment with a sequence identical to the corresponding part of the GBP. The presence of the G1528C mutation in the amplified fragment using patient's DNA strongly suggests that the "gastrin binding protein" gene characterized by Zhang and Baldwin (11) encodes the cDNA for the α-MTP as reported by Hashimoto and coworkers (3).

PCR-RFLP for this mutation is possible since the G1528C mutation creates a restriction site for PstI. Complete restriction of the PCR product at the position of the mutation indicates that only the α-MTP gene was amplified and not the related pseudo-gene. Since this pseudo-gene does not interfere with the PCR-RFLP analysis on genomic DNA and homo- and heterozygous detection is possible, this method is superior to screening for the G1528C mutation on cDNA as described before (9). Therefore this method could be used for prenatal diagnosis by identification of the G1528C mutation which requires only small amounts of chorion villi or amniotic fluid cells.

We re-investigated all patients of our previous study (9) using genomic DNA instead of cDNA for PCR-RFLP. In two patients analysis on genomic DNA clearly showed heterozygosity for the G1528C mutation whereas they were found to be homozygous for the G1528C mutation upon cDNA analysis. This indicates that the mutation in the second allele blocks correct mRNA formation or leads to an unstable mRNA.

Successful expression of the MTP protein in yeast depends on cotransformation of both the α and β subunit for full 3-hydroxyacyl-CoA dehydrogenase activity. The mutant α-MTP (Glu-510→Gln) protein was expressed normally but did not show any long-chain 3-hydroxyacyl-CoA dehydrogenase activity. This provides strong evidence that the common G1528C mutation observed in 87% of the alleles of LCHAD-deficient patients is, indeed, causal. The MTP protein is a heterooctamer and is composed of 4α and 4β subunits. Using gelfiltration chromatography we found an identical native molecular weight for the mutant MTP as compared with the wild type MTP, indicating that the G1528C mutation causes no major changes in the conformation of α-MTP or composition of the MTP protein. Since the mutant MTP has no long-chain 3-hydroxyacyl-CoA dehydrogenase activity the G1528C mutation is probably located in the active site of the dehydrogenase domain of MTP.

Recent data suggest a relationship between certain complications during pregnancy and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (10, 19, 20). This includes acute fatty liver of pregnancy (AFLP) and haemolysis, elevated liver enzymes and low platelets (HELLP)-syndrome. The high frequency of the G1528C mutation in our patient population suggests that analysis of this mutation in blood from women who previously had AFLP or HELLP-syndrome, may be a good

initial method to study the relationship between heterozygosity for LCHAD-deficiency and complications during pregnancy especially since detection of heterozygotes by enzyme analyses in white blood cells is unreliable.

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