Systematic analysis of myotubularins: heteromeric interactions, subcellular localisation and endosome-related functions

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Summary

The myotubularins are a large family of lipid phosphatases with specificity towards PtdIns3*P* and PtdIns(3,5)P_2. Each of the 14 family members bears a signature phosphatase domain, which is inactive in six cases due to amino acid changes at the catalytic site. Fragmentary data have indicated heteromeric interactions between myotubularins, which have hitherto paired an active family member with an inactive one. In this study we have conducted a large-scale analysis of potential associations within the human myotubularin family, through directed two-hybrid screening and immunoprecipitation of epitope-tagged proteins. We have confirmed all previously reported combinations and identified novel heteromeric interactions: MTMR8 with MTMR9, and MTMR3 with MTMR4, the first such combination of enzymatically active MTMs. We also report the capacity of several family members to self-associate, including MTMR3 and MTMR4. Subcellular localisation studies reveal a unique distribution of MTMR4 to endosomal structures, the major site of substrate lipid accumulation. All active MTMs we have tested (MTM1, MTMR2-MTMR4) reduce endosomal PtdIns3*P* levels upon overexpression. Despite this, only MTMR4 exerts any effect on EGF receptor trafficking and degradation, which is more pronounced with a phosphatase inactive form of MTMR4 and requires an intact FYVE domain.

Key words: Myotubularin, Phosphatase, Yeast two-hybrid, Protein-protein interactions, Endosomes

Introduction

The myotubularin (MTM) family constitutes one of the largest and most highly conserved protein-tyrosine phosphatase subfamilies in eukaryotes (Alonso et al., 2004; Clague and Lorenzo, 2005). The consensus CX_5R active site motif is found in the MTM family and the sequence 'CS**D**GW**D**R' is invariant within all of the enzymatically active members. There are eight active members of the family, and six further members that bear inactivating mutations within the catalytic site (Laporte et al., 2003). Each myotubularin possesses an N-terminal PH-GRAM (PH-G) domain and, with one exception, a coiled-coil domain, whereas several distinct C-terminal domains, such as PDZ or FYVE domains are represented within the family (Fig. 1A). Active members possess conserved phosphoinositide 3-phosphatase activity towards PtdIns3*P* and PtdIns(3,5)P_2 (Blondeau et al., 2000; Schaletzky et al., 2003; Taylor et al., 2000; Walker et al., 2001), which are important lipid regulators of endosomal dynamics (Roth, 2004). Mutations in MTM1 lead to myotonic myopathy (Laporte et al., 1996), whereas mutations or truncations in MTMR2 or MTMR13/Sbf2 lead to clinically indistinguishable forms of Charcot-Marie-Tooth syndrome (Azzedine et al., 2003; Berger et al., 2002; Bolino et al., 2000; Senderek et al., 2003). A recent siRNA screen has also suggested that MTMs may regulate cell survival (Mackeigan et al., 2005).

Several interactions between myotubularin family members have now been characterised through identification of co-immunoprecipitating proteins in isolated studies. Thus MTM1 and MTMR2 interact with MTMR12/3-PAP (Nandurkar et al., 2003), MTMR6 and MTMR7 interact with MTMR9 (Mochizuki and Majerus, 2003), and MTMR2 interacts with both MTMR5/Sbf1 and MTMR13/Sbf2 (Kim et al., 2003; Robinson and Dixon, 2005). Furthermore, MTMR2 has been shown to dimerise through its coiled-coil domain (Berger et al., 2003; Kim et al., 2003) and MTM1 can form heptameric ring stuctures (Schaletzky et al., 2003). So far, all heteromeric interactions have paired an active enzyme with an inactive enzyme, which suggests that this could constitute a 'rule' that may have functional significance.

To date there has been no systematic survey of the extent of myotubularin intra-family associations. We have now tested interactions between 12 family members by directed yeast two-hybrid screening (144 combinations) and by co-immunoprecipitation of epitope-tagged proteins expressed in HeLa cells. We confirmed all previously characterised heteromeric interactions and identified several novel interactions, most notably between two active enzymes MTMR3 and MTMR4. We also report data on the subcellular localisation of each protein when overexpressed and the influence that co-expression of binding partners may have on this distribution. MTMR4 shows the clearest localisation to endosomal compartments and expression of a catalytically

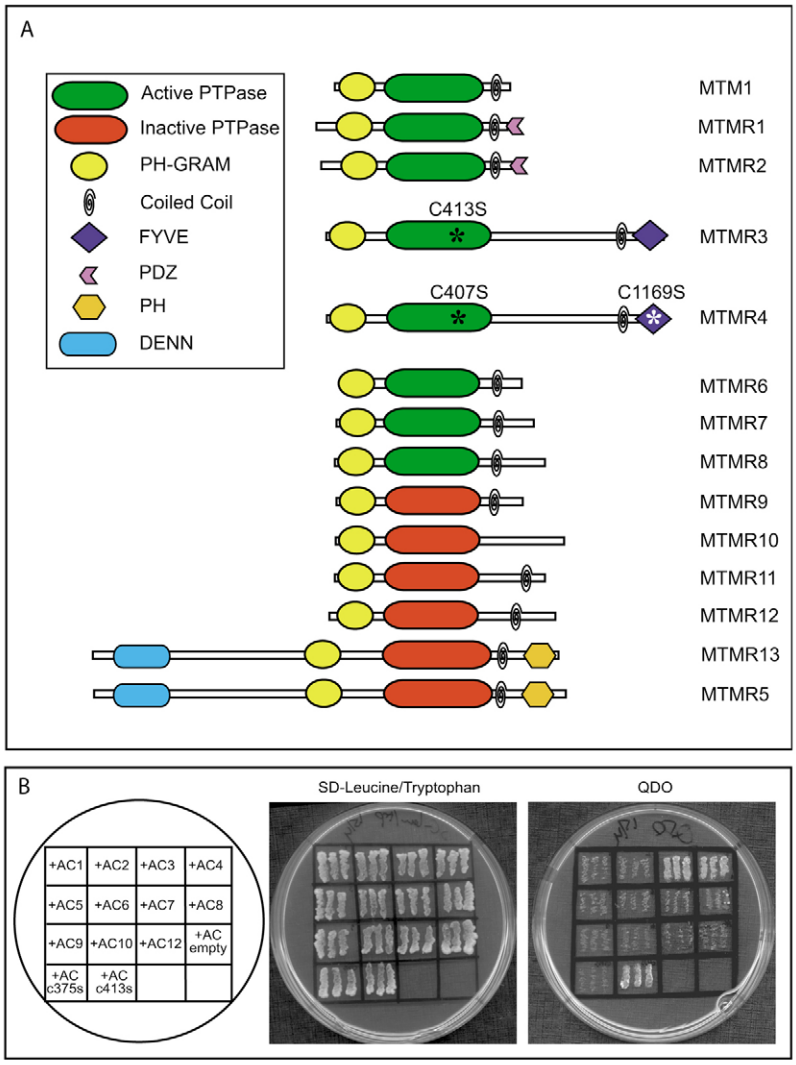

Fig. 1. A directed two-hybrid screen for MTM interactions. (A) Schematic diagram indicating domain structure of the myotubularin family. Point mutations used in this study are indicated by asterisks. (B) Example of a yeast two-hybrid screen for MTMR4 interactions with other MTM family members. Yeast cells were co-transfected with bait-MTMR4 and all different preys (AC). On the selective QDO plate (right), growth (interaction) was observed with MTMR3 and MTMR4 in the prey vectors (see scheme on the left). A catalytically inactive mutant of MTMR3 (C413S) retains binding to MTMR4. As a control, the same co-transformations were plated onto SD-leu/trp (centre).

inactive mutant inhibits the EGF receptor (EGFR) degradation pathway.

Results and Discussion

Various reports have demonstrated heteromeric and homomeric interactions between myotubularin family members (Berger et al., 2003; Dang et al., 2003; Kim et al., 2003; Mochizuki and Majerus, 2003; Nandurkar et al., 2003; Robinson and Dixon, 2005; Schaletzky et al., 2003). Several lines of evidence suggest that these associations are functionally significant: (1) increased enzyme activity through association with an inactive partner (Kim et al., 2003; Mochizuki and Majerus, 2003; Schaletzky et al., 2003); (2) altered subcellular distribution through expression of binding partners (Kim et al., 2003); (3) defects in either MTMR2 and MTMR13/Sbf2 lead to a clinically indistinguishable form of Charcot-Marie-Tooth syndrome (Robinson and Dixon, 2005). To date heteromeric interactions have been detected biochemically through co-immunoprecipitation experiments of both endogenous and overexpressed proteins. Consequently, our knowledge of the specificity of interactions and promiscuity of family members is

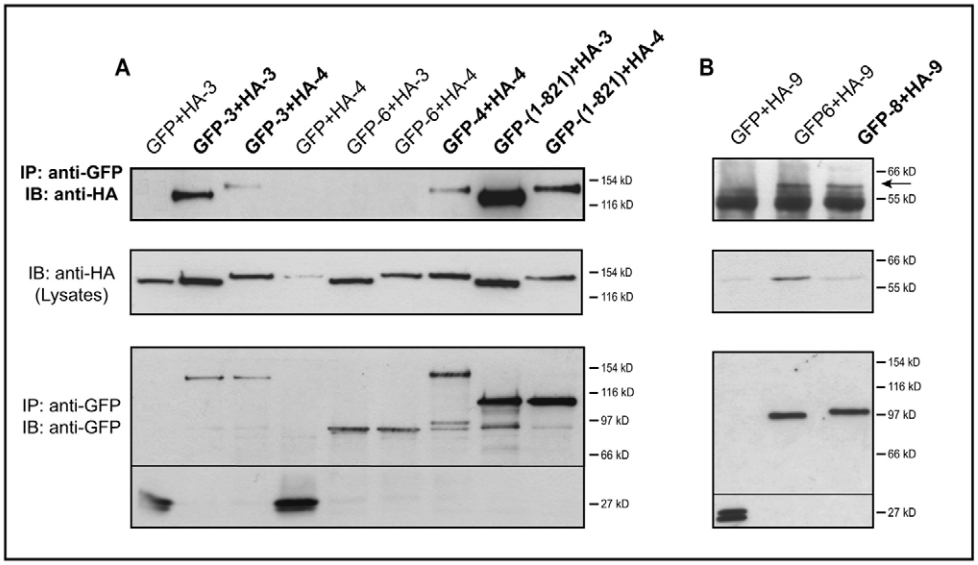

Fig. 2. Co-immunoprecipitation of human myotubularin family members. HeLa cells were transfected with HA-MTMR3, HA-MTMR4 or HA-MTMR9, and different EGFP-tagged myotubularins as indicated. EGFP proteins were immunoprecipitated from cell lysates and processed for western blotting. Corresponding membranes were blotted with an anti-HA antibody (top panels) and confirmed interactions identified in the two-hybrid screen. (A) MTMR3 and MTMR4 interact with each other and with themselves. A truncated form of MTMR3 (1-821) lacking the C-terminal coiled-coil domain preserves this interaction. (B) MTMR8 interacts with MTMR9 (arrow, top panel). Membranes were re-probed with anti-EGFP (bottom panels) and lysates with an anti-HA (middle panels) showing the efficiency of EGFP immunoprecipitation and HA-protein expression levels, respectively.

Table 1. Protein interactions within the myotubularin family

	Directed Y2H bait-prey	Directed Y2H prey-bait	Co-IP	Previous data (by co-IP)
hMTM1	1,12	1,12	1,12	1,12
hMTMR1	Not done	Not done	Not done	
hMTMR2	2,5,12	2,5,12	2,5,12	2,5,12,13
hMTMR3	self-activated	**4**	**3,4**	
hMTMR4	**3,4**	**3,4**	**3,4**	
hMTMR5	2	2	2	2
hMTMR6	9	9	9	9
hMTMR7	9	9	9	9
hMTMR8	self-activated	**9**	**9**	
hMTMR9	6,7,**8,9**	6,7,**9**	6,7,**8**	6,7
hMTMR10	**1,2**	–	**1,2**	
hMTMR11	–	–		
hMTMR12	1,2,**12**	1,2,**12**	1,2	1,2
hMTMR13	Not done	Not done	Not done	2

MTM proteins were sub-cloned into different tagged vectors; pFBT9 (bait vector) and pACT2 (prey vector) for directed yeast-two-hybrid (Y2H) screening, or into pEGFP-C2 and pCMV-HA, for co-immunoprecipitation (co-IP). Previously identified interactions are confirmed by both directed Y2H and co-IP. We also identified new interactions which are shown in bold. Shading indicates active MTMs, no shading indicates inactive MTMs.

fragmentary. We have now sought to systematically survey the interactions between 12 family members through directed yeast two-hybrid screening and follow up co-immunoprecipitation experiments.

Our results are summarised in Table 1. Notably we are able to confirm all hitherto published interactions by two-hybrid screening. In addition we have uncovered two new combinations (Table 1, in bold): MTMR3 with MTMR4 and MTMR8 with MTMR9. A typical plate screening for interactions with MTMR4 is shown in Fig. 1B. Each interaction was confirmed by co-immunoprecipitation following expression of HA- and EGFP epitope-tagged proteins and compared with appropriate controls. Fig. 2A shows a representative experiment of this nature, showing specific interaction between MTMR3 and MTMR4. Previous studies have ascribed a role of the coiled-coil domain in MTM interactions (Berger et al., 2003; Kim et al., 2003; Robinson and Dixon, 2005). Surprisingly, both the MTMR3-MTMR4 and the MTMR3 homomeric interaction is retained following deletion of the C-terminal section of MTMR3 containing both FYVE and coiled-coil domains [MTMR3 (1-821), Fig. 2A]. Fig. 2B illustrates the specific interaction between MTMR9 and a previously identified partner MTMR6, as well as a novel interacting partner MTMR8. MTMR3 and MTMR8 both showed self-activation properties in the bait vector so could not be directly tested for binding with each other by the two-hybrid assay. However, we did not find any interaction of these proteins by co-immunoprecipitation. In fact no interactions of MTMR8 other than with MTMR9 were found by co-immunoprecipitation. Two 'novel' interactions were found in one direction only by two-hybrid – MTMR10 with MTM1 and MTMR2. However, we were unable to confirm these unequivocally in co-immunoprecipitation experiments; at this point they must be seen as potential partnerships.

Our newly established interaction between MTMR3 and MTMR4 is the first report of a heteromeric interaction between two active family members, whilst the other interactions (MTMR8 and MTMR9, and possibly MTMR10 with MTM1 and MTMR2) expand the set of active-inactive combinations. The table of interactions also reveals that with the exception of MTMR11, each member of the MTM family included in this study has at least one heteromeric binding partner.

Previous work, from ourselves and others, has shown the ability of myotubularins to self-associate either into dimers

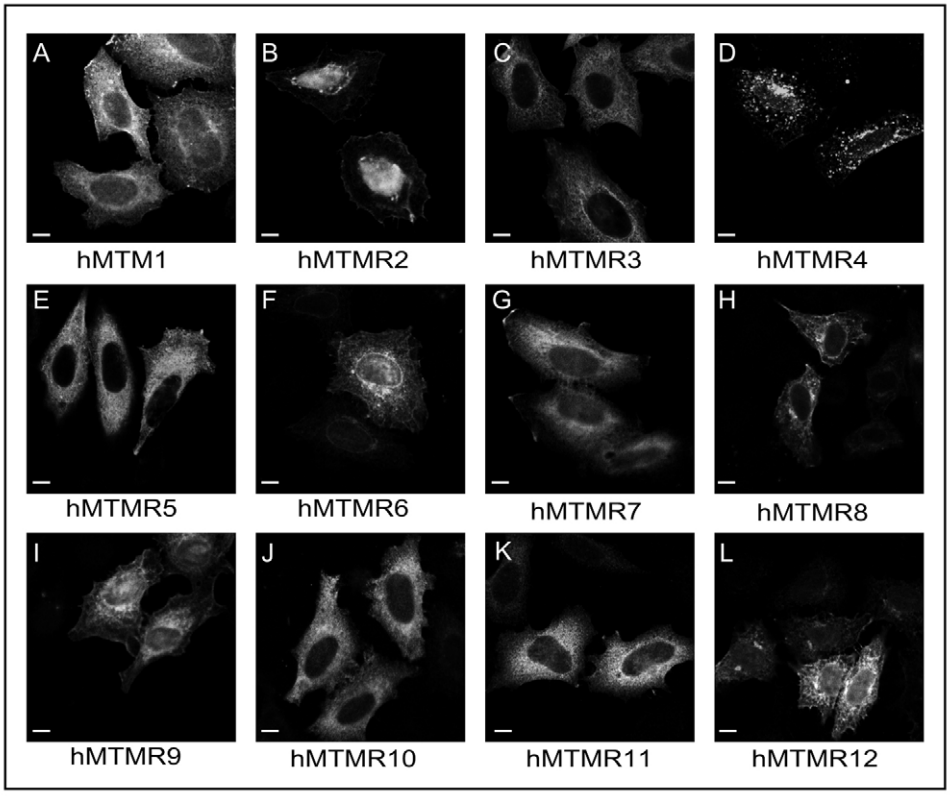

Fig. 3. Subcellular localization of myotubularins in HeLa cells. HeLa cells were transfected for 48 hours with the different EGFP-tagged myotubularins as indicated (A-L). Following fixation, cells were examined by confocal microscopy; representative images are shown. Bars, 10 μm.

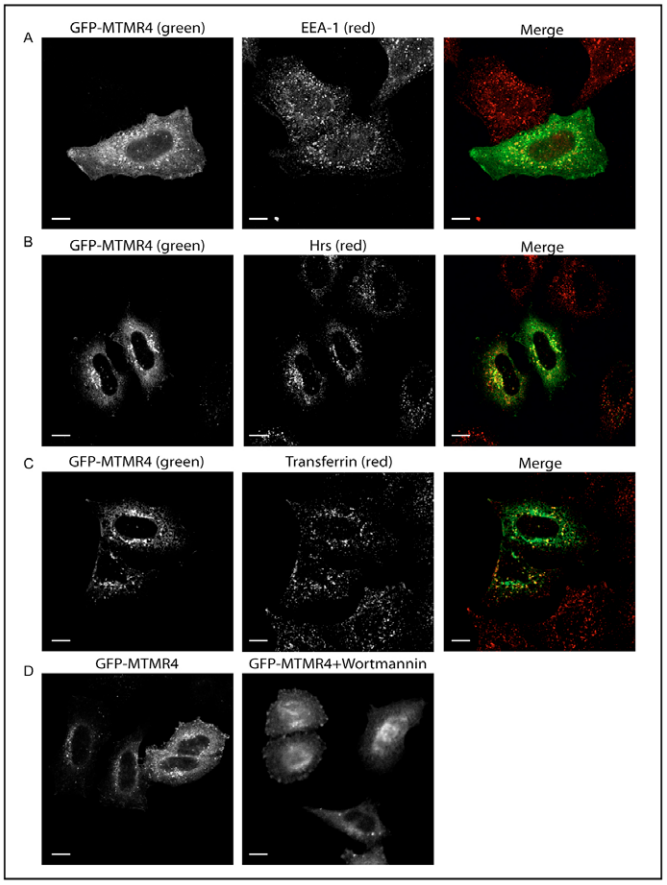

Fig. 4. MTMR4 localizes to endosomes. HeLa cells were transfected with EGFP-MTMR4 and co-stained after fixation with anti-EEA1 or anti-Hrs respectively (A,B). (C) AlexaFluor594-Transferrin was internalised into transfected cells during a 30 minute incubation. (D) Transfected cells were treated with wortmannin for 1 hour before fixation. Bar, 10 μm.

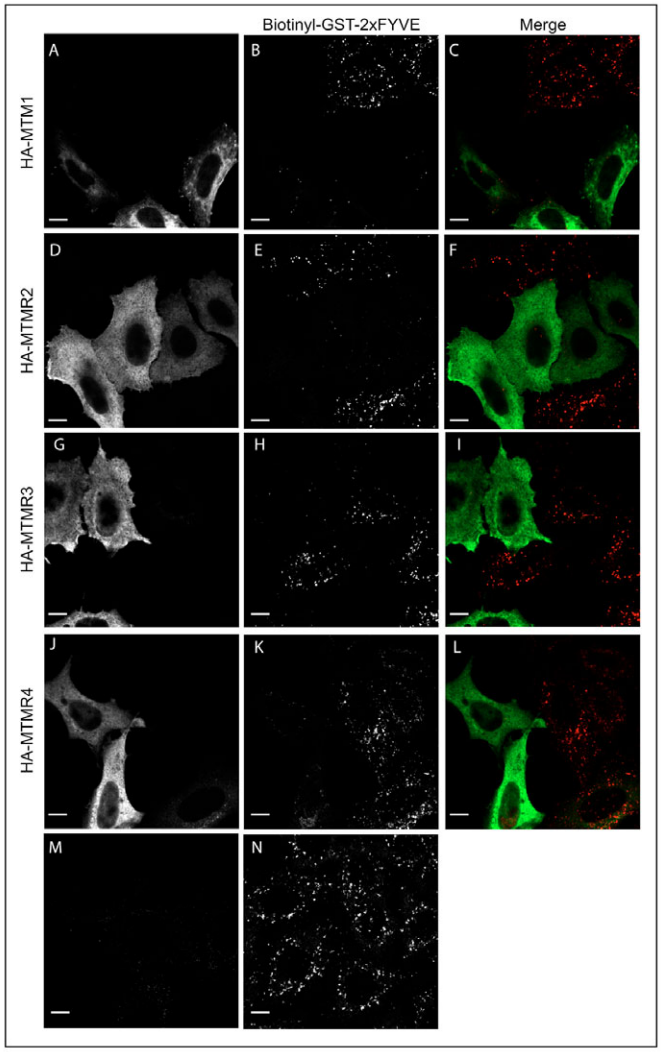

Fig. 5. Dephosphorylation of an endosomal pool of PtdIns3*P* by MTMs. HeLa cells were transfected with MTM1 (A-C), MTMR2 (D-F), MTMR3 (G-I), MTMR4 (J-L), fixed and endosomal PtdIns3*P* was detected using a biotinylated-GST-2xFYVE protein. Control cells are shown in N and wortmannin-treated cells in M. Bars, 10 μm.

(MTMR2) and/or heptameric rings (MTM1) (Berger et al., 2003; Schaletzky et al., 2003). We have confirmed these interactions by directed two-hybrid screening and can add MTMR3, MTMR4, MTMR9 and MTMR12 to the collection of self-associating MTMs (Table 1 and Fig. 2A). On the basis of this study, homo-oligomerisation does not appear to be an absolute requirement for activity across the family, as MTMR6 has well characterised phosphatase activity in the absence of other components (Schaletzky et al., 2003) and as indicated in Table 1 does not self-interact. Conversely, analysis of inactive point mutants [MTM1 (C375S) and MTMR3 (C413S)] indicates that phosphatase activity is not required for homo- or hetero-oligomerisation. Both mutants display the same spectrum of interactions as wild-type protein in the two-hybrid assay.

Given that MTMs share substrate specificity, how can specificity of cellular function be attained? One possible mechanism is through distinct subcellular localisation patterns. We have therefore undertaken the most extensive analysis to date of MTMs subcellular localisation. The intracellular distribution of each MTM epitope-tagged with EGFP is shown in Fig. 3. Most show cytosolic staining, together with specific labelling on subcellular structures with varying morphologies. Similar distributions were also observed for HA-tagged proteins (not shown). We cannot fully discount the possibility of some mislocalisation due to saturation of binding sites following overexpression or enhanced lipid hydrolysis in cases where active enzymes are overexpressed. MTMR6 and MTMR8 clearly stain the nuclear envelope (Fig. 3F,H), whereas uniquely there is a large pool of EGFP-MTMR2 (or HA-MTMR2, not shown) within the nucleus (Fig. 3B). At lower expression levels nuclear localisation is less prominent and the distribution more closely resembles a perinuclear accumulation as described by Kim et al. (Kim et al., 2002). However, nuclear staining of MTMR2 has also been clearly observed in Schwann cells (Bolino et al., 2004).

Much attention has been focused on the possible association of MTMs with endocytic compartments as this is believed to be a site of accumulation of their substrate lipids (Kim et al., 2002; Roth, 2004; Tsujita et al., 2004). Surprisingly, only

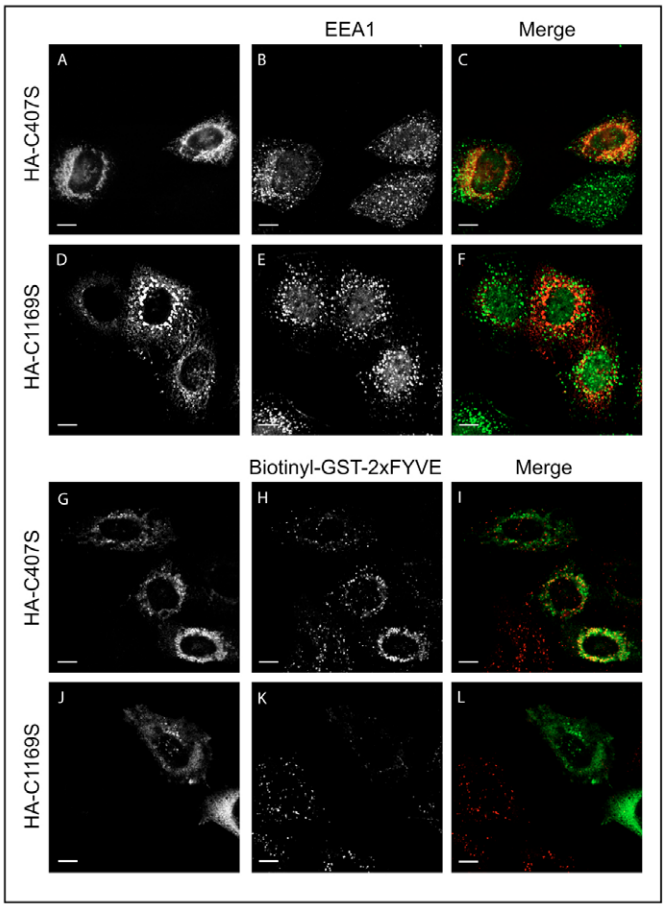

Fig. 6. Localisation and influence upon the endosomal PtdIns3*P* pool following mutant MTMR4 expression. HeLa cells were transfected with HA-MTMR4-C407S (catalytically inactive, A-C and G-I) or HA-MTMR4-C1169S (FYVE domain mutant, D-F and J-L) fixed and stained with anti-HA and EEA1 antibodies (A-F) or anti-HA and biotinyl-GST-2xFYVE probe (G-L) as described in Materials and Methods. Bar, 10 μm.

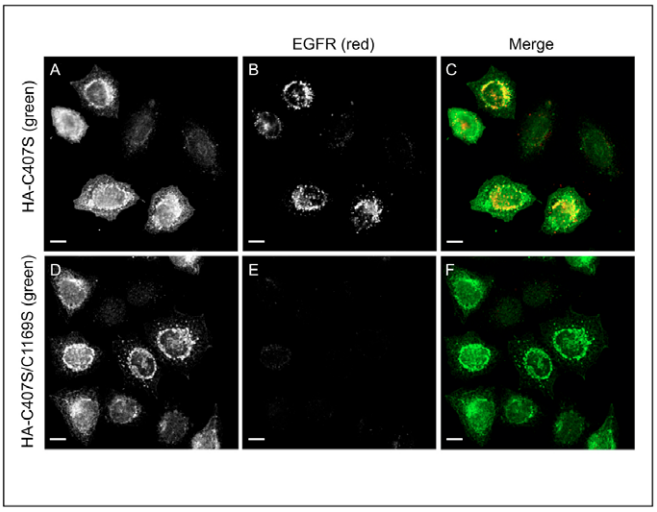

Fig. 7. MTMR4 (C407S) inhibits EGF receptor degradation. HeLa cells were transfected with phosphatase-inactive MTMR4, pHA-C407S (A-C), or with an additional point mutation in the FYVE domain HA-C407S/C1169S (D-F). 100 ng/ml EGF was added to starved cells for 4 hours, which were then fixed and processed for immunofluorescence of EGFR as described in Materials and Methods. Bars, 10 μm. Cells expressing MTMR4-C407S but not its FYVE domain mutant retain EGFR in numerous bright fluorescent punctae.

MTMR4 (panel D) shows a punctate distribution that is characteristic of endosomes. We examined the co-localisation of MTMR4 with endosomal markers and find striking overlap with both EEA1 and Hrs (Fig. 4A,B), two FYVE domain-containing proteins that localise to endosomes through interactions with PtdIns3*P* (Gaullier et al., 1998; Urbé et al., 2000). To clearly establish that MTMR4 punctae are bona fide endosomes we also determined extensive co-localisation with fluorescent transferrin, which has been internalised from the external medium (Fig. 4C). No co-localisation with a late endosome/lysosomal marker, LAMP-2, was observed nor with a Golgi marker (GM130) (data not shown). MTMR4 localisation to endosomes is sensitive to the PtdIns 3-kinase inhibitor wortmannin, consistent with the idea that it may associate with endosomes through interaction of its FYVE domain with PtdIns3*P* (Fig. 4D).

We have examined the effect of overexpression of active MTMs on endosomal PtdIns3*P* levels, as judged by staining with a biotinylated 2xFYVE probe (Gillooly et al., 2000) applied to digitonin permeabilised cells. Expression of MTM1, MTMR2, MTMR3 or MTMR4 all resulted in substantial diminution of endosomal PtdIns3*P* levels as seen for control cells following treatment with wortmannin (Fig. 5). A catalytically inactive mutant of MTMR4 has a more perinuclear distribution than wild-type and shows a modest degree of overlap with EEA1-labelled endosomes (Fig. 6A-C) but does not diminish endosome labelling by the biotinyl-GST-2xFYVE probe (Fig. 6G-I), with which it partially colocalises. By contrast, MTMR4 carrying a point mutant in the FYVE domain (C1169S) showed no overlap with EEA1 but effectively reduced biotinyl-GST-2xFYVE probe labelling (Fig. 6D-F,J-L). Interestingly, the failure to associate with EEA1 endosomes does not render this protein cytosolic, but relocates it to distinct fluorescent punctae. Expression of catalytically inactive myotubularins (MTMR5,MTMR9) had no effect on biotinyl-GST-2xFYVE probe labelling (not shown). Our results differ from a previous report of Kim et al. (Kim et al., 2002), who observed activity of MTM1 but not MTMR2 in similar experiments. We suggest that upon overexpression, MTMs can hydrolyse PtdIns3*P* on endosomes in the absence of specific localisation, through mass action effects. It is unclear why, if MTMR4 hydrolyses PtdIns3*P*, its localisation is wortmannin dependent. It is possible that there may be a residual pool of endosomal PtdIns3*P* below the detection level of the 2xFYVE probe. In this respect it is interesting that Chaussade et al. saw loss of this probe following MTM expression without a major decrease in the cellular mass levels of PtdIns3*P* (Chaussade et al., 2003).

It has been reported that overexpression of MTMR2 may influence the EGFR downregulation pathway, leading to defective receptor degradation and its accumulation in late endosomes (Tsujita et al., 2004). We have carried out a similar experiment with several active MTMs. We see no substantial effect of MTM1, MTMR2 and MTMR3. In some MTMR4-

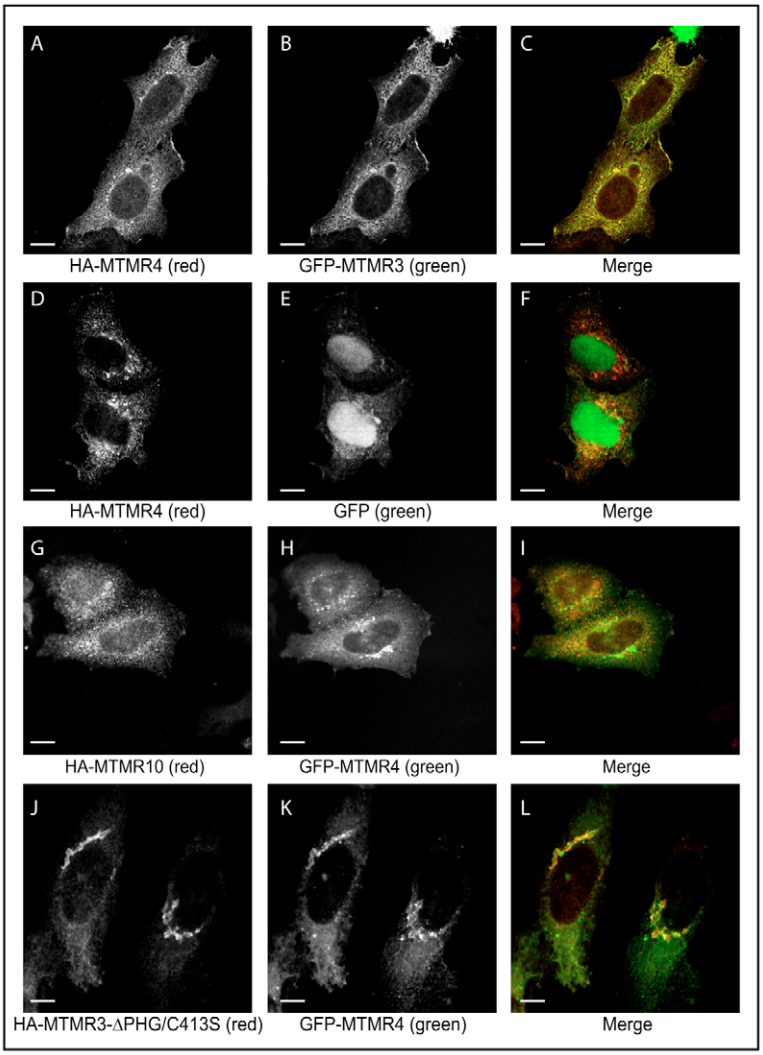

Fig. 8. MTMR4 and MTMR3 interaction leads to redistribution. HeLa cells were co-transfected with the following combinations. (A-C) HA-MTMR4 and EGFP-MTMR3, (D-F) HA-MTMR4 and EGFP-C2 control vector, (G-I) HA-MTMR10 and EGFP-MTMR4, and (J-L) HA-MTMR3-ΔPHG/C413S and EGFP-MTMR4. Merged images are shown on the right. Bar, 10 μm.

expressing cells we noticed a clear inhibition of EGFR degradation 4 hours after acute stimulation. This effect is stronger when the catalytically inactive C407S mutant of MTMR4 is expressed (Fig. 7A-C). It is completely abrogated by incorporation of an additional point mutation in the MTMR4 FYVE domain (C407/1169S) (Fig. 7D-F) and can be recapitulated by expression of the FYVE domain alone at very high levels (not shown). As hydrolysis of PtdIns3*P* by expression of active MTMs to levels below the detection limit of the 2XFYVE probe do not inhibit EGFR trafficking, one may wonder whether this effect is due to sequestration of a hydrolysis-resistant pool by the MTMR4-FYVE domain. Alternatively, MTMR4 may exert an altogether separate inhibitory function that requires its FYVE-domain-dependent association with endosomes.

To what extent do heteromeric interactions influence subcellular distribution? Kim et al. previously showed that co-expression of MTMR5 leads to redistribution of MTMR2. We have previously published extensive characterisation of MTMR3 localisation, which despite possession of a FYVE domain does not associate with endosomal compartments upon overexpression (Lorenzo et al., 2005; Walker et al., 2001). Following our identification of MTMR4 as an MTMR3-binding partner and bona fide endosomal MTM, we asked whether MTMR4 could direct MTMR3 to endosomal compartments (Fig. 8). Our overriding conclusion of this co-expression experiment is that there is a redistribution of both components, neither MTMR4 or MTMR3 is completely dominant. Thus we see reduced endosomal association of MTMR4 when co-expressed with MTMR3 and, conversely, we see some association of MTMR3 with typical MTMR4-associated punctae, which are presumably endosomes (Fig. 8A-C). This redistribution of HA-MTMR4 from endosomes is not seen when EGFP alone (D-F) or a non-interacting partner, MTMR10 (G-I) is expressed. It is formally possible that the inhibitory effects of MTMR4 expression on EGFR degradation could be due to recruitment of endogenous MTMR3. However, we do not favour this idea as overexpression of MTMR3 alone has no effect and co-expression of MTMR3 does not enhance the effects of MTMR4. We have previously characterised a mutant of MTMR3 carrying a deletion within the PH-G domain and an inactivating point mutation at the catalytic cysteine (HA-MTMR3-ΔPHG/C413S), which localises to the ribboned cisternae of the Golgi apparatus (Lorenzo et al., 2005). Co-expression of this mutant with EGFP-MTMR4 leads to the redistribution of MTMR4 to the same Golgi area, but to largely discrete structures, many of which are punctate, in close apposition to the HA-MTMR3-ΔPHG/C413S-labelled Golgi (Fig. 8J-L). We speculate that this represents interaction between MTMR3 and MTMR4 in trans, leading to the tethering of MTMR4-positive vesicles to the Golgi.

In conclusion, our studies have provided at least one binding partner for 11 out of 12 MTMs. These interactions are nevertheless quite specific as no MTM shows wide-scale promiscuity. The interaction between MTMR3 and MTMR4 breaks the general trend of pairing an active with an inactive family member and may redistribute either member. Surveying the subcellular distribution of MTMs has led to the identification of MTMR4 as the first clear example of an MTM localising to endosomal compartments, the site of accumulation of substrate lipids.

Materials and Methods

Plasmids and strains

Human myotubularin cDNA sequences were obtained as follows: MTM1, its catalytically inactive mutant C375S, and MTMR6 from F. Barr (Martinsried, Germany); MTMR12 from H. Nandurkar (Monash University, Australia); MTMR2 and MTMR4 from Kazusa (Acc. nos. AB028996 and AB014547); MTMR3 from T. Nagase (Chiba, Japan); MTMR5 from M. L. Cleary (Stanford University, California); MTMR7 from RZPD (CR749240); MTMR8, MTMR9 and MTMR10 from the MRC mammalian gene collection (BC012399, BC022003, AL832603); MTMR11 (AK097000) from NITE (National Institute of Technology Evaluation, Japan). All cDNA sequences were put into pCR4-Topo vectors (Invitrogen) and sequence verified before sub-cloning into bait (pFBT9, kanamycin resistant version

of pGBT9) and prey (pACT2) vectors for directed yeast two-hybrid screening (Clontech). For expression in HeLa cells, MTM cDNAs were sub-cloned into HA- and EGFP-epitope tagged vectors (Clontech). MTMR3 mutants, ΔPHG/C413S, C413S and truncated MTMR3 (1-821), and MTMR4 mutants C407S, C1169S and C407/1169S, were generated by Quickchange site-directed mutagenesis according to the manufacturer's instructions (Stratagene), sequence verified and then sub-cloned into HA- or EGFP-epitope tagging vectors. All primer sequences are available on request.

Directed yeast two-hybrid assays

All protocols were performed as described in the yeast protocols handbook (Clontech). The yeast two-hybrid (Y2H) reporter strain *Saccharomyces cerevisae* PJ69-4A was transformed with the various bait and prey constructs, or with the empty vectors as controls and plated on synthetic dropout media lacking tryptophan and leucine (SD-Trp/Leu). Three positive colonies of each were tested for the ability to grow on SD-Trp/Leu/His/Ade (QDO), which would indicate an interaction between the bait and prey proteins.

Immunoprecipitation

HeLa cells were transfected using Genejuice (Novagen) and lysed after 48 hours. Immunoprecipitations were performed as described previously (Urbé et al., 2003). The precipitated proteins were separated by SDS/PAGE and transferred to nitrocellulose. Immunoblotting was performed using standard techniques: the membrane was blocked with 5% (w/v) low-fat milk in PBS and washed in PBS containing 0.5% (w/v) Tween-20. Then, they were incubated overnight at 4°C with anti-HA mouse antibody (Cambridge Bioscience, MMS-10IP) and detected using secondary anti-mouse HRP coupled antibody (Sigma, A4416). Membranes were re-probed with sheep anti-EGFP (gift of Ian Prior, University of Liverpool) and anti-sheep HRP antibody (Sigma, A9452).

Immunofluorescence

We used mouse anti-HA antibody (Cambridge Bioscience), rabbit anti-Hrs (Urbé et al., 2000), rabbit anti-EEA1 (Mills et al., 1998), mouse anti-Lamp2 (Hybridoma bank, Iowa), mouse anti-EGFR (R1, CRUK), sheep anti-GM130 (F. Barr, Martinsreid, Germany) and Alexa Fluor 594 Transferrin (Roche). Secondary antibodies were from Molecular Probes (Alexa Fluor 594-coupled) and wortmannin from Sigma (W1628). The GST-2xFYVE probe was produced in bacteria and isolated with glutathione-sepharose (vector, gift of H. Stenmark, Oslo) then biotinylated with Sulfo-NHS-LC-biotin (Pierce). HeLa cells were routinely transfected using Genejuice (Novagen) and left for 48 hours with one change of medium at 24 hours. Cells were fixed with 3% paraformaldehyde (PFA, TAAB, UK) in PBS. Residual PFA was quenched with 50 mM NH_4Cl/PBS. Cells were permeabilised with 0.2% Triton X-100/PBS and blocked with 10% goat serum in PBS, except for GST-2xFYVE experiments in which cells were permeabilised with 20 μM digitonin in 80 mM PIPES pH 6.7, 5 mM EGTA, 1 mM $MgCl_2$ and blocked with 10% BSA. All antibody dilutions were in 5% goat serum and incubation times were 30 minutes at room temperature. Biotin-GST-2xFYVE was used at 40 μg/ml in PIPES buffer/5% BSA. Cover slips were mounted using Mowiol and cells were viewed using a confocal microscope and analysed with the accompanying software.

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