Myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate

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The lipid second messenger phosphatidylinositol 3-phosphate [PI(3)P] plays a crucial role in intracellular membrane trafficking. We report here that myotubularin, a protein tyrosine phosphatase required for muscle cell differentiation, is a potent PI(3)P phosphatase. Recombinant human myotubularin specifically dephosphorylates PI(3)P *in vitro*. Overexpression of a catalytically inactive substrate-trapping myotubularin mutant (C375S) in human 293 cells increases PI(3)P levels relative to that of cells overexpressing the wild-type enzyme, demonstrating that PI(3)P is a substrate for myotubularin *in vivo*. In addition, a *Saccharomyces cerevisiae* strain in which the myotubularin-like gene (YJR110w) is disrupted also exhibits increased PI(3)P levels. Both the recombinant yeast enzyme and a human myotubularin-related protein (KIAA0371) are able to dephosphorylate PI(3)P *in vitro*, suggesting that this activity is intrinsic to all myotubularin family members. Mutations in the *MTM1* gene that cause human myotubular myopathy dramatically reduce the ability of the phosphatase to dephosphorylate PI(3)P. Our findings provide evidence that myotubularin exerts its effects during myogenesis by regulating cellular levels of the inositol lipid PI(3)P.

X-linked myotubular myopathy is a severe congenital disorder in which the muscle cells of affected individuals contain large, centrally placed nuclei and structural features characteristic of fetal myotubes, suggesting that differentiation has been arrested at a step preceding myofiber formation (1–4). The myotubularin gene (*MTM1*), which is mutated in X-linked myotubular myopathy, encodes a protein with sequence similarity to dual specificity protein tyrosine phosphatases (5). Myotubularin contains the Cys-X_5-Arg (CX_5R) active site motif that is the hallmark of the protein tyrosine phosphatase (PTP) superfamily and exhibits dual specificity protein phosphatase activity *in vitro* (6–9). In addition, myotubularin-related proteins are conserved among eukaryotes, suggesting a common substrate or function (5, 9). However, the physiologic target(s) of myotubularin and its essential role in myogenic development have yet to be identified.

Phosphoinositides produced by the actions of phosphatidylinositol (PI) 3-kinases play key roles in a diverse array of cellular processes, including responses to extracellular agonists, growth, survival, cytoskeletal organization, differentiation, and membrane trafficking (10–14). Recently, the *PTEN/MMAC1* gene was identified as a candidate tumor suppressor gene, which mapped to chromosome 10q23, a region frequently mutated in a variety of tumors (15, 16). The *PTEN/MMAC1* gene encodes a protein with similarity to dual specificity protein tyrosine phosphatases (15, 16). Our laboratory has shown that PTEN (phosphatase and tensin homolog) dephosphorylates the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PI (3,4,5)P_3), thus identifying it as the first PTP superfamily enzyme that utilizes an inositol lipid as its physiologic substrate (17). PTEN is highly specific for the D3 position of PI (3,4,5)P_3, and lipid phosphatase activity is critical for its tumor suppressor function, providing a direct correlation between substrate levels and tumor formation (18). Numerous studies have now established a role for PTEN in regulating signaling through PI (3,4,5)P_3-dependent pathways (19, 20).

In the current study, we have undertaken the characterization of myotubularin as a first step toward understanding its involvement in the regulation of myogenic differentiation. During the course of this investigation, we have discovered that myotubularin possesses potent inositol lipid phosphatase activity. We demonstrate here that myotubularin dephosphorylates the lipid second messenger, PI(3)P, *in vitro* and *in vivo*. Although present at relatively low levels in eukaryotic cells, PI(3)P has been shown to play a key role in membrane trafficking/vesicular transport processes and serves as a targeting mechanism for proteins containing a specific PI(3)P-binding motif known as a FYVE domain (21–25). Myotubularin phosphatase activity is highly specific toward PI(3)P, and *MTM1* mutations that cause myotubular myopathy render the enzyme catalytically inactive, suggesting a direct link between myotubular myopathy and PI(3)P levels. Furthermore, two myotubularin-related proteins from yeast and human also dephosphorylate PI(3)P, suggesting that this activity is a property conserved among myotubularin family enzymes. Our findings distinguish myotubularin as the second member of the PTP superfamily to act on a specific phosphoinositide as its physiologic substrate and will provide a basis for the identification of PI(3)P-mediated signaling pathways involved in the regulation of myogenesis.

Materials and Methods

Expression and Purification of Recombinant Proteins. Recombinant human myotubularin was expressed as a fusion protein with N-terminal glutathione *S*-transferase (GST)- and C-terminal 6-histidine tags in *Escherichia coli* BL21 (DE3) CodonPlus cells (Stratagene). An expression construct was created by using the pET-GSTx expression vector (26), which was generously provided by Harry Charbonneau (Purdue University). A PCR product encoding myotubularin (residues 1–603) was inserted into the 5′-*Bam*HI and 3′-*Not*I sites of pET-GSTx. The fusion protein was expressed and purified from soluble bacterial extracts by using successive Ni^{2+}-agarose and glutathione-agarose

Abbreviations: PI(3)P, phosphatidylinositol 3-phosphate; PTP, protein tyrosine phosphatase; pNPP, *para*-nitrophenylphosphate; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; gPIP, glycerophosphoinositide.

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affinity chromatography steps as described (26, 27). The fusion protein was greater than 90% pure as determined by Coomassie staining. Recombinant GST-p61/62n, VHR, and GST-PTEN proteins were expressed and purified as described (17, 28, 29). Site-directed mutagenesis was carried out by using a PCR-based procedure (30). All constructs were confirmed by nucleotide sequencing.

Phosphatase Assays. Phosphatase assays were conducted at 30°C in a buffer consisting of 50 mM sodium acetate, 25 mM bis·Tris, 25 mM Tris (pH 6.0), and 2 mM DTT. Radiolabeled protein substrates and *para*-nitrophenylphosphate (pNPP) were prepared and assayed as described (26). Phosphoinositide substrates used for activity measurements were synthetic D(+)-sn-1,2-di-*O*-octanoyl derivatives obtained from Echelon Research Laboratories (Salt Lake City). Soluble inositol phosphate substrates were obtained from Echelon and Sigma. Phosphoinositide and inositol phosphate substrates were prepared and phosphate release was quantified by using a malachite green-based colorimetric assay for inorganic phosphate as described for Sac1p (27).

Cell Culture, Transfection, and ^{3}H-Inositol Labeling. HEK293 cells were maintained at 37°C and 5% CO_2 in DMEM (GIBCO) containing 10% FCS, 50 units/ml (each) penicillin/streptomycin, and 4 mM glutamine. Vectors for the overexpression of wild-type or a catalytically inactive mutant (C375S) human myotubularin were constructed by inserting the respective cDNA fragments into the *Bam*HI and *Not*I sites of the pCDNA3.1-NF mammalian expression vector. Fusion proteins expressed from this vector contain an N-terminal M2-FLAG epitope tag. For radiolabeling of phosphoinositides, subconfluent cultures of HEK 293 cells (3×10^5 cells per 60-mm plate) were transfected with pCDNA3.1-NF, pCDNA3.1-NF-MTM1, or pCDNA3.1-NF-MTM1 (C375S) by using Fugene transfection reagent (Roche) according to the manufacturer's protocol, and were cultured for 12 h to allow protein expression. Transfection efficiency under these conditions was approximately 60% as determined by enhanced green fluorescent protein (EGFP) immunofluorescence. The media was then replaced with inositol-free DMEM containing 10% dialyzed FCS and antibiotics/glutamine as described above, and 50 μCi/ml ^{3}H-*myo*-inositol (NEN). Cell were cultured an additional 24 h before harvesting. Samples from each transfection were analyzed for protein expression by immunoblotting with M2-FLAG monoclonal antibody.

Yeast Strains, Media, and ^{3}H-Inositol Labeling. The haploid yeast strain GYC121 (MATa *leu2 his3 trp1 ura3*) was used to create the YJR110w null mutant, ΔYJR110w (MATa *leu2 his3 trp1 ura3 YJR110w*Δ::*G418*). Deletion of the YJR110w ORF was carried out by using PCR-based gene disruption with a G418 antibiotic resistance cassette (31). Overnight cultures of GYC121 and ΔYJR110w were grown at 30°C in yeast extract/peptone/dextrose medium. Samples of each culture were used to inoculate fresh cultures in SD medium supplemented with leucine, histidine, tryptophan, uracil, and 10 μCi/ml ^{3}H-*myo*-inositol (NEN). Samples were grown overnight at 30°C, and total cellular lipids were extracted as described (32). Radiolabeled phosphoinositides were deacylated and analyzed by HPLC as described below.

HPLC Analysis of Radiolabeled Phosphoinositides. HEK293 cells radiolabeled with ^{3}H-inositol as described above were resuspended and washed twice with PBS. Total cellular lipids, including 10 μmol (each) of unlabeled PI(3)P and PI(4)P added as carrier lipids, were extracted by using acidified chloroform/methanol, and were deacylated as described by Auger *et al.* (33). Total cellular protein and radioactivity varied less than 5% between samples. After deacylation, greater than 98% of the total radioactivity from the lipid extract was present in the aqueous phase. Radiolabeled glycerophosphoinositides (gPIPs) were separated by using a Partisphere 5-SAX column (Whatman) as described by Auger *et al*. (33) with the following modifications. A discontinuous gradient from 10 mM (pump A) to 1.0 M $(NH_4)_2HPO_4$ (pH 3.8) (pump B) was established as follows: 0% B for 10 min; 0–12.5% B over 30 min; 12.5% B to 100% B over 5 min; 100% B for 5 min; 100% B to 0% B over 1 min; 0% B for 24 min. Radioactivity eluted from the column was quantified by using a continuous-flow in-line scintillation detector (Beckman Instruments). The elution positions of gPI(3)P, gPI(4)P, and gPI(4,5)P_2 were determined by using authentic radiolabeled standards. PI(3)P was prepared by using immunoprecipitated PI-3 kinase, γ-^{32}P-ATP, and phosphatidylinositol as described (32). PI(4)P and PI(4, 5)P_2 were isolated from HEK293 cells radiolabeled with ^{32}P-orthophosphate (NEN) as described (34). Radiolabeled standards were separated by TLC, were deacylated, and were separated by HPLC as described above.

Immunolocalization. A vector for the expression of an EGFP-myotubularin fusion protein was constructed by inserting a PCR product encoding full-length myotubularin into pEGFP-C1 (CLONTECH). HEK293 cells were transfected with pEGFP or pEGFP-MTM1 as described above, and the cells were cultured for an additional 48 h. Cell nuclei were stained with DAPI (Molecular Probes) by using the recommended protocol. EGFP and DAPI staining were visualized by fluorescence microscopy.

Results

Recombinant Myotubularin Is a Highly Efficient PI(3)P Phosphatase. Although previous studies have demonstrated that recombinant myotubularin possesses dual specificity protein phosphatase activity, its relative efficiency toward these substrates has not been reported (7, 8). To this end, we have expressed recombinant human myotubularin in *E. coli* as a fusion protein (GST-MTM1-His_6) with N-terminal glutathione *S*-transferase and C-terminal six-histidine tags for affinity purification. As previously shown, the recombinant myotubularin fusion protein is active toward the artificial PTP substrate *para*-nitrophenylphosphate (pNPP) (8). We have found that myotubularin hydrolyzes pNPP at a pH optimum of 6.0 with a K_m of 21 mM, a k_{cat} of 0.4 s^{-1}, and a k_{cat}/K_m of 20 $s^{-1}\cdot M^{-1}$. These values are similar to those reported for the PTEN inositol lipid phosphatase and the RNA 5′-phosphatase BVP and suggest that, like these enzymes, myotubularin is also an inefficient catalyst with this substrate (17, 35).

Using protein database searches, we made the unexpected observation that the active site sequence of myotubularin (VH-**CSD**GW**DRT**, residues 373–382) bears some similarity to the putative active site region (TN**C**M**D**CL**DRT**, residues 390–399) of the Sac1p phosphoinositide phosphatase from *Saccharomyces cerevisiae*. Sac1p and the related proteins Fig4p, Inp52p, and Inp53p from budding yeast, and human synaptojanin, contain N-terminal *SAC1*-like domains that possess intrinsic inositol lipid phosphatase activity and are believed to play important roles in membrane trafficking (36, 37). Although they share little identity outside the region encompassing the CX_5R motif, the active sites of both Sac1p-like proteins and myotubularin family members contain the catalytic Cys and Arg residues as well as two invariant aspartic acid residues that are located within the X_5 segment. The aspartate residues are of particular interest because acidic amino acids are uncommon in the X_5 sequences of PTPs and structural studies of the PTEN lipid phosphatase have revealed that residues within its X_5 sequence are critical determinants for its specificity toward PI(3,4,5)P_3 (38).

The extremely poor catalytic activity of myotubularin toward pNPP together with its sequence similarity to the active site of

Table 1. Myotubularin phosphatase activity

Substrate	Concentration, μM	Specific activity, mol·min^{-1}·mol^{-1}
PI(3)P	50	4471
PI(4)P	50	3.8
PI(5)P	50	22.8
PI(3,4)P_2	50	7.9
PI(3,5)P_2	50	0*
PI(4,5)P_2	50	0*
PI(3,4,5)P_3	50	2.1
Ins(4)P	50	0*
Ins(1,3)P_2	50	256
Ins(1,4)P_2	50	0*
Ins(1,3,4)P_3	50	0*
Ins(1,4,5)P_3	50	0*
Ins(1,3,4,5)P_4	50	0*
pY-MBP	2.5	0.01
pY-casein	2.5	0.07
pS/T-MBP	2.5	0†
pS-casein	2.5	0†

Values are shown as moles of phosphate released per minute per mole of enzyme and represent the mean of triplicate determinations. Standard error (SEM) of each determination was less than 5%.
*Limit of detection in these assays was 10 pmol of phosphate released.
†Limit of detection in these assays was 0.5 pmol of phosphate released.

an inositol lipid phosphatase suggested that myotubularin should be tested for its ability to dephosphorylate phosphoinositide substrates. As illustrated in Table 1, we show that myotubularin efficiently catalyzes the dephosphorylation of PI(3)P. The specific activity of myotubularin toward PI(3)P is 200-fold greater than that with any of the six other phosphoinositide substrates, indicating that it is highly specific for the D3-position of PI(3)P (Table 1). Using TLC, we have confirmed that myotubularin converts PI(3)P to phosphatidylinositol (data not shown). Inositol 1,3-bisphosphate, the soluble inositol phosphate analog of PI(3)P, is hydrolyzed at a rate approximately 20-fold lower than PI(3)P, indicating a strong preference for the lipid substrate. Moreover, myotubularin did not catalyze the hydrolysis of any other soluble inositol phosphate (Table 1). The phosphatase activity of myotubularin toward synthetic PI(3)P derivatives with varying acyl chain length follow the order $C_8 > C_4 > C_{16}$ (data not shown). To characterize its efficiency as a PI(3)P phosphatase, kinetic parameters have been determined for myotubularin by using a water-soluble (C_4) form of PI(3)P. This substrate is dephosphorylated with a K_m of 43 μM, a k_{cat} of 42 s^{-1}, and a k_{cat}/K_m of 9.8×10^5 s^{-1}·M^{-1}, indicating that myotubularin is a highly efficient catalyst of PI(3)P hydrolysis. In contrast, myotubularin exhibits only weak activity when tested against myelin basic protein or casein phosphorylated on tyrosyl residues, and no activity is detected when using myelin basic protein or casein phosphorylated on seryl/threonyl residues (Table 1). By comparison, the activity of PTP1B, a tyrosine-specific PTP, is 2,200-fold greater than that of myotubularin toward tyrosine-phosphorylated myelin basic protein (39). Myotubularin is sensitive to the PTP inhibitors vanadate and tungstate, and a mutant in which the active site cysteine nucleophile is replaced by serine (C375S) shows no detectable phosphatase activity (data not shown), suggesting that myotubularin employs a catalytic mechanism similar to that of the PTPs (6). When tested under comparable conditions, recombinant myotubularin possesses at least 30-fold greater activity toward PI(3)P than does Sac1p against any phosphoinositides, indicating that myotubularin is an extremely effective phosphatase with this substrate (data not shown).

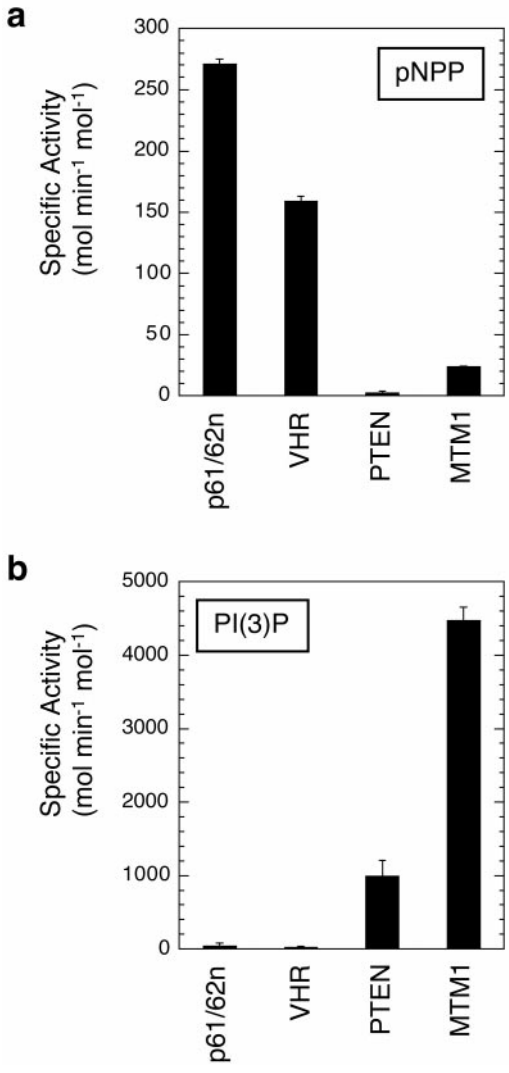

Fig. 1. PI(3)P phosphatase activity is not a general property of protein tyrosine phosphatases. The activities of tyrosine-specific (GST-p61/62n), dual-specific (VHR), and inositol lipid phosphatases (GST-PTEN) were compared with that of myotubularin (GST-MTM1-His_6). (*a*) Phosphatase activity of each enzyme using pNPP as substrate. (*b*) Phosphatase activity toward PI(3)P as substrate. Phosphatase assays were carried out at 30°C as described in *Materials and Methods*. Enzyme specific activity is expressed as moles of phosphate released per minute per mole of enzyme (mean ± SEM).

PI(3)P Phosphatase Activity Is Not a General Property of PTP Superfamily Enzymes. To show that PI(3)P phosphatase activity is not a general property of protein tyrosine phosphatases, we have compared the activity of a tyrosine-specific PTP (GST-p61/62n), a dual specificity PTP (VHR), and a phosphoinositide-specific phosphatase (GST-PTEN), to that of myotubularin using pNPP and PI(3)P as substrates (17, 28, 29). As shown in Fig. 1*a*, pNPP is a poor substrate for myotubularin and PTEN whereas GST-p61/62n and VHR dephosphorylate this substrate efficiently. However, only myotubularin, and to a lesser extent PTEN, use PI(3)P as a substrate (Fig. 1*b*). These results demonstrate that activity toward this lipid is a unique property of myotubularin and not common among PTP superfamily enzymes.

PI(3)P Is a Physiologic Substrate for Myotubularin. Having established that myotubularin could specifically dephosphorylate PI(3)P *in vitro*, we next tested whether it could affect cellular

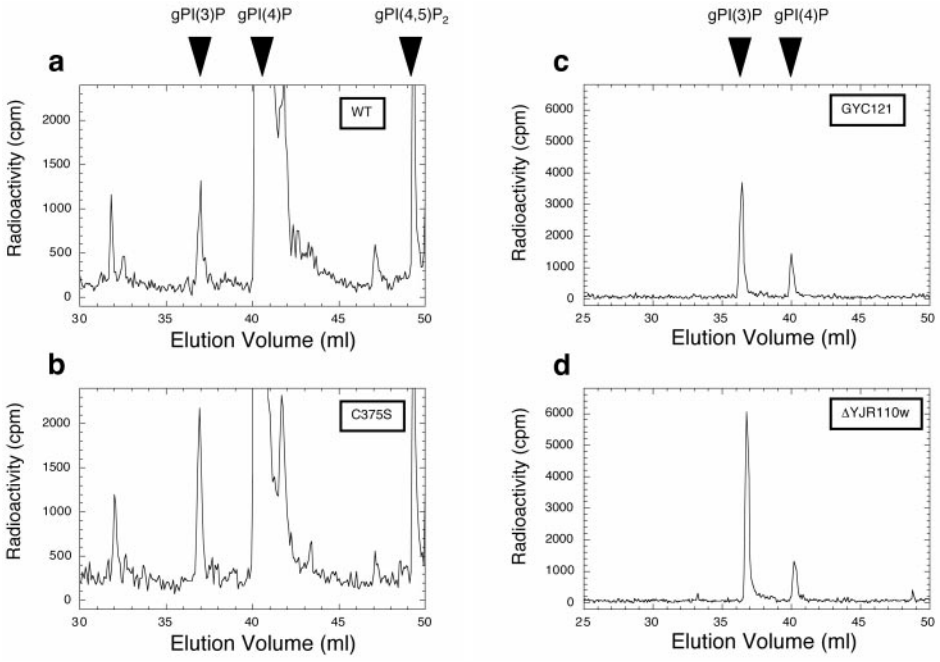

Fig. 2. HPLC analysis of deacylated phosphoinositides from HEK 293 cells and budding yeast. Radiolabeled lipids from HEK293 cells overexpressing wild-type or mutant (C375S) myotubularin, and wild-type (GYC121) or myotubularin homolog null mutant (ΔYJR110w) budding yeast strains, were deacylated and separated by anion exchange chromatography as described in *Materials and Methods*. (*a*) Elution profile of radiolabeled gPIPs from 293 cells overexpressing wild-type (WT) myotubularin. (*b*) Elution profile of radiolabeled gPIPs from 293 cells overexpressing a catalytically inactive myotubularin mutant (C375S). (*c*) Elution profile of radiolabeled gPIPs from wild-type yeast. (*d*) Elution profile of radiolabeled gPIPs from ΔYJR110w yeast. The elution positions of gPI(3)P, gPI(4)P, and gPI(4,5)P_2 reference standards are indicated by arrows. Sample loadings represent equivalent total cellular protein and total radiolabeled phosphoinositides. Chromatographs are representative of four independent experiments.

levels of this lipid. To this end, wild-type or a catalytically inactive myotubularin mutant (C375S) were overexpressed in HEK 293 cells labeled with ^{3}H-inositol. Studies employing PTP active site mutants have shown that these mutants can function as substrate traps, thus elevating levels of their physiological phosphorylated substrates (17, 40). Total cellular lipids from radiolabeled 293 cells were deacylated and analyzed by anion exchange high performance liquid chromatography as described in *Materials and Methods*. Representative elution profiles of deacylated phosphoinositides derived from cells overexpressing wild-type or C375S myotubularin are illustrated in Fig. 2 *a* and *b*, respectively. Overexpression of the C375S mutant consistently elevates PI(3)P levels two-fold compared with wild-type myotubularin, suggesting that this increase can be directly attributed to the C375S mutant protein. The elevated level of PI(3)P in these cells is likely to be caused by protection from dephosphorylation by endogenous myotubularin or other lipid phosphatases. Overexpression of wild-type myotubularin causes a decrease in PI(3)P of approximately 20% compared with levels in cells transfected with empty vector. Both the wild-type and C375S proteins were expressed in equivalent amounts as estimated by immunoblotting (not shown), and levels of phosphatidylinositol, PI(4)P, and PI(4,5)P_2 remain unchanged between cells expressing wild-type or C375S myotubularin. Moreover, PI 3-kinase activity immunoprecipitated from these cells was identical, suggesting that the increase in PI(3)P level in cells overexpressing the C375S mutant was not caused by increased PI 3-kinase activity (data not shown). As expected, wild-type myotubularin immunoprecipitated from these cells possesses robust PI(3)Pase activity whereas the C375S mutant does not (data not shown). These observations, taken together with the specificity of recombinant human myotubularin toward PI(3)P *in vitro*, strongly suggest that PI(3)P is a physiological substrate of this enzyme.

PI(3)P Phosphatase Activity Is Common Among Myotubularin Family Enzymes. To determine whether PI(3)P phosphatase activity is a common characteristic of myotubularin-like phosphatases, we have analyzed phosphoinositide levels in the yeast *S. cerevisiae*. Budding yeast contain a single ORF (YJR110w) that encodes a myotubularin-like protein (5, 8). The active site sequence of YJR110w is identical to that of human myotubularin, including the catalytic Cys and Arg, as well as the two invariant aspartic acid residues characteristic of this family of enzymes. In addition, we have found that a recombinant YJR110w fusion protein possesses *in vitro* PI(3)P phosphatase activity comparable to that of myotubularin (data not shown). Lipids isolated from ^{3}H-inositol-labeled haploid yeast strains bearing a wild-type (GYC121) or null mutant (ΔYJR110w) myotubularin-related allele were deacylated and analyzed as described in *Materials and Methods*. The elution profiles of deacylated phosphoinositides are shown in Fig. 2. The levels of PI(3)P in the YJR110w null mutant strain (Fig. 2*d*) are consistently 2-fold higher than those of the wild-type strain (Fig. 2*c*), suggesting that the ability to use PI(3)P as a substrate is a feature shared by other myotubularin-like proteins. In light of these findings as well as the sequence similarity between myotubularin and YJR110w, we propose the name *YMR1* (yeast myotubularin related) for YJR110w. Our initial characterization of the *ymr1* null mutant strain has revealed no obvious phenotypes, suggesting a nonessential function or possible redundancy among yeast lipid phosphatases. Budding yeast express several Sac1p-like proteins, as well as a PTEN-like phosphatase (YNL128w), which are also thought to play a role in regulating PI(3)P levels (36, 37).

MTM1 Mutations Associated with Myotubular Myopathy Disrupt Myotubularin Phosphatase Activity. As a first step toward understanding the molecular basis of myotubular myopathy, we have examined the effects of *MTM1* mutations associated with severe

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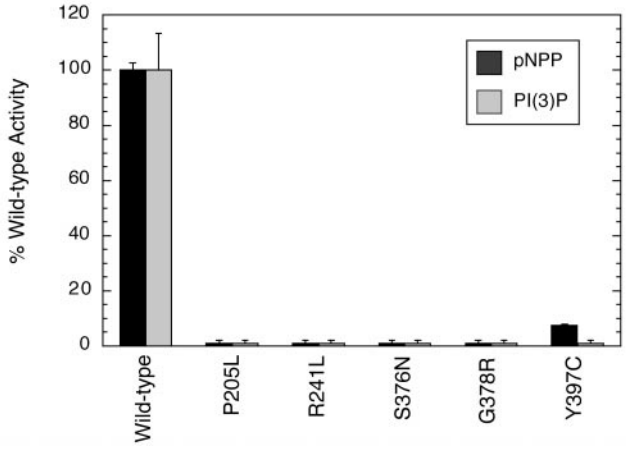

Fig. 3. MTM1 mutations associated with severe myotubular myopathy dramatically decrease the phosphatase activity of myotubularin. Recombinant wild-type or mutant (P205L, R241L, S376N, G378R, Y397C) myotubularin fusion proteins were tested for their ability to dephosphorylate pNPP and PI(3)P substrates. Phosphatase assays were conducted at 30°C as described in *Materials and Methods*. Values are expressed as percent wild-type myotubularin activity (mean ± SEM).

forms of the disorder on myotubularin phosphatase activity (9, 41). Although several of the mutations that have been identified are predicted to affect myotubularin phosphatase activity, a definitive analysis of the enzymatic properties of these mutants toward a physiologic substrate has not been reported. The point mutations chosen for study alter amino acid residues conserved among myotubularin-related proteins and are associated with a severe myotubular myopathy phenotype. PI(3)P and pNPP were used as substrates to test the activity of recombinant wild-type and mutant myotubularin fusion proteins. As shown in Fig. 3, the myotubularin mutants display a dramatic decrease in phosphatase activity toward both substrates, correlating defective phosphatase activity and myotubular myopathy. Notably, the Y397C myotubularin mutant retains some activity toward pNPP whereas it is inactive with PI(3)P as a substrate (Fig. 3). The activity of this mutant is analogous to that of a PTEN phosphoinositide phosphatase mutant, which displays activity toward pNPP but not $PI(3,4,5)P_3$ (18). The argument has been made that this is evidence for $PI(3,4,5)P_3$ being a physiological substrate of PTEN (18). A similar argument can also be made for PI(3)P and myotubularin.

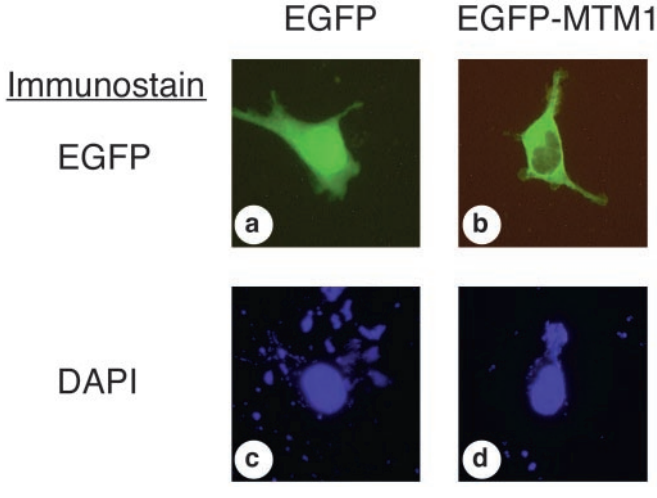

Fig. 4. Myotubularin is localized to the cytoplasm in HEK293 cells. The subcellular distribution of transiently expressed EGFP (*a*) and EGFP-myotubularin fusion protein (EGFP-MTM1) (*b*) was determined by using immunofluorescence microscopy. Nuclei were stained with DAPI (*c* and *d*). An identical pattern of cytoplasmic staining for EGFP and EGFP-MTM1 or with FLAG-tagged myotubularin was observed in both HeLa cells and murine C2C12 myoblasts (not shown).

Immunolocalization of Myotubularin. It has been reported that myotubularin is located in the nucleus of transfected cells (7). A nuclear localization for myotubularin is difficult to correlate with it functioning as a PI(3)P phosphatase because PI(3)P is concentrated on the external surfaces of intracellular lipid vesicles (22). To clarify this issue, we have examined the cellular distribution of transiently expressed myotubularin fused to enhanced green fluorescent protein (EGFP) in mammalian cells. Our data shows that the EGFP-myotubularin fusion protein is predominantly cytoplasmic (Fig. 4*b*) as determined by EGFP immunofluorescence and 6-diamidino-2-phenylindole (DAPI) staining (Fig. 4 *c* and *d*). The control cells expressing EGFP alone exhibit staining in both the cytoplasm and nucleus, as expected (Fig. 4*a*). Identical results were obtained by using EGFP-MTM1 or FLAG-tagged myotubularin and indirect immunofluorescence in both HeLa cells and C2C12 murine myoblasts, indicating that the cytoplasmic localization shown in Fig. 4 is not an artifact resulting from fusion of myotubularin to EGFP (data not shown). Although it is possible that myotubularin subcellular localization has some degree of tissue specificity, the cytoplasmic localization we have observed in three different cell types is consistent with the enzyme having access to cellular PI(3)P.

Discussion

We have provided evidence that myotubularin represents a highly conserved family of phosphatidylinositol 3-phosphatases. More importantly, these findings suggest that the human genetic disorder X-linked myotubular myopathy is a consequence of failure to dephosphorylate PI(3)P. Although the exact role of PI(3)P in myogenic differentiation is unclear, the importance of this lipid second messenger in membrane trafficking raises several intriguing possibilities. For example, precise vesicular trafficking is required for transport of intracellular cargoes to the plasma membrane. Because myogenic development in patients with myotubular myopathy is arrested at a stage before myoblast fusion, it is possible that improper regulation of PI(3)P in

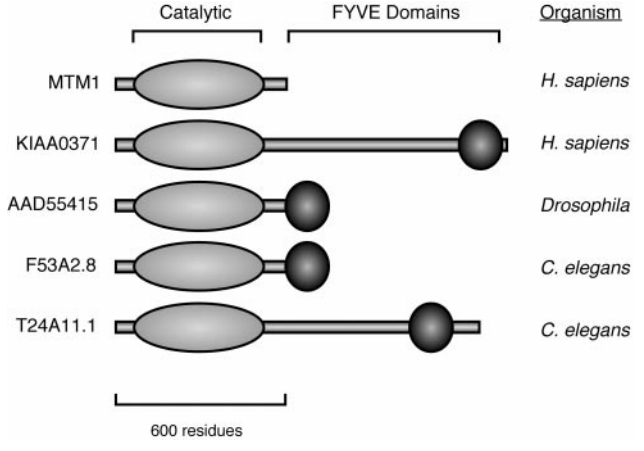

Fig. 5. Myotubularin-like proteins contain C-terminal FYVE domains. The structural features of myotubularin and myotubularin-related proteins from human, *D. melanogaster*, and *C. elegans* are illustrated. N-terminal lipid phosphatase catalytic domains are represented as gray shaded ovals, and C-terminal FYVE domains are shown as shaded black circles.

affected individuals levels may disrupt vesicular trafficking, thus impairing transport to the plasma membrane of cargoes necessary for this fusion process. In addition, previous studies have identified proteins required for vesicular trafficking processes that contain a segment known as a FYVE domain (21). FYVE domains are zinc finger-like domains that specifically bind to PI(3)P, acting as targeting motifs to direct proteins to specific sites within the cell (14, 21, 23–25). Myotubularin may act to regulate the subcellular localization of proteins containing FYVE domains by controlling cellular levels of their target lipid, PI(3)P, thus affecting specific signaling pathways dependent on these effectors. By searching the protein database, we and others have found that several myotubularin-like proteins from human (KIAA0371, KIAA0647), *Drosophila melanogaster* (AAD55415), and *Caenorhabditis elegans* (F53A2.8, T24A11.1) also contain C-terminal FYVE domains (Fig. 5) (25, 42, 43). The function(s) of FYVE domains in myotubularin-like proteins are unknown but are likely to direct these phosphatases to cellular environments in which PI(3)P is concentrated. Future work focusing on the possible involvement of myotubularin and its relatives in membrane trafficking may help to clarify the role of PI(3)P in myogenic differentiation.

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