Muscle-specific alternative splicing of myotubularin-related 1 gene is impaired in DM1 muscle cells

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The myotubularin-related 1 (MTMR1) gene belongs to a highly conserved family of eucaryotic phosphatases, with at least 11 members in humans. The founder member of this gene family, MTM1, is mutated in X-linked myotubular myopathy, a severe congenital disorder that affects skeletal muscle, and codes for myotubularin, a specific phosphatidylinositol 3-phosphate [PI(3)P] phosphatase. MTM1 and MTMR1 are adjacent on the X chromosome, and the corresponding proteins share 59% sequence identity. In the present study, we investigated the putative role of MTMR1 in myogenesis by analysing its expression pattern in muscle cells during differentiation and in skeletal muscle throughout development. We have identified three novel coding exons in the MTMR1 intron 2 that are conserved between mouse and human, are alternatively spliced, and give rise to six mRNA isoforms. One of the transcripts is muscle-specific and is induced during myogenesis both in vitro and in vivo, and represents the major isoform in adult skeletal muscle. We show that the two main MTMR1 protein muscular isoforms, like myotubularin, efficiently dephosphorylate PI(3)P in vitro. We have also analysed whether MTMR1 alternative splicing is affected in skeletal muscle cells derived from patients with congenital myotonic dystrophy (cDM1), in which mRNA splicing disturbances of specific genes are thought to constitute an important pathogenic mechanism. We found a striking reduction in the level of the muscle-specific isoform and the appearance of an abnormal MTMR1 transcript in differentiated cDM1 muscle cells in culture and in skeletal muscle from cDM1 patients. Our results suggest that MTMR1 plays a role in muscle formation and represents a novel target for abnormal mRNA splicing in myotonic dystrophy.

INTRODUCTION

The myotubularin-related 1 (MTMR1) gene belongs to a recently discovered large family of phosphatase genes conserved down to yeast, with 11 members in humans (including 3 phosphatase-inactive members) (1–3). The founder member of this gene family, MTM1, is mutated in X-linked myotubular myopathy (XLMTM), a congenital muscular disorder characterized by severe hypotonia and generalized muscle weakness in affected newborn males, leading, in most cases, to death during the first year of life (4–6). Histopathological studies of the skeletal muscle in these patients have suggested that the disease results from an arrest in late myogenesis, since muscle fibres contain centrally located nuclei surrounded by a halo devoid of myofibrils, which resemble fetal myotubes (7,8). Myotubularin, the MTM1 gene product, was recently shown to act as a phosphatase on the second messenger phosphatidylinositol 3-monophosphate [PI(3)P] in vitro and in yeast (9,10). This lipid is localized on endosomes (11), and regulates intracellular vesicle trafficking and autophagy (12). The MTM1 gene belongs to a phylogenetic subgroup that includes its two closest homologues, MTMR1 and MTMR2 (1,2,13). The latter was recently found mutated in Charcot–Marie–Tooth type 4B, a recessive demyelinating neuropathy (14). Whereas no mutations in the MTMR1 gene have been associated with a human disorder so far, this gene, which arose from an ancient MTM1 duplication and is adjacent to it on Xq28 (15), may share some biological functions with MTM1 and represents a good candidate for phenotype rescue of myotubularin-deficient muscle.

We therefore investigated whether the MTMR1 gene could play a role in myogenesis by analysing its expression pattern.
during muscle differentiation both in vitro and in vivo. We have uncovered a complex alternative splicing in this gene that leads to the synthesis of muscle-specific mRNA isoforms in both human and mouse. MTMR1 splicing pattern is not affected in XLMTM muscle cell cultures, but we show that it is strikingly altered both in cultured muscle cells and in skeletal muscle from patients with congenital myotonic dystrophy (cDM1), a disease with RNA splicing disturbances that shares some clinical and pathological features with X-linked myotubular myopathy (7,16,17).

RESULTS

Alternative splicing in the MTMR1 gene involves three novel coding exons

Mutations in the MTM1 gene are responsible for XLMTM, a severe muscular disorder, indicating that myotubularin plays a specific and important role in muscle formation or function that contrasts with its apparently ubiquitous expression. The MTM1 gene and its close homologue MTMR1 are adjacent in Xq28 (and in the syntenic region of the mouse X chromosome) (15). In order to understand the muscle specificity of MTMR1 function, we have compared the expression of MTM1 and MTMR1 (or their murine counterparts, Mtm1 and Mtmr1) during myogenesis. We studied Mtm1 and Mtmr1 mRNA levels, using semiquantitative RT–PCR, in the mouse myoblast line C2C12, which can be induced to fuse and differentiate into myotubes by shifting to a low serum concentration. We observed an upregulation of Mtm1 expression in C2C12 cells by >7-fold after 4 days in differentiating medium (Fig. 1), similar to a recent report (18). A somewhat more complex pattern was observed for Mtmr1, with a decreased expression in the initial phase of differentiation (from 6 to 48 hours), followed by an increase reaching, at 4 days, 2–3 times the initial level in myoblasts (Fig. 1).

Since alternative splicing is a well-known mechanism of regulation of gene function in muscle, we have systematically searched for the presence of alternative transcripts of the Mtm1 and Mtmr1 genes in C2C12 cells, by RT–PCR analysis of overlapping fragments encompassing the coding sequences. Whereas no mRNA variants were detected for the Mtm1 coding sequence (data not shown), analysis of Mtmr1 transcripts revealed the presence of four alternative mRNA isoforms, corresponding to an amplicon overlapping exons 2–5 (Fig. 2A). These amplification products were cloned and sequenced, revealing three novel protein-coding sequences of 24, 27 and 51 nucleotides present in various configurations in the alternative isoforms. These sequences are located between exons 2 and 3. Examination of human MTM1 intron 2 (the largest intron of the gene, ~19 kb, GenBank accession no. AF002223) showed the presence of three highly conserved sequences flanked by consensus splice sites, which we named exons 2.1, 2.2 and 2.3 (Fig. 2B and C). These exons were later identified in the mouse Mtmr1 genomic sequence deposited in databases (GenBank accession no. AF125314). The novel exons encode small peptides of 8 (2.1), 9 (2.2) and 17 (2.3) amino acids, which overall show 91% sequence identity between mouse and humans, and share no homology with known protein motifs (as searched in the Profile, Patterfind, Pratt and Pattinprot databases). They contain highly charged amino acids, either basic ones in both peptides 2.1 and 2.2 or acid ones in peptide 2.3 (Fig. 2C). According to these results, the MTMR1 gene contains 19 exons instead of the 16 described so far (15).

MTMR1 alternative splicing is regulated during myogenesis

We investigated by RT–PCR the presence of Mtmr1/MTMR1 mRNA isoforms during differentiation of muscle cells in culture, using the mouse C2C12 cell line and human fetal myoblasts. In both cell types, two major isoforms were observed in myoblasts prior to fusion, corresponding to the absence (isof orm A) or presence (isof orm B) of exon 2.1 (Figs 2A and 3). A third isof orm (C), which includes both exons 2.1 and 2.2, was detected very early (6 hours) after induction of mouse myoblast differentiation, and its level increased by about ~20-fold to become the major isof orm after 4 days of differentiation (when myotubes are predominant) (Fig. 3). The decreases in isof orms A and B and the parallel greater increase in isof orm C account for the pattern observed in Figure 1, where the total Mtmr1 message was analysed. Isof orm C also accumulates in human differentiating myoblasts, although to a lesser extent (which may reflect the lower percentage of myotubes in such cultures). The appearance of
Figure 2. Alternative splicing in the \textit{MTMR1} gene. (A) RT–PCR analysis of \textit{Mtmr1} between exons 2 and 5 from C2C12 myoblasts and myotubes after 48 hours in differentiation medium. Myoblasts contain mainly isoforms A (346 bp) and B (370 bp, including exon 2.1). Isoform C is predominant in myotubes (397 bp, with the presence of both exons 2.1 and 2.2), together with a minoritary band corresponding to isoform D (448 bp, which contains isoforms 2.1, 2.2 and 2.3). In the negative control (–RT), the reverse transcriptase was omitted from the reaction. M, size marker. (B) Genomic organization of the \textit{MTMR1} gene between exons 2 and 3. Diagram showing the nucleotide position of the novel exons 2.1, 2.2 and 2.3 (boxes) in intron 2 of both mouse (GenBank accession no. AF125314) and human (GenBank accession no. AF002223). (C) Nucleotide sizes of exons 2.1, 2.2 and 2.3, and exon/intron boundary sequences, are indicated. Peptide sequences translated from exons 2.1, 2.2 and 2.3 reveal the presence of highly charged amino acids. Sequence comparisons between mouse (m) and human (h) peptides 2.1, 2.2 and 2.3 show 87.5%, 100% and 88.2% of identity, respectively.
isoform C in muscle cell cultures correlates with the induction of embryonic myosin heavy-chain expression, an early myogenic marker, as opposed to the late upregulation of dystrophin mRNA (Fig. 3). PCR amplification of MEF2A (myocyte-specific enhancer factor 2A) isoforms was used as an internal control of muscle-specific alternative splicing (19).

We also analysed the presence of alternative Mtmr1 transcripts in vivo during skeletal muscle development and in other tissues. Isoform C was already detected at low levels at E13.5 in skeletal muscle, and gradually increased to become the major isoform around birth (Fig. 4A and C). In contrast, the relative proportion of Mtmr1 isoforms A and B diminish progressively, and they are present at very low levels in the adult tissue. Isoform D (containing exons 2.1, 2.2 and 2.3) was also induced during late embryonic muscle development, but remained at a low level thereafter. The pattern in heart is remarkably similar to that in skeletal muscle (Fig. 4B). In human muscle, we also observed an almost complete switch from isoform A in a 7-week embryonic muscle to isoform C in the adult tissue (Fig. 4A). Mtmr1 mRNA isoforms A and B were also found in other mouse tissues (Fig. 4B), with transcript B being relatively more abundant in adult liver and lung than in the corresponding embryonic tissues, and representing the predominant isoform in the adult brain. Two additional brain-specific transcripts were found, one containing exon 2.3 alone (isoform E) and another with both exons 2.1 and 2.3 (isoform F) (Fig. 4B and C). Thus, expression of isoform C appears to be specific to skeletal muscle and heart development, in mouse and human.

MTMR1 isoforms A and C have phosphoinositide phosphatase activity and similar subcellular localization

Myotubularin was shown to actively dephosphorylate the lipid PI(3)P in vitro and in vivo in yeast (9,10). We investigated whether MTMR1 protein isoforms display the same activity in vitro towards this substrate. We transiently expressed in HEK293 cells constructs encoding either the MTM1 protein or N-terminal FLAG-tagged MTMR1 isoforms A and C. The expressed proteins were immunoprecipitated using either an antibody against myotubularin (1G6) or an anti-FLAG antibody, and assayed for phosphatase activity using synthetic di-C8 fluorescent PI(3)P. Myotubularin and both MTMR1 isoforms A and C were able to similarly dephosphorylate this substrate (Fig. 5A). Thus, the inclusion of a 17-amino-acid stretch in the N-terminal part of MTMR1 (isoform C) gives rise to a functional phosphatase with no apparent change in its substrate specificity towards PI(3)P. Similar in vitro activity was also very recently demonstrated for the MTMR2, MTMR3 and MTMR6 proteins (18,20), as well as for MTMR1 isoform A (18).

We have studied the impact of these additional peptide sequences on the subcellular localization of MTMR1 isoforms. We found that mouse MTMR1 isoform A has a similar subcellular localization to its human and mouse MTM1 homologues (Fig. 5B, and not shown). The inclusion of 17 amino acids in the N-terminal part of MTMR1 isoform C does not modify this localization either in COS cells (not shown) or in C2C12 myoblasts and myotubes (Fig. 5B), indicating that this peptide does not target the protein to a different subcellular localization.
Figure 4. MTMR1 mRNA isoforms in several tissues. (A) Splicing events in the Mtmr1 gene during myogenesis. RT–PCR from mouse skeletal muscle (exons 2–5, left panel) at different developmental stages, from embryonic day (E) 13.5 to postnatal day (P) 11 and adult life. The relative proportion of Mtmr1 isoforms C and D increases progressively, and transcript C becomes predominant in adult muscle. RT–PCR analysis from human quadriceps muscle originating from a 7-week-old fetus and a 26-year-old control (exons 2–5, right panel) demonstrates the existence of the same Mtmr1 isoforms. Transcript C is also predominant in adult skeletal muscle. (B) Mtmr1 alternative splicing in mouse liver, lung, heart and brain at embryonic day (E) 13.5 and adults. The position of isoform B and brain-specific Mtmr1 transcripts E and F is shown. (C) Schematic representation of Mtmr1 alternative splicing in skeletal muscle and brain. The major isoforms in both embryonic and adult tissue are shown. Nucleotide position corresponding to exon 2–3 boundary in mouse Mtmr1 cDNA (GenBank accession no. AF073997).
Figure 5. (A) Both MTMR1-A and MTMR1-C exhibit PI(3)P 3-phosphatase activity. FLAG-tagged mouse MTMR1-A (R1-A) and MTMR1-C (R1-C) were transiently expressed in HEK293 cells and immunoprecipitated using anti-FLAG antibody. Wild-type human MTM1 was also expressed in these cells and immunoprecipitated using anti-MTM1 1G6 antibody. As control (lane C), protein extracts from untransfected HEK293 cells were used for immunoprecipitation using 1G6 antibody. Immunoprecipitated proteins were submitted to phosphatase assay using di-C8 fluorescent PI(3)P as described in Materials and Methods. A representative thin-layer chromatogram of phosphatase assay reaction products is shown. The positions of di-C8 fluorescent lipids are shown (PI, phosphatidylinositol). Phosphatase assays with immunoprecipitates from untransfected HEK293 cells using anti-FLAG antibody gave similar negative results to lane C (not shown).

(B) Subcellular localization of MTMR1 isoforms A and C. Examples of localization and co-localization of MTMR1 isoforms in transfected COS cells (top panels) and C2C12 myoblasts (middle panels) and myotubes (bottom panels). Mouse MTMR1-A co-localizes with human MTM1 in the cytoplasm and at the plasma membrane (top). MTMR1-A and MTMR1-C co-localize in C2C12 myoblasts (middle). The subcellular localizations of these two MTMR1 isoforms and MTM1 are also similar in multinucleated myotubes (bottom). Note the visible filamentous staining in some cells above the nuclei.
Figure 6. Alternative splicing of MTMR1 in congenital DM1 muscle cells. (A) Human fetal cDM1 myoblasts (hDM1) were grown in proliferative medium (Mb) and induced to differentiate for several hours in medium containing 5% FCS. The same reverse-transcribed RNAs were used for PCR amplification of several myogenic markers. Semiquantitative RT–PCR analysis of myosin heavy chains [embryonic (MHCe) and perinatal (MHCp)] and dystrophin was used to monitor the degree of differentiation of muscle cells. Induction of MTMR1 and MEF2A alternative splicing is shown. MTMR1 isoforms B and C are reduced, with transcript C (arrow) being relatively more decreased. An abnormal MTMR1 transcript (*, isoform G) appears in these cells. Tissue-specific MEF2A alternative splicing is not altered in cDM1 cells. (B) RT–PCR experiments from myoblasts (Mb) and myotubes grown for 4 days in differentiating medium (Mt), originating from four different congenital DM1 patients (hDM1), three normal persons (hCON), and one XLMTM patient. MTMR1 splicing is impaired only in cDM1 patients, as shown in (A). (C) Alternative splicing of MTMR1 in skeletal muscle from 15- and 26-week-old cDM1 fetuses (hDM1) and age-matched un-affected fetuses (hCON). The abnormal MTMR1 isoform G (*) is shown in both skeletal muscle and muscle cell cultures (4 days in differentiating medium) originating from cDM1 patients.
compartment under basal conditions. All the constructs showed a cytoplasmic signal together with labelling of the plasma membrane at ruffles, as described for the human homologues (20). The cytoplasmic localization does not appear diffuse, and enlargements clearly shows filamentous staining in some cells and above nuclei (Fig. 5B). The filamentous localization of MTMR1 isoforms and myotubulin in myotubes suggests that they may associate to the cytoskeletal network; however, recent studies have shown that myotubulin does not co-localize with known cytoskeletons (21).

**MTMR1 alternative splicing is impaired in congenital DM1 muscle cells**

As the pattern of MTMR1 splicing is profoundly altered during myogenesis, we decided to investigate whether it is affected in two severe neonatal muscle diseases: X-linked myotubular myopathy and the congenital form of myotonic dystrophy (cDM1). The two diseases share some clinical and pathological features, i.e. severe neonatal hypotonia and presence of muscle fibres containing centrally located nuclei that resemble fetal myotubes (8,16). It has been demonstrated that alternative splicing of cardiac troponin T and insulin receptor mRNAs is strikingly altered in skeletal muscle and muscle cultures derived from patients with myotonic dystrophy (22,23). Similar observations were made for the mRNAs encoding the microtubule-associated tau protein in brain from DM1 patients and from a mouse model of the disease (24,25). These manifestations are an indirect consequence of the expanded CTG repeat in the 3'-untranslated region (UTR) of the DMPK gene that causes the disease. We analysed the splicing pattern of MTMR1 mRNA in muscle cultures isolated from the quadriiceps of four congenital DM1 fetuses. The shift upon differentiation from isoform A to C was found to be strikingly impaired in the four cultures (Fig. 6A and B), while muscle-specific alternative splicing of the MEF2A (myocyte-specific enhancer factor 2A) transcription factor did not appear to be altered (compare Figs 6A and 3). In addition, a novel MTMR1 alternative transcript was detected at low levels in cDM1 but not in control cultures (Fig. 6A and B). This minor isoform was cloned and sequenced, and corresponds to the unique inclusion of exon 2.2 (isoform G, Fig. 4C). Since the differentiation and maturation of cDM1 skeletal myoblasts were recently shown to be delayed in culture (26,27), we analysed in parallel the expression of several myogenic markers. We found that upregulation of embryonic MHC mRNA, an early-induced gene, was similar to that observed in control cultures. Induction of later markers of differentiation (perinatal MHC and dystrophin) was indeed delayed and/or reduced (compare Figs 6A and 3). Thus, induction of MTMR1 mRNA isoforms and embryonic MHC splicing, two early differentiation-induced events, appear to be differentially affected in cDM1 muscle cultures. These results indicate that the alterations of MTMR1 alternative splicing in cDM1 are not an indirect consequence of a defect in myoblast fusion and maturation, but rather result from a direct and selective effect on RNA splicing. We also examined the MTMR1 splicing pattern in XLMTM myoblasts upon differentiation in vitro, and observed that it is similar to that in control cultures. Thus, the MTMR1 splicing pattern appears to be unaffected by the deficiency in MTM1 expression (Fig. 6B). We then investigated whether the abnormal MTMR1 isoform G exists in vivo, by comparing the expression of MTMR1 transcripts in skeletal muscle originating from five cDM1 transcripts and five normal fetuses aged between 15 and 37 weeks. We found the presence of transcript G in all cDM1 fetal samples (Fig. 6C, and not shown), suggesting that splicing disturbances in the MTMR1 gene also occur in human muscle affected by the disease.

**DISCUSSION**

The MTMR1 gene, together with MTMR2, is the closest homologue of the MTM1 gene that is mutated in the severe congenital muscle disorder X-linked myotubular myopathy (6). MTM1 and MTMR1 are adjacent to each other on Xq28. MTMR2 is also implicated in a human disorder (Charcot–Marie–Tooth 4B neuropathy), and thus appears to be necessary for Schwann cell rather than muscle function (14). Mutations in the MTMR1 gene, the only other member of this MTM1 family subgroup in the human genome, have not been described so far. In particular, mutational analysis of the MTMR1 gene in the rare patients with a myotubular myopathy phenotype where MTM1 mutations were not detected (some of them within X-linked pedigrees), gave negative results (28), even after analysis of the newly identified exons (A. Buj-Bello, unpublished work).

In the present study, we have obtained evidence for a role of the MTMR1 gene in myogenesis by analysing its expression pattern during muscle differentiation in vitro and in vivo. We show the existence of muscle-specific alternative splicing that affects the coding sequence and is highly conserved between mouse and human. The expression of this novel transcript, named isoform C, is induced early during myoblast differentiation in vitro and during skeletal muscle formation in vivo, and becomes the major mRNA isoform in both adult skeletal and cardiac muscles. We confirm that the MTMR1 protein, like myotubulin, acts as a PI(3)P phosphatase in vitro, as recently reported (18). This enzymatic function is conserved amongst other members of the MTM1 gene family (18,20). We have further shown that the substrate specificity of the MTMR1 protein is unchanged by the inclusion of 17 amino acids in its N-terminal part (isoform C). In addition, this small peptide does not target the protein to a different subcellular compartment, and does not disrupt or add any known localization domains. The recently identified GRAM domain (‘glucosyltransferases, Rab-like GTPases and myotubulins common domain’), located from amino acids 96 to 165 in mouse Mtmr1, starts eight amino acids after the additional sequences. The structure of this domain is found in several proteins known to regulate membrane-associated processes (29). The inclusion of the novel peptide may alter the conformation of the adjacent GRAM domain or alternatively regulate MTMR1 function by modulating its interaction with putative partners. Another alternative splicing event has been described for the human MTMR1 gene at exon 13 in brain, leading to a shorter protein missing the C-terminal part (15). Although the SET-interacting domain (SID) is still present in this alternative transcript (1), the functional PDZ (PSD-95, Discs-large, ZO-1) binding site is deleted (30).
We have also shown that alternative splicing of MTMR1 pre-mRNA is impaired in differentiating myoblasts derived from patients with congenital myotonic dystrophy. The induction of isoform C is severely reduced and an abnormal isoform G appears in cDM1 myotubes. It has been recently shown that in vitro differentiation and maturation of cDM1 myoblasts is delayed (26). This is unlikely to be responsible for the altered splicing of MTMR1, since induction of other early markers of differentiation, such as MEF2A splicing and embryonic MHC expression, appeared normal in cDM1 cultures. Moreover, an abnormal isoform appeared only in cDM1 muscle cell cultures. Importantly, this transcript was also found in skeletal muscle originating from cDM1 fetuses. Alternative splicing of selected genes is thought to constitute a major pathogenic target in DM1 (17). Abnormal expansion of the CTG repeat in the 3′-UTR region of the DMPK gene leads to accumulation of DMPK transcripts in the nuclei of DM1 muscle cells (31,32), and to an increased steady-state level of CUG-binding protein (CUG-BP) (23). This results in aberrant regulation of CUG-BP-dependent splicing events, as demonstrated for cardiac troponin T mRNA (22), and more recently for insulin receptor mRNA (23). The latter alteration is thought to contribute to the phenotype of insulin resistance in DM1 patients. Alterations in the splicing of chloride channel (CIC-1) mRNA have also been cited (33,34). Thus, MTMR1 is one of only four genes for which abnormal splicing has been reported in muscle tissue or cells in culture from DM1 patients. Alterations of splicing of the tau gene have been described in brain from patients or from a mouse DM1 model (24,25).

CUG-BP was originally thought to require binding to CUG repeats in its target pre-mRNAs (22). We found no CTG repeats (only isolated CTG motifs) in the intronic sequences surrounding MTMR1 exons 2.1 to 2.3, suggesting that non-CUG-containing intronic sequences may be the target of CUG-BP or other related proteins. Accordingly, a 110-nucleotide region without CUG repeats located in the insulin receptor intron 10 binds to CUG-BP and is required for aberrant splicing of exon 11 in cells overexpressing expanded CUG-RNA (23). Recent studies have shown that the preferred site for CUG-BP and a related protein, ETR-3, is constituted by UGU motifs rather than CUG, and that the 110-nucleotide region located in intron 10 of the insulin receptor contains TGT repeats and only one CTG motif (35). Indeed, an intronic sequence 39 bases 3′ to human exon 2.1 contains a TGT motif (TGTTGTGTTCTGT, position 126983, GenBank accession no. AF002223) that may regulate the inclusion of this exon in MTMR1 isoform C. A reduced efficiency in the splicing of exon 2.1 would explain the appearance of isoform G (containing only exon 2.2) and the reduced expression of isoform B observed in cDM1 muscle cells. Transfection studies using MTMR1 minigenes would clarify the functional relevance of this TGT motif in exon 2.1 inclusion.

The role of MTMR1 in myogenesis remains to be elucidated using functional studies and/or by the generation of knockout mice. The relevance of MTMR1 to the pathogenesis of myotonic dystrophy will also require analysis of its splicing pattern in skeletal muscle and heart from patients with juvenile or late-onset forms of the disease (or from mouse models of DM1), and studies of the effect of expression of the various isoforms in DM1 myoblast cultures. It will also be interesting to investigate the effect of MTMR1 mis-splicing on intracellular PI(3)P metabolism. MTMR1 overexpression may correct MTM1 deficiency in skeletal muscle, and thus open new genetic approaches for XLMTM therapy.

**MATERIAL AND METHODS**

**Human skeletal muscle**

Biopsies from quadriceps muscles were obtained during autopsies, in accordance with the French legislation on ethical rules. Fetal muscles were taken for diagnostic purposes from therapeutic abortuses or from premature or caesarean stillbirths. Five congenital DM1 (cDM1) and five unaffected fetuses were included in this study. Molecular diagnosis of the cDM1 fetuses showed that all of them have >1900 CTG repeats in the DMPK gene. Control biopsies revealed no histopathological changes.

**Mouse tissues**

CD1 mice were killed either by decapitation (embryos, P0 and P11 mice) or CO₂ inhalation (adults). Skeletal muscle from hindlimbs and other tissues were dissected and immediately frozen in liquid nitrogen.

**Cell cultures**

Human myoblast populations were isolated from three normal and four cDM1 muscle biopsies (with >1800 CTG repeats) as described previously (36). Myoblasts were grown in HAM's F10 medium (Gibco) supplemented with 50 μg/ml gentamycin (Biomedia) and 20% fetal calf serum (FCS; Biomedia). For myoblast differentiation, growth medium was removed from subconfluent cultures and replaced by DMEM (Gibco) containing 10 μg/ml of insulin and 100 μg/ml of transferrin (Sigma). C2C12 myoblasts were grown in DMEM (Gibco) supplemented by 20% FCS (Gibco) and 40 μg gentamycin, and differentiation was induced by decreasing serum concentration in the medium to 5%. COS cells were grown in DMEM (Gibco) containing 5% FCS (Gibco). All cultures were incubated at 37°C in a humid air atmosphere containing 5% CO₂.

**RT–PCR analysis**

Total RNA was extracted from muscle cells and tissues using Trizol reagent (Life Technologies), according to the manufacturer's instructions. RNA (5 μg) was reverse-transcribed in a total volume of 50 μl using dNTPs (1 mM), MgCl₂ (2.5 mM), DTT (5 mM), RNAsin (100 U; Promega), random hexamer (0.1 μg), oligo dT (0.1 μg), M-MLV reverse transcriptase (100 U; Gibco-BRL) in RT buffer (50 mM Tris–HCl pH 8.3, and 40 mM KCl). Eight overlapping sets of primers were used for screening the entire Mtmr1 open-reading frame in both C2C12 myoblasts and myotubes. Similarly, five overlapping sets of primers covering most of the Mtmr1 coding sequence (from exon 2 to 14) were used for RT–PCR analysis (not shown).

The sets of primers used for PCR amplification of mouse Mtmr1 and human MTM1 exons 2–5 were as follows: the forward primer was the same for both species,
5′-catgttgatggtgtaacag-3′ and the reverse primer was 5′-aattatccaggctgctgt-3′ for mouse and 5′-aatggtctagctgcggct-3′ for human. PCR was performed in a total volume of 25 μl, using 1.5 μl of cDNA (for semiquantitative RT–PCR, this amount was normalized with the amplification of Hprt cDNA) for 35 cycles at 94°C, 55°C and 72°C (10 s each). Primers for amplification of mouse Mef2A were 5′-cttgaattcaatgctggtg-3′ and 5′-tgcaatggctcagaga-3′, and for amplification of human MEF2A they were 5′-gtgaattcagctgaaacag-3′ and 5′-gctggtcaggtatagct-3′, both for 35 cycles. MTMR1 and MEF2A PCR products were loaded in either 3% agarose gels (Fig. 2A), 5% polyacrylamide gels (Figs 3, 4A, 4B, 6A and 6B) or 12.5% polyacrylamide gels (GeneGel Excel 12.5/24 kit, Amersham Pharmacia; Fig. 6C). The sets of primers used for semiquantitative RT–PCR were as follows: Mtm1 exons 10–12, 5′-gaggatgatgtagtgac-3′ and 5′-gatcgacatgctagttt-3′ for 25 cycles; Mtm1 exons 5–8, 5′-acagacgcatgggtgat-3′ and 5′-aacaatctgctggtgag-3′ for 28 cycles; mMHCe, 5′-acagcaaggaacagagga-3′ and 5′-cctgctgtgtcctcag-3′ for 24 cycles; hMHCe, 5′-gaataatccggagaagc-3′ and 5′-aagttcaggctttgc-3′ for 24 cycles; hMHCp, 5′-gcaatggccttcagttcag-3′ and 5′-gattatggcagcagtcg-3′ for 25 cycles; Mtm1 exons 8, 5′-gagtaaagctcttcctagc-3′ and 5′-cttgattggaaatactggtgc-3′, both for 30 cycles; human HPRT, 5′-tattttgctcctctcttcc-3′ and 5′-tctttggatcttttttc-3′ for 25 cycles; mouse Hprt, 5′-gaggaacctcttcctttc-3′ and 5′-gattatggcagcagttc-3′ for 25 cycles.

Plasmids, constructions and sequence analysis

The full-length open reading frame (ORF) of the human MTM1 gene (Genbank accession no. U460204) was subcloned as described into the pCS2 eukaryotic expression vector (9). A 2.6 kb full-length cDNA of the mouse Mtmr1 gene (isoform A), isolated from an E10 mouse embryo cDNA library (13), was subcloned into the pTL10–FLAG expression vector (derived from the pSG5 vector, which contains a SV40 promoter; Dr Devys, IGMC). The FLAG epitope is located at the N-terminal region of MTMR1. MTM1 isoform C was obtained from PTL10–FLAG–MTMR1-A by PCR cloning using overlapping primers that contain exon 2.1 + 2.2 sequences. The sequences of Mtmr1 cDNA isoforms between exons 2 and 5 (ABI 310 sequencer) were obtained after cloning column-purified PCR fragments (NucleoSpin, Machery-Nagel) into the pGEM-T vector (Promega). Sequences for Mtmr1 isoforms B to F are deposited in the GenBank database, accession numbers: AY099894–AY099898.

Cell transfection, immunoprecipitations and phosphatase assays

HEK293 cells were grown in Dulbecco +5% FCS with 400 µg/ml gentamycin. MTM1, MTM1-A or MTM1-C cDNAs (1 µg) were transfected in HEK293 cells plated in six-well plates using Effectene (Qiagen) according to the manufacturer’s instructions. After 24h expression, cells were washed once in cold PBS and lysed in RIPA buffer (20 mM Tris, pH 7.4, 90 mM NaCl, 4 mM EDTA, 1% triton X-100 and antiprotease cocktail). After a preclearing of the lysate with protein A for 30 min, the antibody [either anti-MTM1 1G6 antibody (IGMC) or M2 anti-FLAG antibody (Sigma)] were added to the supernatant with 30 µl of protein A and incubated for 3 h at 4°C. The immunoprecipitates were then washed five times with lysis buffer, divided in two and washed two more times with phosphatase buffer without DTT (37) and assayed for lipid phosphatase activity. Half of the immunoprecipitates were analysed by western blot using either 1G6 (1:5000) or anti-FLAG (1:5000) antibodies (not shown).

Phosphatase assays for immunoprecipitated proteins were carried out using synthetic di-C8-fluorescent PI(3)P (Echelon Research Laboratories, Salt Lake City, UT, USA) as described by Taylor and Dixon (37). Briefly, immunoprecipitated proteins were incubated for 30 min at 30°C in 30 µl of buffer containing 50 mM ammonium acetate (pH 6.0) and 2 mM diethiothreitol with 1 µg di-C8–NBD–PI(3)P. Reaction products were dried under nitrogen, resuspended in 10 µl of methanol/2-propanol/glacial acetic acid (5/5/2) and spotted onto a thin-layer chromatogram (Silica G60, Merck). The chromatogram was resolved in a solvent system consisting of chloroform/methanol/acetic/glacial acetic acid/water (70/50/20/20). Fluorescent lipids were visualized by ultraviolet light.

Immunofluorescence microscopy

COS cells and C2C12 myoblasts and myotubes were grown onto a 22 mm × 22 mm glass coverslip, transiently transfected with 1 µg of DNA using Ex-Gen 500 (Euromedex, France), washed 12 h after transfection and allowed to grow for another 24 h. Cells were fixed with 4% paraformaldehyde and permeabilized in PBS with 0.3% Triton X-100. Subcellular localization of the different constructs was assessed using monoclonal antibodies 1G6 for human MTM1 (1/1000, IGMC) and anti-FLAG antibodies for MTMR1 constructs (1/1000, IGMC). Cy3-labelled goat anti-mouse or biotin-conjugated donkey anti-rabbit were used at 1/400 (Jackson Immunoresearch Laboratories), together with fluorescein (DTAF)-conjugated streptavidin (Immunotech) for single and co-localization. Fluorescence was examined under a DMLB microscope (Leica).

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