Myotubularin and MTMR2, Phosphatidylinositol 3-Phosphatases Mutated in Myotubular Myopathy and Type 4B Charcot-Marie-Tooth Disease*

Myotubularin is the archetype of a family of highly conserved protein-tyrosine phosphatase-like enzymes. The myotubularin gene, MTM1, is mutated in the genetic disorder, X-linked myotubular myopathy. We and others have previously shown that myotubularin utilizes the lipid second messenger, phosphatidylinositol 3-phosphate (PI(3)P), as a physiologic substrate. We demonstrate here that the myotubularin-related protein MTMR2, which is mutated in the neurodegenerative disorder, type 4B Charcot-Marie-Tooth disease, is also highly specific for PI(3)P as a substrate. Furthermore, the MTM-related phosphatases MTMR1, MTMR3, and MTMR6 also dephosphorylate PI(3)P, suggesting that activity toward this substrate is common to all myotubularin family enzymes. A direct comparison of the lipid phosphatase activities of recombinant myotubularin and MTMR2 demonstrates that their enzymatic properties are indistinguishable, indicating that the lack of functional redundancy between these proteins is likely to be due to factors other than the utilization of different physiologic substrates. To this end, we have analyzed myotubularin and MTMR2 transcripts during induced differentiation of cultured murine C2C12 myoblasts and find that their expression is divergently regulated. In addition, myotubularin and MTMR2 enhanced green fluorescent protein fusion proteins exhibit overlapping but distinct patterns of subcellular localization. Finally, we provide evidence that myotubularin, but not MTMR2, can modulate the levels of endosomes. PI(3)P. From these data, we conclude that the developmental expression and subcellular localization of myotubularin and MTMR2 are differentially regulated, resulting in their utilization of specific cellular pools of PI(3)P.

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Myotubularin (MTM1)1 is a dual specificity protein-tyrosine phosphatase (PTP)-like enzyme that is mutated in X-linked myotubular myopathy, a severe congenital disorder in which muscle cell development is compromised (1–3). Myogenesis in affected individuals is arrested at a late stage of differentiation/maturation following myocyte formation, and the muscle cells have characteristic large centrally located nuclei (1). The MTM1 protein is the first characterized member of one of the largest families of dual specificity PTPs yet identified (reviewed in Refs. 4 and 5). The MTM family includes at least eight putative catalytically active proteins as well as four forms that are predicted to be enzymatically inactive (4–7). The inactive MTM proteins contain substitutions at specific residues that are required for catalysis by PTP superfamily enzymes and may function as interaction modules (4–8). Phylogenetic analysis of MTM family proteins indicates that they can be further divided into at least four distinct subgroups, which include the catalytically active MTM1/MTMR1/MTMR2, MTMR3/MTMR4, and MTMR6/MTMR7/MTMR8 enzymes, as well as the SBF1/LIP-STYX/MTMR10/3-PAP inactive forms (4–7). Our laboratory and others have previously shown that myotubularin specifically dephosphorylates the D3 position of the inositol lipid, phosphatidylinositol 3-phosphate (PI(3)P) (9, 10). Recent reports have also shown that MTMR3 and MTMR4 (also referred to as FYVE-DSP1 and FYVE-DSP2, respectively) can utilize PI(3)P as a substrate (11, 12). Together, these findings suggest that all of the active MTM family members may function to regulate cellular PI(3)P levels. Although it is well established that PI(3)P can serve as a targeting motif for membrane-trafficking and signaling proteins that contain lipid binding modules such as FYVE, pleckstrin homology, and Phox homology domains (13–20), the precise physiologic roles of MTM family phosphatases as regulators of PI(3)P are unclear.

In addition to MTM1 mutations identified in X-linked myotubular myopathy, a second MTM-related gene has been associated with a human genetic disease. Mutations in the MTMR2 gene are causative for the neurodegenerative disorder, type 4B Charcot-Marie-Tooth (CMT) syndrome, which is a hereditary demyelinating peripheral neuropathy that results from improper Schwann cell development (21–23). Patients with type 4B CMT exhibit abnormal focally folded myelin in the neural sheath (21). The difference in the pathologies of myotubular myopathy and type 4B CMT are particularly intriguing in light of the fact that MTM1 and MTMR2 are both expressed in adult skeletal muscle and neuronal tissues and are highly similar (64% identity, 76% similarity) (24). Although their specific physiologic roles are not known, it is clear from the different pathologies manifested in myotubular myopathy and CMT dis-
ease that MTM1 and MTMR2 are not functionally redundant. The lack of functional overlap between these proteins may have several possible explanations. Although they are highly similar at the protein level, MTM1 and MTMR2 may have distinct substrate preferences. Alternatively, although they are expressed in both adult skeletal muscle and neuronal tissues, MTM1 and MTMR2 may play important roles during specific developmental stages and thus be expressed differently during maturation of specific tissues. Finally, they may both act on PI(3)P but be localized to distinct subcellular compartments, thus regulating different cellular pools of this lipid. Consistent with this notion, a recent study has demonstrated that PI(3)P can be detected at multiple discrete sites within internal vesicular structures, including endosomes and endosomal carrier vesicles as well as the nucleolus (25).

In the current study, we have undertaken the enzymatic characterization of MTM family phosphatases to determine whether utilization of the inositol lipid, PI(3)P, as a substrate is common among these enzymes. As a first step toward understanding the molecular basis of MTM function in the human genetic disorders, myotubular myopathy and type 4B CMT, we have also conducted a detailed comparison of the enzymatic properties of recombinant MTM1 and MTMR2. Our results demonstrate that like MTM1, MTMR2 can act on PI(3)P, providing a direct link between inositol lipid regulation and type 4B CMT. In addition, we have analyzed the subcellular localization and expression of MTM1 and MTMR2 during myogenic differentiation to determine whether these factors might contribute to their functional regulation. Finally, we present evidence that MTM1 and MTMR2 are likely to act on distinct cellular pools of PI(3)P.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**COS-1 and C2C12 cells were maintained at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. C2C12 cell differentiation was induced in Dulbecco’s modified Eagle’s medium containing 5% horse serum, 50 units/ml penicillin, and 50 μg/ml streptomycin for up to 5 days at 37 °C with 5% CO2.

**Vectors for the expression of bacterial recombinant His-tagged fusion proteins used for phosphatase assays were expressed in Escherichia coli BL21 (DE3) Codon Plus (Stratagene) and purified using Ni2+-agarose affinity resin as previously described for SacIcp (28).** FLAG-tagged MTM1 proteins used for phosphatase assays were expressed in COS-1 cells and purified using anti-FLAG M2 affinity resin (Sigma) as previously described for MTMR6 (27). C2C12 wild-type or Myotubularin-deficient myotubes were transfected with GST-2 FYVE protein was expressed in Escherichia coli DH5α cells and purified over glutathione-agarose affinity resin. Briefly, cells harboring the pGEX-2X FYVE plasmid were grown in 2X YT medium containing 100 μg/ml ampicillin to an A600 of 0.7, and then protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (0.5 mM) for 2 h at 37 °C. The cells were harvested by centrifugation and resuspended in PBS (pH 7.4) containing 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml (each) aprotinin, leupeptin, and pepstatin (30 ml of lysis buffer per liter of cultured cells). The cells were disrupted by sonication, and Triton X-100 was added to 0.5% (v/v). The crude lysate was centrifuged for 30 min at 18,000 × g to remove unbroken cells and insoluble debris. The soluble fraction was incubated with glutathione-agarose affinity resin (1 ml of 50% slurry in PBS/liter of culture) for 2 h at 4 °C. The resin was washed three times for 5 min each with lysis buffer containing 0.5% Triton X-100, followed by two 5-min washes in lysis buffer without detergent. The GST-2X FYVE fusion protein was eluted from the resin for 2 × 30 min with 0.5 mM of lysis buffer containing 25 mM reduced glutathione (pH 7.4) and filtered through a 0.2-μm syringe filter. Free glutathione was removed by gel filtration chromatography, and the reaction products were visualized under UV light (27).

**Phosphatase Assays—**All phosphoinositide and soluble inositol phosphate assays in this work were obtained from Echelon Research Laboratories (Salt Lake City, UT). Phosphatase assays using FLAG-tagged MTM1 proteins immunoprecipitated from COS-1 cells were carried out on anti-FLAG M2 affinity resin in reaction buffer containing 1.5 μg of di-C6-NBD-phosphatidylinositol 3-phosphate (Echelon) as described (27). Samples were separated by thin layer chromatography, and the reaction products were visualized under UV light (27). Phosphatase assays with bacterial recombinant MTM1, MTMR1, MTMR2, and MTMR6 using synthetic di-C6-phosphoinositide or soluble inositol phosphate substrates were carried out at 30 °C, and phosphate release was determined using a malachite green-based assay for inorganic phosphate as described (28). The artificial protein substrates phosphatase substrates myelin basic protein and casein were phosphorylated with [γ-32P]ATP on tyrosyl or seryl/threonyl residues and used as substrates for recombinant MTM1 and MTMR2 as previously described (9).

**Northern Blot Analysis—**Total RNA was isolated from C2C12 cells with TRIzol reagent as recommended by the manufacturer (Invitrogen). RNA samples (15 μg) were then electrophoresed through a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The blots were hybridized with digoxigenin-labeled probes, and mRNAs corresponding to murine MTM1 and MTMR2 were detected using a digoxigenin chemiluminescence detection kit (Roche Molecular Biochemicals). A murine MTM1 cDNA probe corresponding to the 3′-untranslated region (283 bp) was amplified by PCR using expressed sequence tag clone AW911859 (Research Genetics/Invitrogen) as a template. A cDNA probe corresponding to the murine MTMR2 5′-region (nucleotides 5 to 3′- HindIII sites of pET21a in-frame with the six-histidine tag.

Vectors for the expression of N-terminally FLAG-tagged MTM1 wild type and C375S mutant proteins in mammalian cells have been previously described (9). Mammalian expression vectors for N-terminally FLAG-tagged MTMR1, MTMR2, MTMR3, and MTMR6 proteins were generated by inserting cDNA fragments containing open reading frames encoding each of these proteins into the 5′-BamHI/3′-NotI, 5′-NheI/3′-KpnI, 5′-NheI/3′-XbaI, and 5′-BamHI/3′-XbaI, respectively, of pcDNA3.1-NF (9).

**Bacterial Protein Expression and Purification—**Bacterial recombinant MTM1 and MTMR2 C-terminally His-tagged fusion proteins used for phosphatase assays were expressed in E. coli and purified as described (9). A cDNA fragment containing the complete open reading frame of MTMR6 was inserted into the 5′-BglII and 3′-KpnI sites of the pEGFP-C1 vector (CLONTECH) to create pEGFP-MTMR2. A vector for bacterial expression of tandem FYVE domains from the murine hepatectogen factor-regulated tyrosine kinase substrate (Hrs) protein fused to GST (GST-2X FYVE; see Ref. 25) was the generous gift of Dr. Kathleen Collins (University of Michigan).

**Protein Expression and Purification—**Bacterial recombinant MTM1 and MTMR2 C-terminally His-tagged fusion proteins used for phosphatase assays were expressed in COS-1 cells and purified using anti-FLAG M2 affinity resin (Sigma) as previously described for MTMR6 (27). C2C12 wild-type or Myotubularin-deficient myotubes were transfected with GST-2 FYVE protein was expressed in Escherichia coli DH5α cells and purified over glutathione-agarose affinity resin. Briefly, cells harboring the pGEX-2X FYVE plasmid were grown in 2X YT medium containing 100 μg/ml ampicillin to an A600 of 0.7, and then protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (0.5 mM) for 2 h at 37 °C. The cells were harvested by centrifugation and resuspended in PBS (pH 7.4) containing 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml (each) aprotinin, leupeptin, and pepstatin (30 ml of lysis buffer per liter of cultured cells). The cells were disrupted by sonication, and Triton X-100 was added to 0.5% (v/v). The crude lysate was centrifuged for 30 min at 18,000 × g to remove unbroken cells and insoluble debris. The soluble fraction was incubated with glutathione-agarose affinity resin (1 ml of 50% slurry in PBS/liter of culture) for 2 h at 4 °C. The resin was washed three times for 5 min each with lysis buffer containing 0.5% Triton X-100, followed by two 5-min washes in lysis buffer without detergent. The GST-2X FYVE fusion protein was eluted from the resin for 2 × 30 min with 0.5 mM of lysis buffer containing 25 mM reduced glutathione (pH 7.4) and filtered through a 0.2-μm syringe filter. Free glutathione was removed by gel filtration chromatography, and the reaction products were visualized under UV light (27).
RESULTS AND DISCUSSION

The association of MTM1 and MTMR2 with the respective genetic disorders myotubular myopathy and type 4B Charcot-Marie-Tooth syndrome is of significant interest because although they are highly similar proteins and exhibit overlapping expression patterns in human tissues, they do not appear to be functionally redundant (2, 21–23). As a first step toward understanding the functional differences between MTM1 and MTMR2, we analyzed their enzymatic properties to determine whether they possessed similar activity and/or substrate specificity. Recombinant MTM1 and MTMR2 fusion proteins were expressed and purified as described under “Experimental Procedures,” and their phosphatase activity was tested toward a panel of substrates including phosphoinositides, soluble inositol phosphates, and radiolabeled artificial protein substrates. As shown in Table I, the activity of MTMR2 was indistinguishable from that of MTM1 over the panel of substrates tested. Both enzymes dephosphorylated PI(3)P >50-fold more efficiently than any other substrate, demonstrating that they are extremely poor protein phosphatases.

The identification of MTM1 and MTMR2 as PI(3)P-specific phosphatases led us to question whether activity toward this inositol lipid is a conserved property among other active myotubularin family enzymes. We therefore tested the ability of the myotubularin family phosphatases MTM1, MTMR1, MTMR2, MTMR3, and MTMR6, which represent each of the three active MTM subgroups (see Ref. 4), to function as PI(3)P-specific inositol lipid phosphatases. FLAG epitope-tagged MTM proteins were transiently overexpressed in COS-1 cells and immunoprecipitated with anti-FLAG affinity resin, and their ability to dephosphorylate PI(3)P was determined using a qualitative inositol lipid phosphate assay that employs a water-soluble fluorescent substrate (di-C6-NBD-phosphatidylinositol-3-phosphate) combined with thin layer chromatography as previously described (27). As shown in Fig. 1A, human MTM1, MTMR1, MTMR2, MTMR3, and MTMR6 immunoprecipitates each converted PI(3)P to phosphatidylinositol (Fig. 1A, lanes 3–7, respectively). As expected, immunoprecipitates from cells transfected with vector alone (Fig. 1A, lane 1) or a vector encoding the catalytically inactive MTM1 C375S mutant (Fig. 1A, lane 2) showed no activity toward PI(3)P. The expression of MTM proteins in the lysates of transfected cells used for anti-FLAG immunoprecipitation and lipid phosphate assays was confirmed by immunoblotting with anti-FLAG antibody (Fig. 1B). Because both MTM1 and MTMR2 are highly specific for PI(3)P as a substrate (Table I), we wanted to determine how the substrate preferences of MTM1 and MTMR6 might compare with those of MTM1 and MTMR2. To accomplish this, we performed lipid phosphate assays using bacterial recombinant MTM1 and MTMR6 proteins with the complete panel of phosphoinositide substrates. As shown in Fig. 2, both MTMR1 and MTMR6 were also highly specific for PI(3)P as a substrate. It should be noted that the specific activities of recombinant MTM1 and MTMR6 toward PI(3)P were ~30- and 100-fold lower than those of MTM1 and MTMR2 (Fig. 2, Table I) but are similar to those observed for recombinant MTMR3 and MTMR4 (11). A recent report has also suggested that MTMR3 may function to regulate levels of the inositol lipid, PI(3,5)P2.
have been unable to detect activity toward PI(3,5)P₂ utilizing FLAG-tagged MTMR3 immunoprecipitated from mammalian cells (not shown). It is unclear whether this discrepancy may be the result of differences in the preparations of MTMR3 used or in lipid phosphatase assay conditions. Regardless, the results presented here not only identify MTMR1, MTMR2, and MTMR6 as PI(3)P-specific inositol lipid phosphatases but further support the hypothesis that PI(3)P is likely to be a common substrate among all of the active MTM proteins. Furthermore, the finding that MTMR2 is specific for PI(3)P as a substrate suggests a possible link between type 4B CMT and phosphoinositide signaling.

Having established that MTM1 and MTMR2 are both specific and efficient catalysts toward PI(3)P, we next asked whether other factors such as subcellular localization, developmentally regulated expression, or utilization of distinct substrate pools might be responsible for the differences in their function(s). To analyze their subcellular localization, MTM1 and MTMR2 were expressed as EGFP fusion proteins in COS-1 cells and visualized by fluorescence microscopy. As expected, cells expressing EGFP alone exhibited staining in both the nucleus and cytoplasm (Fig. 3A). EGFP-MTM1 (Fig. 3B) and EGFP-MTMR2 (Fig. 3C) displayed a cytosolic staining pattern, with the highest staining intensity in the perinuclear region. In contrast to EGFP-MTMR2, EGFP-MTM1 staining was also detected in large membrane projections and at the cell periphery (Fig. 3B). This staining pattern is similar to that observed previously for an MTM1 putative substrate-trapping mutant protein, which localized to plasma membrane extensions in HeLa cells (10). The dramatic difference in the morphology of COS-1 cells transfected with either EGFP-MTM1 or EGFP-MTMR2 fusion protein expression constructs also provides evidence of their distinct properties. EGFP-MTM1 overexpression resulted in the formation of membrane projections (Fig. 3B), whereas EGFP-MTMR2 did not (Fig. 3C). Although the precise molecular basis of this phenomenon is unclear, it highlights a difference between the effects of MTM1 and MTMR2 overexpression. Collectively, these findings suggest the possibility that MTM1 and MTMR2 may perform different functions by targeting to different subcellular environments.

We have also examined the expression of MTM1 and MTMR2 during myogenic differentiation in murine C2C12 myoblasts, which can be induced to differentiate into myotubes by growth in low mitogen medium. To accomplish this, proliferating C2C12 cells at confluence were cultured to confluence in high mitogen medium (Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum), and differentiation was then induced for 5 days by switching the cells to low mitogen medium (Dulbecco’s modified Eagle’s medium containing 5% horse serum). Differentiation of C2C12 cells was monitored visually by the formation of multinucleated myotubes and by analyzing the expression of a myogenic differentiation marker, myosin heavy chain, which commenced ~40 h after the cells were switched to DMSO-containing differentiation medium.
MTM1 and MTMR2 Phosphoinositide Phosphatases

FIG. 4. Divergent expression of MTM1 and MTMR2 during myogenic differentiation. C2C12 myoblast cells were grown to confluence and induced to differentiate in low mitogen medium. A, immunostaining of myosin heavy chain as a marker for myogenic differentiation. B, at the indicated times, total RNA was isolated and separated on an agarose-formaldehyde gel, and Northern blot analysis was performed using digoxigenin-coupled cDNA probes specific for murine MTM1 or MTMR2. The mRNAs corresponding to MTM1 and MTMR2 were visualized using alkaline phosphatase-conjugated anti-digoxigenin antibody coupled with chemiluminescence. Ethidium bromide staining of the same gel was used to monitor nucleic acid loading in each lane.

Low mitogen media (Fig. 4A). To probe the expression of MTM1 and MTMR2 during myogenic differentiation, total RNA was isolated from day 0 to day 5 following the change to low mitogen medium, and the levels of MTM1 and MTMR2 transcripts were analyzed by Northern hybridization using transcript-specific probes. As shown in Fig. 4B, MTM1 expression was up-regulated during the differentiation of C2C12 cells, whereas MTMR2 expression steadily declined over the same period. Identical results were obtained using quantitative RT-PCR (not shown). These data are consistent with a role for MTM1 at a late step of myogenic differentiation/maturity and suggest that the expression of MTM1 and MTMR2 is differentially regulated during myogenic development.

In addition to their differential expression, another possible explanation for the lack of functional redundancy between MTM1 and MTMR2 is that they may regulate different subcellular pools of PI(3)P. To test this hypothesis, we used a biotinylated bacterial recombinant GST-2×FYVE fusion protein as a probe to specifically label intracellular PI(3)P. FYVE domains are modified zinc finger-like domains that specifically recognize and bind PI(3)P, thus serving to target proteins to distinct sites within cells (13–15). Previous work has shown that a biotinylated GST fusion protein containing tandem FYVE domains binds specifically to PI(3)P with high affinity (25). As expected, COS-1 cells stained with biotinylated GST-2×FYVE probe exhibited a punctate staining pattern representing endosomal structures where PI(3)P is abundant (Fig. 5A) (13, 25).

Treatment of COS-1 cells with the phosphatidylinositol 3-kinase inhibitor, wortmannin (100 nM) (B). Cells overexpressing EGFP-MTM1 (C–E) or EGFP-MTMR2 (F–H) fusion proteins were also examined. At 24 h post-transfection, cells were fixed overnight at 4°C with 4% paraformaldehyde and examined by confocal fluorescence microscopy. Green fluorescent staining for EGFP-MTM1 and EGFP-MTMR2 is shown in C and F, respectively. Red fluorescent staining for Cy3-conjugated streptavidin bound to the biotinylated GST-2×FYVE fusion protein is shown in D and G, respectively. In the merged images (E and H), the yellow color indicates colocalization.

FIG. 5. MTM1 can dephosphorylate an endosomal pool of PI(3)P. A recombinant biotinylated GST-2×FYVE probe was used to localize and quantify endogenous PI(3)P in COS-1 cells. As controls, untransfected cells were analyzed following treatment for 20 min without (A) or with the PI3-kinase inhibitor, wortmannin (100 nM) (B). Cells overexpressing EGFP-MTM1 (C–E) or EGFP-MTMR2 (F–H) fusion proteins were also examined. At 24 h post-transfection, cells were fixed overnight at 4°C with 4% paraformaldehyde and examined by confocal fluorescence microscopy. Green fluorescent staining for EGFP-MTM1 and EGFP-MTMR2 is shown in C and F, respectively. Red fluorescent staining for Cy3-conjugated streptavidin bound to the biotinylated GST-2×FYVE fusion protein is shown in D and G, respectively. In the merged images (E and H), the yellow color indicates colocalization.
becomes unrestricted, rendering it capable of depleting endosomal PI(3)P. We therefore conclude that MTM1 and MTMR2 are likely to regulate different subcellular pools of PI(3)P by virtue of their subcellular localization and differential expression during development.

We have provided the first evidence that MTMR2, a phosphatase mutated in type 4B Charcot-Marie-Tooth syndrome, utilizes the lipid second messenger, PI(3)P, as its physiologic substrate. Because mutations in the MTMR2 gene associated with type 4B CMT would disrupt the phosphatase activity of MTMR2, it is probable that this disease results from improper regulation of PI(3)P. Although the exact mechanisms by which failure to dephosphorylate PI(3)P contributes to myotubular myopathy and type 4B CMT are unclear, the identification of this lipid as a specific target for MTM1, MTMR2, and other MTM family phosphatases represents a first step toward understanding their roles in human neuromuscular diseases. The similarity of MTM1 and MTMR2 protein sequences and enzymatic properties has also led us to question what factors might underlie the apparent lack of functional redundancy between these phosphatases. Collectively, our findings suggest that MTM1 and MTMR2 can be regulated not only by tissue-specific expression during development, but also by their subcellular localization and use of specific cellular pools of PI(3)P. It will now be important to identify downstream effectors of PI(3)P whose function is regulated by MTM1 or MTMR2 in order to pinpoint the signaling pathways that are affected by mutations associated with myotubular myopathy and type 4B CMT.

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