FEBS Letters 585 (2011) 1722–1728

journal homepage: www.FEBSLetters.org

Receptor mediated endocytosis 8 is a novel PI(3)P binding protein regulated by myotubularin-related 2

Besa Xhabija [a], Gregory S. Taylor [b], Akemi Fujibayashi [c], Kiyotoshi Sekiguchi [c], Panayiotis O. Vacratsis [a,*]

[a] Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada N9B3P4
[b] Department of Biochemistry and Molecular Biology, University of Nebraska, Nebraska Medical Center, Omaha, NE, USA
[c] Division of Protein Chemistry, Institute for Protein Research, Osaka University, Osaka, Japan

ARTICLE INFO

Article history:
Received 24 January 2011
Revised 17 March 2011
Accepted 7 April 2011
Available online 15 April 2011

Edited by Lukas Huber

Keywords:
Myotubularin
Endocytosis
Mass spectrometry
Phosphoinositide phosphatase
Receptor mediated endocytosis
Epidermal growth factor receptor

ABSTRACT

Myotubularin related protein 2 (MTMR2) is a member of the myotubularin family of phosphoinositide lipid phosphatases. Although MTMR2 dephosphorylates the phosphoinositides PI(3)P and PI(3,5)P2, the phosphoinositide binding proteins that are regulated by MTMR2 are poorly characterized. In this study, phosphoinositide affinity chromatography coupled to mass spectrometry identified receptor mediated endocytosis 8 (RME-8) as a novel PI(3)P binding protein. RME-8 co-localized with the PI(3)P marker DsRed-FYVE, while the N-terminal region of RME-8 is required for PI(3)P and PI(3,5)P_2 binding in vitro. Depletion of PI(3)P by MTMR2 S58A or wortmannin treatment attenuated RME-8 endosomal localization and co-localization with EGFR on early endosomes. Our results suggest a model in which the localization of RME-8 to endosomal compartments is spatially mediated by PI(3)P binding and temporally regulated by MTMR2 activity.

Structured summary of protein interactions:
EGFR and **LAMP1** colocalize by fluorescence microscopy (View interaction)
RME8, **EGFR** and **Rab5** colocalize by fluorescence microscopy (View interaction)
RME8 and **EGFR** colocalize by fluorescence microscopy (View interaction)

1. Introduction

The phosphatidylinositol phosphate (PIP) isoforms PI(3)P and PI(3,5)P_2 are known to serve as membrane targeting ligands for proteins that are essential for cellular membrane trafficking processes. These lipid–protein interactions function in translocating signaling proteins to discrete membrane locations where they can properly respond to extracellular stimuli [1]. The signaling pathways are subsequently turned off through the dephosphorylation of the PIPs.

The MTMR family of lipid phosphatases, composed of active and inactive subgroups, represents the largest protein tyrosine phosphatase (PTP) subfamily conserved from yeast to humans [2,3]. Initially, MTM1 was the first family member shown to dephosphorylate the D3 position of PI(3)P in vitro and in vivo [4,5]. Subsequently, all active members tested have been shown to also be PI(3)P phosphatases [6,7]. Furthermore, there is accumulating evidence that MTMs can also utilize PI(3,5)P_2 as a physiologic substrate [6,8,9]. Therefore, MTMs are thought to antagonize effector molecules that utilize PI(3)P or PI(3,5)P_2 as targeting ligands and/or allosteric activators.

Mutations in *mtmr2*, on chromosome 11q22, have been shown to cause the neurodegenerative disorder, Charcot-Marie-tooth disease 4B1 (CMT4B1) [10]. CMT4B1 is an autosomal recessive aggressive form of CMT, characterized by abnormally folded myelin sheaths, inadequate nerve signaling to muscles, and eventual muscle weakness and atrophy [11]. While the pathophysiological consequence resulting from the loss of myotubularin related protein 2 (MTMR2) function is well established, how MTMR2 participates in trafficking events remains poorly understood. Recently we have characterized an N-terminal MTMR2 phosphorylation site at position Ser^{58} that dramatically regulates MTMR2 endosomal localization and thus access to its lipid substrates [12]. A phosphorylation

Abbreviations: MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; TOF, time of flight; PTP, protein tyrosine phosphatase; RME-8, receptor mediated endocytosis 8; Hsc70, heat shock cognate 70; MTMR2, myotubularin related protein 2; PIP, phosphatidylinositol phosphate; PI(3)K, phosphoinositide 3-kinase; EGFR, epidermal growth factor receptor; CMT4B1, Charcot-Marie tooth disease 4B1

* Corresponding author. Fax: +1 519 9737098.
E-mail address: vacratsi@uwindsor.ca (P.O. Vacratsis).

0014-5793/$36.00
doi:10.1016/j.febslet.2011.04.016

deficient variant (MTMR2 S58A) displays strong endosomal localization and an enhanced ability to deplete PI(3)P from endosomal vesicles signifying that reversible phosphorylation is a critical mechanism regulating the activities of MTMR2.

The biological pathways affected by MTMR proteins remain inadequately characterized. This task has proven difficult due to the poor understanding of PIP binding domains that specifically interact with the PIP isoforms that MTMRs target [1]. In this study, we utilized a proteomic approach using immobilized PIP isoforms to identify novel PIP binding proteins. Herein, we describe the identification of receptor mediated endocytosis 8 (RME-8) as a novel PI(3)P binding protein whose lipid binding activity is affected by MTMR2.

2. Materials and methods

2.1. Identification of novel PIP binding proteins by mass spectrometry

Immobilized PIP isoforms (Echelon) were prepared for affinity chromatography according to the manufacturer's protocol. RT4-D6P2T Schwann cells (6×10^7) were grown to 80% confluency, lysed and the soluble lysate filtered. The cellular lysates was pre-cleared with PI control beads followed by the loading of 10 mg of total protein to either PIP, PI(3)P, PI(3,5)P_2, or PI(5)P resin. The lipid pulldown was performed by batch method overnight at 4 °C. Following rigorous washing conditions the bound proteins were eluted in SDS–PAGE loading buffer and analyzed by SDS–PAGE and silver staining. Protein bands of interest were excised and in-gel digested with trypsin. The extracted pools of tryptic peptides were then analyzed by matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) for protein identification as described previously [13].

2.2. Cell culture and transfection

HeLa and HEK293 cells were utilized for their reproducible transfection efficiency and were maintained in DMEM/ F-12 supplemented with 10% FBS, 1% penicillin/streptomycin and maintained at 37 °C with 5% CO_2. Cells were transiently transfected with expression vectors encoding FLAG tagged-MTMR2 S58A [12] and GFP-RME-8 variants [14] using FuGene HD Transfection Reagent (Roche) according to the manufacturer's protocol. The DsRed-FYVE construct was created by PCR amplification of the sequence encoding the FYVE domain of EEA1 followed by cloning into the pDsRed expression vector (Clonetech). Where indicated, wortmannin treatment (1 μM) was for 1 h at 37 °C.

2.3. PIP pulldown

HEK293 cells were transiently transfected with GFP-RME-8 variants for 24 h and lysed in modified RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 76 mM NaCl, 2 mM EGTA, 10% Glycerol) supplemented with protease inhibitors PMSF (1 mM) and aprotinin (1 mM). Cell lysates were incubated with PI(3)P, PI(3,5)P_2 and PIP conjugated resin (Echelon Research Laboratories) overnight at 4 °C. The samples were washed 3 times with 10 mM HEPES pH 7.4, 150 mM NaCl, 0.25% NP-40 and resuspended in SDS–PAGE loading dye. The protein samples were separated on an 8% SDS–PAGE, transferred to a PVDF membrane and immunoblotted with goat anti-GFP (Rockland) as the primary antibody and rabbit-anti goat HRP (Rockland) as the secondary antibody. Proteins were visualized using Super Signal West Femto reagent (Thermo scientific).

2.4. Immunoflourescence and image acquisition

Following transient transfection, Hela cells were fixed at room temperature with 3.7% paraformaldehyde in PBS. Cells were then permeabilized with 0.15% Triton X-100 in PBS at room temperature for 2 min and blocked for 1 h in 5% BSA (Sigma). Cells were incubated with mouse anti-FLAG antibody (Sigma–Aldrich) or rabbit anti-EGFR (Santa Cruz Biotechnology) in TBST. Following washing, cells were incubated with either Alexa 568 goat anti-mouse, Alexa 350 donkey anti-rabbit (Invitrogen) or fluorescein goat anti-rabbit (Vector laboratories) secondary antibodies. All the incubations were performed at room temperature for 1 h followed by three 5 min washes in TBST. Hoechst 33342 (Molecular Probes) was used to stain the nuclei and Slowfade Antifade kit was used to mount the slides (Molecular Probes) according to the manufacturer's protocol. Images were captured with a Q-imaging CCD camera on a Leica DMIRB microscope using the Northern Eclipse software and Abobe Photoshop 7.0.

3. Results and discussion

3.1. Isolation of the RME-8/Hsc70 complex from Schwann cells using PI(3)P affinity chromatography

Knowledge of proteins affected by the lipid phosphatase activity of MTMR2 is poorly defined. To identify putative targets of MTMR2, a pull down assay was performed using disease relevant Schwann cells and conjugated PI(3)P and PI(3,5)P_2 beads. Beads conjugated with the enzymatic products of MTMR2, PI and PI(5)P, were also included. The PIP beads were incubated with equal concentrations of cellular lysate from rat RT4-D6P2T Schw-

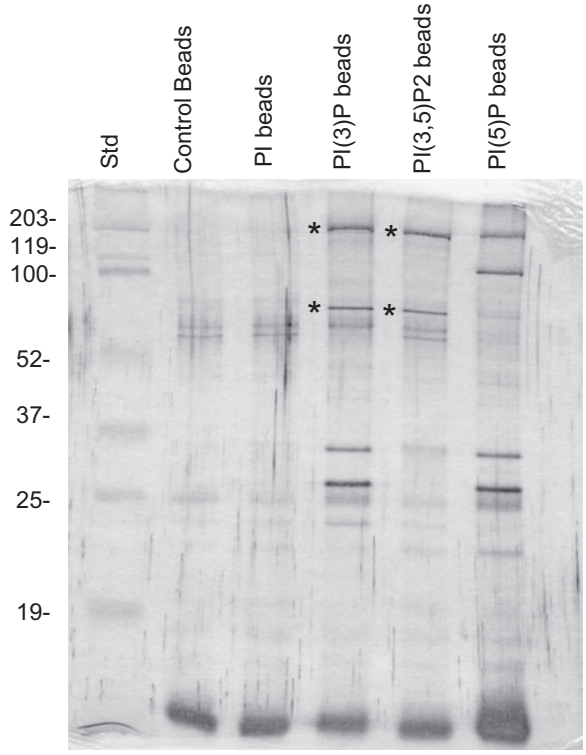

Fig. 1. PIP lipid pull down from Schwann cells. Cell lysates were prepared from rat RT4-D6P2T Schwann cells (6×10^7). Approximately 10 mg of total protein was subjected to affinity chromatography on the indicated immobilized PIP resin. Following stringent washing conditions, the samples were resolved on a 12% SDS–PAGE gel and silver stained. Asterisks indicate the protein bands of interest which were excised and identified by mass spectrometry.