MTMR9 Increases MTMR6 Enzyme Activity, Stability, and Role in Apoptosis*

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Myotubularin-related protein 6 (MTMR6) is a catalytically active member of the myotubularin (MTM) family, which is composed of 14 proteins. Catalytically active myotubularins possess 3-phosphatase activity dephosphorylating phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-bisphosphate, and some members have been shown to form homomers or heteromeric complexes with catalytically inactive myotubularins. We demonstrate that human MTMR6 forms a heteromer with an enzymatically inactive member myotubularin-related protein 9 (MTMR9), both in vitro and in cells. MTMR9 increased the binding of MTMR6 to phospholipids without changing the lipid binding profile. MTMR9 increased the 3-phosphatase activity of MTMR6 up to 6-fold. We determined that MTMR6 is activated up to 28-fold in the presence of phosphatidyserine liposomes. Together, MTMR6 activity in the presence of MTMR9 and assayed in phosphatidyserine liposomes increased 84-fold. Moreover, the formation of this heteromer in cells resulted in increased protein levels of both MTMR6 and MTMR9, probably due to the inhibition of degradation of both proteins. Furthermore, co-expression of MTMR6 and MTMR9 decreased etoposide-induced apoptosis, whereas decreasing both MTMR6 and MTMR9 by RNA interference led to increased cell death in response to etoposide treatment when compared with that seen with RNA interference of MTMR6 alone. Thus, MTMR9 greatly enhances the functions of MTMR6.

Myotubularin proteins are a family of 14 proteins with the canonical dual specificity protein tyrosine phosphatase active site CX3R motif (1–3). Eight members of the myotubularin family possess catalytic activity, dephosphorylating phosphatidylinositol 3-phosphate (PtdIns-3-P)4 and phosphatidylinositol 3,5-bisphosphate (PtdIns-3,5-P2) at the D-3 position, and six members are not catalytically active because they lack the conserved cysteine residue in the protein tyrosine phosphatase motif that is required for activity. Interest in this group of proteins originated from the genetic evidence linking myotubularin, the founding member of this family, to myotubular myopathy, an X-linked disorder characterized by severe hypotonia and generalized muscle weakness (4). Subsequently, mutations in MTMR2 and in its inactive binding partner MTMR13 were linked to a subset of Charcot-Marie-Tooth disease type 4B, a demyelinating neurodegenerative disorder (5, 6).

Despite near identical substrate specificity, biochemical and genetic evidence supports the hypothesis that myotubularin proteins are not redundant and have unique functions within cells (2, 7–9). The mechanisms by which loss of function of myotubularin proteins produce diseases are not known. Current evidence supports the hypothesis that each myotubularin protein regulates a specific pool of PtdIns-3-P and/or PtdIns-3,5-P2, which in turn regulates a variety of cellular functions. Differences in tissue expression and subcellular localization play a role in the specificity of different myotubularins (10–15).

The functions of myotubularin proteins are altered by the formation of heteromers between catalytically active and inactive members of the family. The initial biochemical purification of MTM1 demonstrated the presence of MTM1 homodimers and MTM1-3-phosphatase adapter protein (3PAP) heteromers (16), which was later described as MTMR12 (15, 17). MTMR2 was found to form heteromers with MTMR5 (13) and MTMR13 (18), and MTMR7 formed heteromers with MTMR9 (19). In each case, a catalytically active myotubularin protein interacted with an inactive protein. Heteromerization generated two important effects: increased catalytic activity of the active component (13, 15, 19, 20) and targeting of the heteromer to specific subcellular locations (15). Mutations in the inactive member MTMR13 result in a similar phenotype in patients as the mutations in its catalytically active binding partner MTMR2, indicating an indispensable role for the catalytically inactive subunit (21).

Myotubularin proteins can be grouped into subfamilies based on homology. Closely related MTMR6, MTMR7, and MTMR8 comprise such a subfamily. We have previously char-
acterized the interaction between mouse MTMR7 and MTMR9 proteins (19). In this report, we characterize the interaction between human MTMR6 and MTMR9. MTMR6 and MTMR9 have been shown to form a heteromeric complex in mouse and Caenorhabditis elegans (19, 22). MTMR6 has been shown to inhibit the activity of a calcium-activated potassium channel (type KCa3.1) (23, 24). Two screening experiments implicate MTMR6 as a regulator of apoptosis. By RNA microarray analysis, increased MTMR6 expression was observed in B cell chronic lymphoid leukemia cells with increased resistance to irradiation-induced apoptosis (25), whereas in an RNA interference screen in HeLa cells, decreased MTMR6 expression promoted apoptosis (26).

Here we show that MTMR6 interacts with MTMR9 in vitro and in human cells. This interaction increases the phospholipid binding and enzymatic activity of MTMR6 in vitro. Co-expression of either subunit in cells dramatically increased the protein levels of the individual binding partners, suggesting that heteromer formation increases the stability of the proteins. Finally, MTMR9 was found to potentiate the effects of MTMR6 on apoptosis.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals**—All chemicals and reagents, unless specifically noted, were purchased from Sigma-Aldrich.

**Cell Culture, Transfection, and Treatment**—HeLa cells were maintained in culture using 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium. Unless noted, transfection was conducted by using Lipofectamine 2000 (Invitrogen). RNAi transfections were done using a Nucleofector kit (Amaxa, Gaithersburg, MD). The RNAi duplexes were obtained from Ambion (Austin, TX), and sequences are: control RNAi (luciferase) duplex, sense 5′-CUUACGCCUGAGUACCUUCGAdTdTdT3′; antisense, 5′-UCGAAUGACUCAGCGUAAGDtTdTdT-3′; MTMR6 RNAi (R6-1), sense, 5′-GGAACTATGGCCACTCATG-3′; antisense, 5′-TTTATGCGCCATTTCCATC3′; MTMR9 RNAi (R9-1), 5′-CAAGGAGGTGCTTTTGAAT3′. The specificity and efficacy were determined using quantitative reverse transcription-PCR (data not shown). There was about 50% reduction upon the RNAi treatment in both MTMR6 and MTMR9 followed by a stop codon in pcDNA4/TO plasmid. MTMR6 and MTMR9 were also subcloned into a pGEX-6p-1 vector (Amersham Biosciences) with a GST tag on the N terminus. All constructs were verified by DNA sequencing. Expression and purification of human MTMR6 and MTMR9 were conducted in Sf9 cells as described previously (19).

**GST Binding Assays, Co-immunoprecipitation Assays, and Western Blot Analysis**—For GST binding assays, GST, or a GST fusion protein (100 µg) was bound to glutathione beads (Amersham Biosciences) and incubated with 500 ng of MTMR9-FLAG or MTMR6-FLAG in assay buffer (25 mM Hepes, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, 0.4% Triton X-100, and Complete Protease Inhibitor Mix (Roche Applied Science) on a rotator at 4 °C for 1 h. In assays containing both MTMR6-FLAG and MTMR9 (FLAG-tagged) for 24 h, were conducted as described (28). Rabbits polyclonal antibodies against MTMR6 and MTMR9 were raised against a peptide spanning amino acid residues 519–546 in MTMR6 and 211–231 in MTMR9.

**Immunofluorescence Microscopy**—Twenty-four hours after transfection, HeLa cells grown on coverslips were fixed as described previously (29) and then washed with Tris-buffered saline and solubilized with 0.5% Triton X-100 in phosphate-buffered saline for 10 min. Primary antibody and secondary antibody were diluted in phosphate-buffered saline, 0.1% Triton X-100, 5% BSA. Cells were incubated for 1 h with primary and for 30 min with secondary antibodies at 37 °C. Following antibody incubations, washes were performed by dipping coverslips into phosphate-buffered saline 30 times. Cells were mounted in Prolong mounting medium (Molecular Probes). Images were taken by using an Olympus IX70 inverted microscope and processed with the Metamorph software (Molecular Devices, Sunnyvale, CA).

**Phospholipid Binding Using PIP Strip and PIP Array**—PIP strip and PIP array membranes (Echelon Biosciences) were blocked in Tris-buffered saline-T (0.05% Tween 20 in Tris-buffered saline) + 3% fatty acid-free BSA (Sigma) for 1 h and then incubated with 0.5 µg/ml protein for 2 h at room temperature. In assays containing both MTMR6-FLAG and MTMR9-FLAG proteins, the two proteins were incubated together for 30 min at 4 °C before being applied to the membrane. The membrane was incubated with anti-FLAG antibody and then with horseradish peroxidase-conjugated anti-IgG mouse antibody (Pierce). Both primary and secondary antibodies were diluted in Tris-buffered saline-T + 3% fatty acid-free BSA, and the membranes were washed three times with Tris-buffered saline-T + 3% fatty acid-free BSA after each step. Bands were visualized using SuperSignal West Pico chemiluminescent substrate.

**3-Phosphatase Activity Assay**—For determination of pH optima, [32P]PtdIns-3-P (∼20,000 cpm/assay), unlabeled
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PtdIns-3-P, and PS were dried under nitrogen in a siliconized microcentrifuge tube and resuspended in 25 mM buffer (MES for pH 5.5, 6.0, and 6.5, MOPS for pH 7.0, 7.5, and 8.0, or CHES for pH 8.5 and 9.0), containing 125 mM sodium chloride, 1 mM dithiothreitol, and 50 μg/ml BSA followed by brief sonication. Enzyme assays were performed in a reaction volume of 50 μl containing 1 μM PtdIns-3-P and 100 μM PS. The activity assay was performed as described previously (30).

For kinetic studies, enzyme assays were performed in a reaction volume of 50 μl containing 0.1 μM PtdIns-3-P and 100 μM carrier lipids as described. Reactions were started by the addition of 100 ng of enzyme and carried out for 0, 10, 20, 30, 40, 50, and 60 s at 37 °C and were terminated by the addition of 500 μl of 10% trichloroacetic acid.

**Protein Stability Assay**—HeLa cells were transfected with either MTMR6 alone or MTMR6 + MTMR9 using Lipofectamine 2000 (Invitrogen). For stably transfected HEK-293 TRex cells overexpressing MTMR6 or MTMR9, 0.5 μg/ml tetracycline was added into the medium 6 h after the transient transfection was conducted with vector alone or MTMR6 or MTMR9. Twenty-four hours after transfection, cycloheximide (150 μg/ml) was added to halt protein synthesis. Cells were harvested at the indicated time points. Total protein concentrations of cell lysates were measured, and immunoblotting was conducted. Quantification was performed by using the ImageJ software.

**Apoptotic Cell Staining**—HeLa cells treated with RNAi were grown on 6-well plates. Thirty-six hours later, cells at 90–95% confluence were treated with 100 μM etoposide for 8 h. Apoptotic cells were detected using the APOPercentage apoptotic kit (Accurate Chemical, Westbury, NY) according to the manufacturer’s instruction. The cells that are undergoing apoptosis selectively import the APOPercentage dye that accumulates on the cell.

**FACS**—Four million HeLa cells were transfected with a total of 4 μl of RNAi oligonucleotides (100 μM): control (luciferase) RNAi (4 μl), MTMR6 RNAi (2 μl of R6 RNAi and 2 μl of control RNAi), MTMR9 RNAi (2 μl of R9 RNAi and 2 μl of control RNAi), and MTMR6 + R9 RNAi (2 μl of R6 RNAi and 2 μl of MTMR9 RNAi). Apoptotic cells were detected with an annexin V-FITC apoptosis detection kit (Calbiochem) and analyzed on a BD FACSscan.

**RESULTS**

**Human MTMR6 and MTMR9 Proteins Form a Heteromeric Complex**—Previously, it was demonstrated that mouse MTMR7 interacts with MTMR9 resulting in enhanced 3-phosphatase activity of MTMR7, and MTMR6 interacts with MTMR9 in mouse and *C. elegans* (19, 22). In addition, MTM1 and MTMR2 have been shown to form homodimers as well as heteromers with catalytically inactive myotubularin proteins (12, 16). To investigate whether the human orthologs of MTMR6 and MTMR9 interact, we cloned by PCR the human MTMR6 (NM_004685) and MTMR9 (NM_015458) cDNAs based on sequences available from the GenBank database, from a human whole brain cDNA library. Human MTMR6 and MTMR9 proteins were expressed in Sf-9 insect cells, and the respective proteins were purified using an anti-FLAG affinity column (Fig. 1A, left panel). Both MTMR6 and MTMR9 appear to form homodimers as the band corresponding to the molecular weight of a dimer is recognized by the MTMR6- and MTMR9-specific antibodies in a Western blot (Fig. 1A, right panel).

We verify that human MTMR6 directly interacts with MTMR9 in vitro by GST binding assay and co-immunoprecipitation experiments. GST-MTMR6 pulled down FLAG-MTMR9, suggesting that the heteromer forms between MTMR6 and MTMR9 (Fig. 1B). The reciprocal experiment using FLAG antibody demonstrated that GST-MTMR6 co-immunoprecipitated with FLAG-MTMR9 (Fig. 1C). To further confirm this interaction in the cell, we co-expressed HA-MTMR6 and FLAG-MTMR9 in HeLa cells and immuno...
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When MTMR6 was preincubated with MTMR9 for 30 min, the lipid binding of MTMR6 was significantly increased in an MTMR9 concentration-dependent manner (Fig. 3B). Thus, the interaction of MTMR9 with MTMR6 significantly enhances the binding properties of MTMR6 toward negatively charged phospholipids.

Phospholipid Binding of MTMR6 Is Increased by MTMR9—Given the interaction of MTMR6 with MTMR9, we determined whether the complex formation had an effect on lipid binding of MTMR6 and MTMR9. Lipid overlay assays were performed with purified recombinant MTMR6 and MTMR9 using PIP strips. MTMR6 bound all monophosphorylated phosphatidylinositols (PtdIns-3-P, PtdIns-4-P, and PtdIns-5-P), PtdIns-3,5-P2, phosphatidic acid, and PS; however, MTMR9 did not demonstrate any significant binding to phospholipids (Fig. 3). Similar results were observed using a PIP array. The affinity of MTMR6 binding to individual monophosphorylated phosphatidylinositols was similar to but significantly greater than that of polyphosphorylated phosphatidylinositols (Fig. 3A); of the polyphosphorylated phosphatidylinositols, MTMR6 bound to its substrate Ptd-3,5-P2 with the greatest affinity.

Catalytic Activity of MTMR6 Is Increased by MTMR9—We determined the pH optimum for MTMR6 catalytic activity to be 7.0, differing from the previously published pH optimum of 6.5 (31). The pH optimum of MTMR6 was not changed by the addition of MTMR9 (data not shown). To investigate the effect of the MTMR6 and MTMR9 interaction on the 3-phosphatase activity of MTMR6, we determined the first-order rate constant, as measured by the release of [32P]PO4 from PtdIns-3-P (Table 1). Given the strong lipid binding of MTMR6 to PS (Fig. 3), we initially examined the 3-phosphatase activity in the presence of increasing amounts of PS in liposomes. By increasing PS from 0 to 50% and then to 100%, the first-order rate constant of MTMR6 increased to 1.8- and 28.1-fold of the level without PS, respectively. In the presence of MTMR9, the catalytic activity of MTMR6 increased under both PS concentrations tested; the activity increased by 1.7-fold for 0% PS, 6.4-fold for 50% PS, and 3-fold for 100% PS. In the presence of MTMR9 and 100% PS, MTMR6 activity is increased by 84-fold when compared with that seen in 50% phosphatidycholine, 50% phosphatidylethanolamine in the absence of MTMR9. These results show the
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A.

| Phospholipid | MTMR6 | MTMR9 |
|-------------|-------|-------|
| Phosphatidylinositol 4,5-bisphosphate | PtdIns-3,4,P_2 | PtdIns-4,5,P_2 |
| Phosphatidylinositol 4,5-trisphosphate | PtdIns-3,4,5,P_3 | PtdIns-3,4,5,P_3 |
| Phosphatidylserine | Blank | Blank |

TABLE 1

First-order rate constant of MTMR6

| Phospholipid | 1% PtdIns-4-P | 1% PtdIns-5-P |
|-------------|--------------|--------------|
| PC/PS | 4.1 | ND |
| MTMR6 | 7.1 | ND |
| MTMR6+MTMR9 | 48.3 | 344 |

FIGURE 3. MTMR6 phospholipid binding is increased by MTMR9. A, recombinant MTMR6-FLAG (0.5 μg/ml) or MTMR9-FLAG proteins (0.5 μg/ml) were used to probe the PIP strip. Bound protein is indicated by Western blot with the appropriate antibody. Phospholipids bound by MTMR6 are indicated in the middle panel. MTMR9 did not bind to any lipid on the membrane (far right panel). A schematic diagram of the PIP Strip membrane with the phospholipids is indicated on the left. PtdIns-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate. B, a schematic diagram of the PIP array membrane containing serial dilutions of the indicated phosphatidylinositol phosphates (in picomoles) are shown on the left. MTMR6-FLAG (0.5 μg/ml) and MTMR6-FLAG + MTMR9-FLAG were used to probe the PIP arrays. The ratio of MTMR6 to MTMR9 is as noted. Bound proteins were visualized by anti-FLAG antibody.

MTMR6 and MTMR9 Protein Stability Is Increased by the Formation of the Complex—In transfected cells, significantly higher levels of either MTMR6 or MTMR9 protein were always observed in the presence of its binding partner (Fig. 4, B and D, 0 time points). In other words, co-expression of both MTMR6 and MTMR9 resulted in much higher protein levels for both proteins than overexpression of MTMR6 or MTMR9 alone when the same amount of plasmid DNA was used for transfection and equal amounts of total protein were loaded. RNAi of MTMR6 reduced protein expression 20–40%, but a much greater reduction in the level of MTMR6 (up to 90%) was seen when RNAi of both proteins was carried out as seen in Fig. 4A (lanes 2 and 6). The levels of MTMR9 protein were further reduced by a combination of RNAi oligonucleotides targeting both binding partners (Fig. 4A, compare lanes 4, 5, and 6). This also suggests that the formation of the MTMR6 and MTMR9 complex stabilizes the proteins, possibly by decreasing the degradation rates. To test this, we chased MTMR6 in cycloheximide-treated HeLa cells in the presence or absence of MTMR9 and observed a higher protein level and slower turnover rate of MTMR6 in cells co-expressing both proteins. Based on the intensity of Western blots, the half-life of MTMR6 was increased from ~40 min to 4 h when MTMR9 was co-expressed in HeLa cells (Fig. 4B and C). To rule out the possibility of signal over saturation on the Western blot, we artificially loaded 3-fold more total protein of the MTMR6 only samples and analyzed it after a very light exposure. As shown in Fig. 4C, in the absence of MTMR9, the level of MTMR6 decreased drastically more than the similarity of MTMR6 protein level at the zero time point. We next asked whether MTMR9 stability was also enhanced when MTMR6 was present using a stably transfected
with control RNAi as seen in Fig. 5C.

DISCUSSION

In this report, we verify that human MTMR6 and MTMR9 form a functional heteromeric complex and characterize this interaction. Importantly, we found that MTMR9 influences MTMR6 function at multiple levels, including increasing its stability. We demonstrate that MTMR6 co-localizes with MTMR9 in a perinuclear cellular distribution. Complex formation also increased lipid binding and the catalytic activity of MTMR6. Finally, we show that heteromer formation played a role in regulating apoptosis.

MTMR6 was found to bind to all monophosphorylated phosphatidylinositols, with a weaker affinity for PtdIns and PtdIns-3,5-P₂, a known myotubularin substrate. Interestingly, PS and phosphatidic acid also bind to MTMR6. MTMR9 did not bind lipids; however, complex formation between MTMR6 and MTMR9 enhanced the lipid binding of MTMR6 without changing the lipid binding profile. We obtained an MTMR6 binding profile different from that previously published possibly due the fact that Choudhury et al. (32) used only the pleckstrin homology/GRAM (glucosyltransferases, Rab-like GTPase activators, and myotubularins) domain, whereas in this study, the entire MTMR6 protein was used.

The catalytic activity of active myotubularin proteins increases when they are in a heteromeric complex with inactive binding partners. This increased activity was previously demonstrated for the MTMR7-MTMR9 complex, the MTM1-MTMR12 complex (15), the MTMR2-MTMR5 complex (13), and the MTMR2-MTMR13 complex (20). MTMR6 activity increased significantly with an increasing percentage of PS in substrate liposomes; elevating PS from 0 to 100% increased MTMR6 activity 28-fold. This is consistent with the strong PS binding observed in the lipid overlay assay, supporting the importance of PS in MTMR6 activity. The highest concentra-
tion of PS in the cell is at the inner leaflet of the plasma membrane with 15–30% PS (33–35); therefore, a site of increased MTMR6 activity may be the plasma membrane. This is consistent with the findings of Srivastava et al. (23) and Choudhury and co-workers (24, 32) that MTMR6 localized to the plasma membrane dephosphorylated PtdIns-3-P, thereby inhibiting a
Ca\(^{2+}\)-activated K\(^+\) channel, KCa3.1. Interestingly, from our high magnification immunofluorescence images, in addition to co-localizing with MTMR6, MTMR9 seems to distribute along the plasma membrane (Fig. 2). It would be of interest to characterize the effect of MTMR9 on the MTMR6-mediated regulation of the Ca\(^{2+}\)-activated K\(^+\) channel.

MTMR9 increased MTMR6 activity under all conditions examined, resulting in 2–6-fold increases. The highest increase, 6.4-fold, was in 50% PS liposomes with the remaining conditions in the 2–4-fold range. Acting together, PS and MTMR9 can potentially increase MTMR6 activity 84-fold. Given these data, we postulate that MTMR9 functions to increase the lipid binding of MTMR6, recruiting MMTR6 to the membrane, and in the presence of both MTMR9 and local PS, significantly activating MTMR6, resulting in a decreased local pool of PDtns-3-P.

In addition to increasing the enzymatic activity and lipid binding, we demonstrated that MTMR6 and MTMR9 are stabilized by complex formation with each other. The stability studies suggested that the turnover rate of both proteins was significantly decreased when the complex was formed. The half-life of MTMR6 is increased about 6-fold with concomitant overexpression of MTMR9. Reciprocally, the complex was also able to significantly protect MTMR9 from degradation. This phenomenon has not been previously described in the myotubulin family of proteins, and it would be interesting to investigate whether complex-forming subunits of other family members affect each other’s turnover rate. Indeed, Robinson and Dixon (18) demonstrated a roughly 2–4-fold range. Acting together, PS and MTMR9 can potentially protect MTMR9 from degradation. This phenomenon has not

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