Myotubularin Regulates the Function of the Late Endosome through the GRAM Domain-Phosphatidylinositol 3,5-Bisphosphate Interaction*

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Myotubularin and related proteins constitute a large and highly conserved family possessing phosphoinositide 3-phosphatase activity, although not all members possess this activity. This family contains a conserved region called the GRAM domain that is found in a variety of proteins associated with membrane-coupled processes and signal transduction. Mutations of myotubularin are found in X-linked myotubular myopathy, a severe muscle disease. Mutations in the GRAM domain are responsible for this condition, suggesting crucial roles for this region. Here, we show that the GRAM domain of myotubularin binds to phosphoinositide with the highest affinity to phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2). In patients with myotubular myopathy, mutations in the myotubularin GRAM domain eliminate this binding, indicating that the PtdIns(3,5)P2 binding ability of the GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularin) domain is crucial for the functions of myotubularin in vivo. Stimulation of epidermal growth factor receptors recruits myotubularin to the late endosomal compartment in a manner dependent on the phosphoinositide binding. Overexpression of myotubularin inhibits epidermal growth factor receptor trafficking from late endosome to lysosome and induces the large endosomal vacuoles. Thus, our data suggest that myotubularin phosphatase physiologically functions in late endosomal trafficking and vacuolar morphology through interaction with PtdIns(3,5)P2.

In eukaryotic cells, D3-phosphorylated phosphoinositides such as phosphatidylinositol 3-phosphate (PtdIns3P)1 play key roles in the vesicular trafficking through direct interaction with phosphoinositide-binding domains such as the PH domain, FYVE finger domain, and PX (Phox) domain found in effector proteins that control vesicular trafficking (1–3). Previous studies have revealed that PtdIns3P binding is essential for the recruitment/activation of these effector proteins at unique membrane sites (4, 5). PtdIns(3,5)P2 was one of the phosphoinositide species identified recently in both yeast and mammalian cells (6). PtdIns(3,5)P2 is thought to be involved in osmotic stress responsiveness and essential for the maintenance of vacuole size and homeostasis in yeast (7). Recently, it was reported that PtdIns(3,5)P2 is necessary for late endosomal trafficking in yeast (8). However, the mechanisms for cellular PtdIns(3,5)P2 regulation are unknown. Intracellular levels of these phosphoinositide species are strictly regulated by enzymes that dephosphorylate at the D3-position of the inositol ring. Myotubularin and its related proteins (myotubulin-related proteins; MTMRs) constitute a large and highly conserved subfamily of dual specific phosphatases that were recently revealed to be phosphoinositide 3-phosphatases (9–11). Among those proteins, myotubularin is encoded by the MTM1 gene, which is mutated in X-linked myotubular myopathy (12), whereas MTMR2 is associated with neurodegenerative disorder Charcot-Marie-tooth disease type 4B (13). Myopathy patients have severe hypotonia at birth, and most of them die from hypoventilation within the first months of life (14). This disease is characterized by the presence of disorganized skeletal muscle fibers that contain centrally located nuclei, resembling myotubes. Recent work has reported that myotubularin is essential for skeletal muscle maintenance but not for myogenesis in mice (15). However, the cellular functions of myotubularin are still obscure. MTMRs also share a conserved region of about 70 amino acids, called the GRAM domain, which has also been found in glucosyltransferases, GTPase-activating proteins of the Rab small GTPases, and other adaptor proteins associated with membrane-coupled processes (16). Some mutations in the GRAM domain of myotubularin cause X-linked myotubular myopathy, suggesting the importance of this domain (17–19). A very recent study reported that the GRAM domain of MTMR2 binds to PtdIns(3,4,5)P3, PtdIns(3,5)P2, and PtdIns5P-related proteins; VRP, verapamil; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; EGFP, enhanced GFP; GRAM, glucosyltransferases, Rab-like GTPase activators and myotubularins; FYVE, Fab1p, YOTB, Vac1p, and EEA1.

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Fig. 1. Interaction between the GRAM domain and PtdIns(3,5)P₂. An ELISA phosphoinositide binding assay with GST fusion proteins was performed. (a), the schematic structure of myotubulin. (b), each well of a microtiter plate was coated with 2.0 μg of total phospholipid containing 10% of the indicated phospholipid and then overlaid with GST, GST fusions of phospholipase C-1 PH, Akt/PKB PH, and myotubulin GRAM. Amounts of bound proteins were calculated as described under “Experimental Procedures.” Results from three independent experiments are represented as mean values ± S.E. (error bars). PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns5P, phosphatidylinositol 5-phosphate. (c), membrane overlay assay. Binding of either GST-myotubulin or GST to immobilized phosphoinositides was assessed by overlay assay using PIP arrays (Echelon Bioscience). (d), the ELISA assay was carried out using GST-GRAM domains of MTMR2 and VRP. (e), liposome binding assay with the GRAM domain. Liposomes (PC/PE = 4:1; total 100 μg) containing various amounts of PtdIns(3,5)P₂ were mixed with GST-GRAM. Proteins in the supernatant (s) and precipitate (p) were visualized by SDS-PAGE and stained with Coomassie Brilliant Blue (upper figure). Binding of GST-GRAM domains of myotubulin, MTMR2, and VRP to liposomes containing 5% of PtdIns or PtdIns(3,5)P₂ (lower figure). Data are representatives of three independent experiments. (f), comparison of wild type and mutants of myotubulin GRAM in phosphoinositide binding. Graphs show the binding ability of wild type (open circle) and mutants (V49F (closed circle); R69C (open square); L70F (closed square); L87P (open triangle)) to different amounts of PtdIns or PtdIns(3,5)P₂. The results are presented by the average of three independent experiments.
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P2 is essential for full function of this phosphatase. We demonstrated that overexpression of myotubulin inhibits epidermal growth factor receptor (EGFR) trafficking from the late endosome to the lysosome and induces large endosomal vacuoles. Significantly, GRAM domain mutants, as well as phosphatase inactive mutants seen in myopathy patients, do not induce these effects. Thus, our data indicate that through interaction with PtdIns(3,5)P2, myotubulin functions in late endosomal trafficking and vacuolar formation.

**EXPERIMENTAL PROCEDURES**

**DNA Construction**—Human myotubulin (8–112), MTMR2 (49–155), and VRP GRAM-(21–155), and VRP GRAM-(21–112) were obtained by reverse transcriptase-PCR. Obtained cDNA sequences were verified and subcloned into pGEM-T (Amersham Biosciences). Full-length cDNA of human myotubulin was obtained by reverse transcriptase-PCR. After the sequence was verified, it was subcloned into pEGFP-C1 (Clontech), pCMV Myc, and pEF-Bos Myc. Site-directed mutagenesis was carried out by PCR with mutated primers. Myotubulin ΔGRAM was generated by deleting the first 119 amino acids of myotubulin.

**ELISA Lipid Binding Assay**—All synthetic phosphoinositides with C16 fatty acids were purchased from Cell Signals Inc., whereas other phospholipids (PA, PC, PE, PS, and PE) were obtained from Sigma. Lipid vesicles (PE/PC = 1/1; total 2 μg) containing the indicated ratio (weight percentage) of phospholipids resuspended in ethanol were coated on a 96-well microtiter plate (Immulon 2;Dynatech) and dried at room temperature. The wells were then blocked with phosphate-buffered saline containing 5% bovine serum albumin before incubation with the respective glutathione S-transferase (GST) fusion proteins (1.0 μg/ml) for 45 min. After washing with phosphate-buffered saline containing 0.05% Tween 20, bound protein was detected by reaction of the substrate, orthophenylendiamine dihydrochloride, with glutathione conjugated to peroxidase (Sigma). The colorimetric reaction was measured at 492 nm in an ELISA plate reader (Bio-Rad).

**Lipid Overlay Assay**—Flp arrays were purchased from Echelon Bioscience Inc. In brief, arrays were incubated overnight at 4 °C with GST fusion protein (0.5 μg/ml) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) with fatty acid-free bovine serum albumin (Sigma). Membranes were washed and incubated with monoclonal antibody, GST antibody (Sigma). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody and visualized using an ECL kit (Amersham Biosciences).

**Measurement of PtdIns(3,5)P2 Levels in EGF-stimulated COS-7 Cells**—COS-7 cells were cultured in serum-deprived medium for 24 h and then in orthophosphate-free Dulbecco’s modified Eagle’s medium for 15 min. After radiolabeling with 5 μCi of [32P]orthophosphate for 2 h, cells were stimulated with 100 ng/ml human recombinant EGF (Invitrogen) for 0, 20, 40, 60, and 120 min. Cells were washed three times with ice-cold phosphate-buffered saline, and phosphoinositides were extracted with the addition of 1.5 ml of methanol followed by the same volume of chloroform:methanol (1:2, v/v). After brief sonication, the sample volume of chloroform and then 1 ml of ethyl ether was added. The organic phase was pooled and dried under nitrogen gas. The phosphoinositides were deacylated with methanol and acetic acid (1:1, v/v) for 2 h. After filtration, the sample volume of chloroform was reduced to 1 ml. The organic phase was deproteinized with 1.5 ml of 0.1 M sodium tungstate solution (20% vol/vol) for 5 min. The aqueous phase was a mixture of 20% methanol, 45.7% methanol, 11.4% butanol, and 5% water. The samples were submitted to Flscillation Analyser (PerkinElmer Life Sciences).

**Transfections—**COS-7 cells were transfected by either the calcium phosphate method or Lipofectin using LipofectAMINE (Invitrogen) and examined 24 h after transfection. Transfected cells on coverslips were fixed in 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and immunostained with various antibodies. For EGF stimulation, after starvation, cells were incubated with EGF (50 ng/ml, Invitrogen) at 37 °C for the indicated times. For treatment with wortmannin, cells were incubated with wortmannin (100 μM Sigma) for 15 min before EGF stimulation or fixation. Coverslips were examined using confocal microscopy (Bio-Rad).

**Electron Microscopy**—Conventional electron microscopy was performed as described previously (34). Briefly, COS-7 cells grown on plastic coverslips were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h. After washing in the buffer, cells were post-fixed in 1% OsO4 for 60 min, washed in distilled water, incubated with 15% ethanol for 10 min, and stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections were doubly stained with uranyl acetate and lead citrate.

**RESULTS AND DISCUSSION**

**The GRAM Domain Binds to PtdIns(3,5)P2**—We examined the possibility that the GRAM domain of myotubulin and related proteins could bind to phosphoinositides. We used an ELISA assay where phospholipids composed of phosphatidyethanolamine (PE), phosphatidylcholine (PC), and each phosphoinositide were coated on ELISA plates and overlaid with a GST fusion protein of interest. We first confirmed that GST alone showed no affinity to any of the phospholipids in this assay (Fig. 1a). Specific binding was observed between the PH domain of phospholipase C-61 and PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (23, 24), indicating the validity of this assay (Fig. 1a). The GRAM domain of myotubulin was found to bind to the phospholipid containing PtdIns(3,5)P2 with the highest affinity (Fig. 1a). PtdIns(3,4,5)P3 and PtdIns(3,5)P2 were also favored to some degree. Furthermore, PH domains did not bind PtdIns(3,5)P2 at all in this assay, confirming that the binding detected for the GRAM domain was not an artifact. Interaction between the GRAM domain and PtdIns(3,5)P2 was also examined by overlay assay where each phosphoinositide was spotted on the membrane without any basal lipid as in the ELISA assay. Although the GRAM domain still bound strongly to PtdIns(3,5)P2, binding to other phosphoinositides was also observed (Fig. 1b). In this assay, GST alone did not bind to any phosphoinositide (Fig. 1b). We also carried out the same ELISA assay using GRAM domains from MTMR2 and VRP and found they also bound to PtdIns(3,5)P2 most strongly (Fig. 1c). Moreover, to confirm this interaction in a membranous environment, we performed a liposome binding assay. Although the GRAM domain of myotubulin, MTMR2, and VRP did not co-sediment liposomes containing PtdIns, co-sedimentation between liposomes containing PtdIns(3,5)P2 and the GRAM domain was observed (Fig. 1d). These findings indicate that the evolutionally conserved GRAM domain is designed for binding to PtdIns(3,5)P2.

Among GRAM-containing proteins, myotubulin is the product of the MTM1 gene, the gene responsible for X-linked myotubular myopathy. Four cases of missense mutation in the GRAM domain (V49F, R69C, L70F, L87P) have been reported to be associated with the disease (18, 19). We generated these point mutants and compared their phosphoinositide binding abilities. As shown in Fig. 1e, the binding ability was significantly reduced for the V49F, a mutation that is associated with a more severe phenotype. Mutations of R69C, L70F, or L87P,
FIG. 2. GRAM-PtdIns(3,5)P₂ binding is essential for translocation of myotubularin to the late endosomal compartment after EGF stimulation. 

a, COS-7 cells were transfected with GFP-myotubularin. After starvation, cells were incubated with EGF (50 ng/ml) for the indicated time periods. After the cells were fixed, the intracellular localization of GFP-myotubularin and EGFR was visualized by immunofluorescence. Yellow indicates co-localization of both proteins. 

b, increased PtdIns(3,5)P₂ content in response to EGF stimulation. COS-7 cells were labeled with [³²P]orthophosphate and stimulated with EGF. Lipid labeling values were corrected to a reference value of 2.0 × 10⁷ cpm of total [³²P]-labeled lipid in each experiment. Data are representatives of three independent experiments. 

c, overexpression of PIKfyve recruits myotubularin to the perinuclear area, where they co-localize. Yellow indicates co-localization of both proteins. 

d, the localization of GFP-myotubularin mutants at 40 min after EGF stimulation was compared with that of EGFR. Both mutants showed cytosolic patterns but no co-localization with EGFR. 

e, quantitative representation of EGF dependent recruitment of myotubularin to the late endosomal compartments. After treatment with EGF for 40 min, cells that changed the localization of the indicated proteins to the late endosomal compartments were counted and represented as percentages relative to all cells observed (total of >50 cells) from three independent experiments.
which are associated with a milder phenotype, reduced the binding to a lesser extent. These findings suggest that phosphoinositide binding ability of the GRAM domain is crucial for functions of myotubularin in vivo.

The Essential Role of the GRAM Domain Is in Translocation to the Late Endosome—Because our results redefined myotubularin as both a phosphoinositide 3-phosphatase and a PtdIns(3,5)P2-interacting protein, and because previous studies have indicated essential roles for PtdIns3P and PtdIns(3,5)P2 in endosomal trafficking (3, 25), we next exam-
FIG. 4. Large endosomal vacuoles induced by myotubularin overexpression. a, each myotubularin protein was overexpressed in COS-7 cells as in Fig. 3. Cells were stimulated with EGF for 40 min and then fixed and stained with anti-EGFR (red). WT, wild type. b, electron microscopy of large vacuoles induced by myotubularin overexpression. Arrows indicate internal vesicles. Bar, 1.6 μm. c, quantitative representation of large vacuolar formation by expression of various proteins. Cells that induced the formation of large vacuoles (with diameters more than 1.5 μm) were counted and represented as percentage relative to all cells observed (total of > 50 cells) from three independent experiments.
ined the possibility that myotubularin is involved in intracellular vesicular trafficking. The localization of the GFP-tagged form of myotubularin was monitored after EGF stimulation in COS-7 cells. In non-stimulated cells, EGFR retained at the plasma membrane, whereas distribution of GFP-myotubularin is mainly cytosolic (Fig. 2a). 20 min after EGF stimulation, EGFR internalization was observed by the significant co-localization with the EEA1-positive early endosome (not shown). GFP-myotubularin, however, was still distributed throughout the cytosol at this time point (Fig. 2e), indicating that myotubularin is not involved in the EGFR trafficking from the plasma membrane to the early endosome. However, after 40 min of EGF stimulation, myotubularin was observed to translocate together with the internalized EGFR (Fig. 2a) to the late endosomal compartment shown by its partial colocalization with LAMP1, a marker protein for the late endosome/lysosome (not shown). We also confirmed that cellular PtdIns(3,5)P₂ levels were increased in response to EGF stimulation and peaked at 40 min after EGF stimulation (Fig. 2b), indicating the possibility that translocation of myotubularin is dependent on interactions between the GRAM domain and PtdIns(3,5)P₂. Moreover, to clarify whether intracellular localization of myotubularin is affected by PtdIns(3,5)P₂ in vivo, we overexpressed PIKfyve, a phosphoinositide-5-kinase shown to produce PtdIns(3,5)P₂ both in vivo and in vitro (26). As shown in Fig. 2c, overexpression of PIKfyve recruited cytosolic myotubularin to the perinuclear area where they co-localized, suggesting that the localization of myotubularin is determined by PtdIns(3,5)P₂. To evaluate the importance of interaction between the GRAM domain and phosphoinositides, we further examined the effect of mutations in myotubularin on its translocation. Importantly, myotubularin ΔGRAM in which the whole GRAM domain is deleted did not translocate to the late endosomal compartment after EGF treatment (Fig. 2d). The same result was observed with myotubularin V49F, in which the mutated GRAM domain was unable to bind PtdIns(3,5)P₂ (Fig. 2d). This indicates that the lack of PtdIns(3,5)P₂ binding ability by a point mutation is comparable with the loss of the entire GRAM domain. Moreover, cell pretreatment with the PI3-kinase inhibitor wortmannin prevented the recruitment of myotubularin to this compartment subsequent to EGF stimulation (Fig. 2e), supporting the idea that the myotubularin translocation is dependent on a D3-phosphoinositide.

**Myotubularin Negatively Regulates EGFR Degradation**—The translocation of myotubularin to the EGFR-positive late endosomal compartment suggested its roles in the degradation pathway of EGFR. To assess the effect of myotubularin on EGFR trafficking, we overexpressed myotubularin in COS-7 cells with the pEF-Bos vector that carries a powerful promoter main-containing protein, has been found to bind PtdIns(3,5)P₂, facilitating the dephosphorylation of the phosphoinositide. Moreover, point mutations in the GRAM domain, reported in severe cases, eliminate this binding, indicating that GRAM-PtdIns(3,5)P₂ interaction is crucial for functions of myotubularin in vivo. In yeast, the formation of PtdIns(3,5)P₂ from PtdIns3P is catalyzed by Fab1p, a PtdIns3P-5-kinase necessary for maintenance of normal vacuolar morphology and functions (7). Fab1P as well as PIKfyve, a mammalian homologue of Fab1p, are reported to localize to late endosome/multivesicular bodies (30), suggesting that localization of PtdIns(3,5)P₂ may also be to this compartment. According to this hypothesis, myotubularin probably localizes to the late endosome via interaction with PtdIns(3,5)P₂. In addition, recent studies have revealed that PIKfyve suppressed the vacuolar enlargement in mammalian cells, indicating that it functions to maintain the proper size of vacuolar organelles (31). Thus, our data strongly suggest that myotubularin physiologically functions in the late endosomal trafficking and vacuolar morphology by controlling PtdIns(3,5)P₂ and show the indispensable roles of the GRAM domain in its cellular functions. Recently, Ent3p, a yeast epsin N-terminal homology (ENTH) domain-containing protein, has been found to bind PtdIns(3,5)P₂ through its ENTH domain and function in the late endosome (32), indicating that this lipid plays important roles in sorting of membrane proteins at late endosomal compartment throughout yeast to mammals. However, the precise mechanism of how PtdIns(3,5)P₂ regulates sorting of membrane proteins remains to be solved in future.
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