Analysis of phosphoinositide binding domain properties within the myotubularin-related protein MTMR3

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Summary

The myotubularins are a large family of phosphoinositide-specific phosphatases with substrate specificity for PtdIns3*P* and PtdIns(3,5)P_2. In addition to an N-terminal PH-GRAM (PH-G) domain and a signature catalytic domain shared with other family members, MTMR3 contains a C-terminal FYVE domain. We show that the FYVE domain of MTMR3 is atypical in that it neither confers endosomal localisation nor binds to the lipid PtdIns3*P*. Furthermore the FYVE domain is not required for in vitro enzyme activity of MTMR3. In contrast, the PH-GRAM domain is able to bind to phosphoinositide lipids, of which the allosteric regulator PtdIns5*P* is the preferred partner. Consequently, generation of PtdIns5*P* at the plasma membrane by ectopic expression of the bacterial phosphatase IpgD leads to a translocation of MTMR3 that requires the PH-G domain. Deletion of the PH-G domain leads to loss of activity of MTMR3 in vitro, and surprisingly, when combined with an active site mutation, accumulates the protein on the Golgi complex.

Key words: Myotubularin, PH domain, FYVE domain, Phosphoinositides, Phosphatase, Golgi

Introduction

The myotubularins are a family of phosphoinositide 3-phosphatases that display distinct substrate specificity against PtdIns3*P* and PtdIns(3,5)P_2 (Blondeau et al., 2000; Schaletzky et al., 2003; Taylor et al., 2000; Walker et al., 2001). There are eight active members of this family, and six further members that bear inactivating mutations within the catalytic site (Laporte et al., 2003). Myotubularin is the prototypical member of this family to which it lends its name. It was identified as a gene on the X chromosome, which when mutated, led to a defect in myotube maturation and a condition known as myotubular myopathy (Laporte et al., 1996). Mutation of myotubularin-related protein 2 (MTMR2) leads to a neuropathy called Charcot-Marie-Tooth syndrome 4B1 for which the underlying cause appears to be a defect in myelination of the nerves (Bolino et al., 2000).

Each myotubularin protein has an N-terminal domain that was originally proposed to represent a novel domain termed a GRAM (Glucosyl transferase, Rab-like GTPase activator and myotubularins) domain (Doerks et al., 2000). We noticed the possibility that this domain in MTMR3 may overlap with a PH domain fold (Walker et al., 2001) and this has now been confirmed by the crystal structure of MTMR2, which reveals an N-terminal PH domain flexibly linked to the catalytic domain (Begley et al., 2003). For clarity we will refer to this domain in MTMR3 as the PH-GRAM (PH-G) domain (Fig. 1). PH domains have been implicated in recognition of phosphotyrosinated proteins and in the specific recognition of phosphoinositides (Rebecchi and Scarlata, 1998). A wide variety of relative specificities for different phosphoinositides amongst the PH domain family is observed (Dowler et al., 2000).

The crystal structure of MTMR2 reveals the conserved catalytic domain to be much larger (375 amino acids) than many PTP superfamily domains (Begley et al., 2003). Intrinsic to this catalytic domain are sequences previously proposed to be distinct domains, the rac interaction domain (RID) and SET interaction domain (SID). Downstream of the phosphatase domain, lies a predicted coiled-coil domain that has been shown to run parallel and mediate dimerisation of MTMR2 (Berger et al., 2003). This domain may also mediate heterodimer formation, for which recently identified examples include MTMR2/MTMR5 (Kim et al., 2003), MTMR7/MTMR9 (Mochizuki and Majerus, 2003) and MTM1/MTMR12 (3-PAP) (Nandurkar et al., 2003).

Several of the myotubularins contain a C-terminal domain, which would be predicted to govern associations at a membrane surface, such as the PDZ domain (MTM1, MTMR1, MTMR2), PH domain (MTMR5) or the FYVE domain (MTMR4, MTMR3). The FYVE domain has been proposed to specifically interact with PtdIns3*P* and, through this interaction, direct proteins to early endosomes (Gaullier et al., 1998; Patki et al., 1998). Examples of proteins for which this principal has been clearly established include EEA1 (Dumas et al., 2001) and Hrs (Raiborg et al., 2001; Sankaran et al., 2001; Urbé et al., 2000), although in both cases supplementary interactions are required for efficient protein targeting.

Myotubularins can be allosterically activated by the non-substrate lipid PtdIns5*P* (Schaletzky et al., 2003). It has been

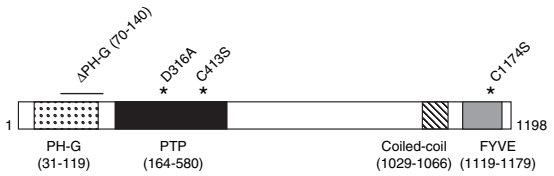

Fig. 1. Domain structure of MTMR3. MTMR3 contains, from the N-terminus, PH-Gram (PH-G), phosphatase (PTP), coiled-coil and FYVE domains. Deletion and point mutations used in this study are indicated.

postulated that the PH-G domain may represent the allosteric binding site. The phosphoinositide binding properties of the PH-G domain are now coming under scrutiny. Lipid overlay assays have indicated some selective binding of MTM1 and MTMR2 to phosphoinositides (Berger et al., 2003; Tsujita et al., 2004).

MTMR3 is a ubiquitously expressed myotubularin, which shows both cytosolic and reticular localisation upon overexpression. Mutation of the catalytic cysteine (C413S) promotes the formation of vacuolar structures that resemble autophagosomes; they contain both cytosol and internal membranes that label strongly for the protein (Walker et al., 2001). In this paper we have analysed the function of the PH-G and FYVE domains of MTMR3 with respect to phosphoinositide binding, enzyme activity, subcellular localisation and promotion of vacuole formation.

Materials and Methods

Plasmids and strains

The ORF for human MTMR3 was amplified by PCR from pBluescipt-KIAA0371 (cDNA, a kind gift of T. Nagase, Chiba, Japan), and subcloned into an HA-epitope tagging vector (pcDNA3.1-HA) for mammalian cell expression and either a GST or His_6-tag vector for Sf9 cell expression, pAcG2T (Pharmingen) or pAcHis (modified from pAcSG2, a gift from Francis Barr, Martinsried, Germany). C413S, C1174S, C413S/C1174S, D316A mutants were generated by site-directed mutagenesis (QuickChange, Stratagene) and then subcloned into appropriate expression vectors. The MTMR3-FYVE domain (amino acids 1073-1198) bacterial expression construct was generated by PCR amplification and subcloned into pGEXT-4T2. MTMR3 (ΔPH-G) was generated by deleting amino acids 70-140 of MTMR3 by PCR mutagenesis and was subcloned into appropriate expression vectors. A tandem 2×PH-G-MTMR3 domain was generated by duplicating amino acids 31-119 of MTMR3 coupled through a ten amino acid flexible linker (QGQGSRIQLE) and then, subcloned into pGEXT-4T2 or pRFP (Campbell et al., 2002), a kind gift of Roger Tsien (San Diego, USA). Generation of GST-EEA1-FYVE domain has been previously described (Mills et al., 1998). pEGFP-IpgD was a kind gift from Bernard Payrastre (Toulouse, France). All primer sequences are available upon request.

Sf9 cell infection and protein production

Sf9 cells were used for amplification of virus and protein production of all full-length MTMR3 proteins. All cell culture reagents were from Life Technologies (UK) unless stated otherwise. Baculovirus production and transfection were carried out with the BaculoGold system (Pharmingen) according to the manufacturer's instructions. Infected Sf9 cells (three 15 cm dishes) were harvested after 3 days, washed, and resuspended in 5 ml lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 μM $ZnCl_2$, 1 mM DTT, before disruption by probe sonication. Isolated domains were produced in bacteria (BLR cells). GST-tagged proteins were batch purified with glutathione-sepharose (Pharmacia) and His_6-tagged proteins were purified on Ni^{2+}-NTA columns (Pharmacia), both according to manufacturer's instructions. Protein-containing eluates were dialysed overnight against 20 mM HEPES, pH 7.2, 100 mM KCl, 1 μM $ZnCl_2$ and 0.2 mM DTT. Purity of the protein preparations was assessed by SDS-PAGE.

Phosphatase activity assay

Phosphatase assays were carried out using a Malachite Green assay essentially as previously described (Maehama et al., 2000; Walker et al., 2001). Lipid stock solutions were dried under argon in glass vials (2500 pmoles per experimental point) and rehydrated in assay buffer (20 mM HEPES, pH 7.2, 100 mM KCl, 1 μM $ZnCl_2$ and 2 mM DTT) with vigorous shaking. Enzyme (GST or His_6-tagged protein) was added to 50 μl lipid suspensions in a 96-well plate and incubated at room temperature with gentle shaking. At the end of the incubation, two volumes of Malachite Green solution were added and absorbance was measured at 650 nm using a Multiskan Spectrum plate reader (Thermo Lab Systems).

Immunofluorescence

Anti-HA antibodies were from Covance (HA), GRASP55 antibodies were a gift of Francis Barr (Martinsried, Germany). Secondary antibodies were from Molecular Probes (Alexa Fluor 488 and 594-coupled) and Sigma (HRP-coupled). HeLa cells were routinely transfected using calcium phosphate and left for 48 hours with one change of medium at 24 hours. Cells were fixed with 3% paraformaldehyde (TAAB, UK) in PBS. Residual paraformaldehyde 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. All antibody dilutions were in 5% goat serum and incubation times were 20-30 minutes at room temperature. Coverslips were mounted using Mowiol and cells were viewed using a BioRad LaserSharp confocal microscope (Z-sections were taken at 260 nm steps and analysed with the accompanying software).

Lipid overlay assay

For lipid-protein overlay assays we used Echelon strips (Echelon, P-6001; 100 pmoles/spot) or Hybond-C extra membrane (Amersham Life Sciences), which were spotted with di-C16 synthetic lipids (Cell Signals; 500 pmoles/spot) and air-dried for 1 hour at room temperature as described (Dowler et al., 2002). Strips/membranes were blocked in blocking buffer (2% BSA, 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.05% Tween-20) for 1 hour at room temperature and incubated with 12.5 nM purified GST or His_6 fusion protein in 3 ml blocking buffer for 2 hours (for GST-proteins) or overnight (for His_6 proteins) at 4°C. They were washed six times for 5 minutes in washing buffer (0.5 mM $MgCl_2$, 0.1% Tween-20 in TBS) and protein bound to the lipids was detected with an anti-GST (Sigma), or anti-Penta-His (Qiagen) HRP coupled antibody for 1 hour. Chemiluminescence was detected using an ECL reagent (Pierce).

Liposome binding assays

GST-2×PH-G domain binding to liposomes was determined essentially as described (Hayes et al., 2004). Lipid matrix composed of 20% PE (Sigma, egg yolk) and (80-X)% PC (Sigma, egg yolk) was combined with Xmol% phosphatidylinositol (d16 chains, Cell Signals) and trace amounts of [^{3}H]PC. This suspension was mixed,

dried under a steady stream of argon and rehydrated with vigorous shaking in 1 ml buffer A (25 mM HEPES-KOH pH 7.4, 0.5 mM $MgCl_2$, 250 mM Raffinose and 1 mM DTT) to form multilamellar liposomes (1 mg lipid/ml). These were extruded five times through 0.2 μm pore size polycarbonate filters to generate unilamellar liposomes. ^{3}H unilamellar liposomes were counted and adjusted to equal concentrations. Three volumes of Binding Buffer (100 mM Sorbitol, 40 mM HEPES-KOH, pH 7.1, 30 mM KCl, 0.5 mM $MgCl_2$ and 1 mM DTT) were added and the suspension was pelleted at 108,000 ***g***, 4°C, for 20 minutes, and resuspended in binding buffer. Unilamellar liposomes (6.7 mg/ml) were incubated with GST-2×PH-G (12.5 nM) for 20 minutes at 20°C, and then pelleted at 108,000 ***g***, for 20 minutes at 4°C. Protein binding was detected by western blotting with anti-GST peroxidase antibody (Sigma) using enhanced chemiluminescence reagent. Liposome binding of isolated GST-FYVE domains was assayed using [^{3}H]PC radiolabelled liposomes containing 1 mol% phosphoinositide as previously described (Mills et al., 2001; Schiavo et al., 1997).

Results

Phosphoinositide binding activities

MTMR3 is unusual for a FYVE domain-containing protein in that it does not localise to endosomal compartments (Walker et al., 2001). Despite the fact that the sequence conforms to a canonical FYVE domain, we have been unable to detect phosphoinositide binding of the isolated GST-FYVE domain (amino acids, 1073-1198) using lipid overlays and a liposome-binding assay that would detect PtdIns3*P* interaction. The GST-FYVE domain of EEA1 (amino acids, 1098-1411) provided a positive control for both assays (Fig. 2A,B) (Mills et al., 2001), and GST protein alone did not show lipid binding (not shown).

The PH-G domain (amino acids, 31-119) of MTMR3 showed phosphoinositide lipid binding properties as judged by lipid overlay assays and binding to liposomes. Using a tandem repeat of the PH-G domain, the lipid overlay assay binding

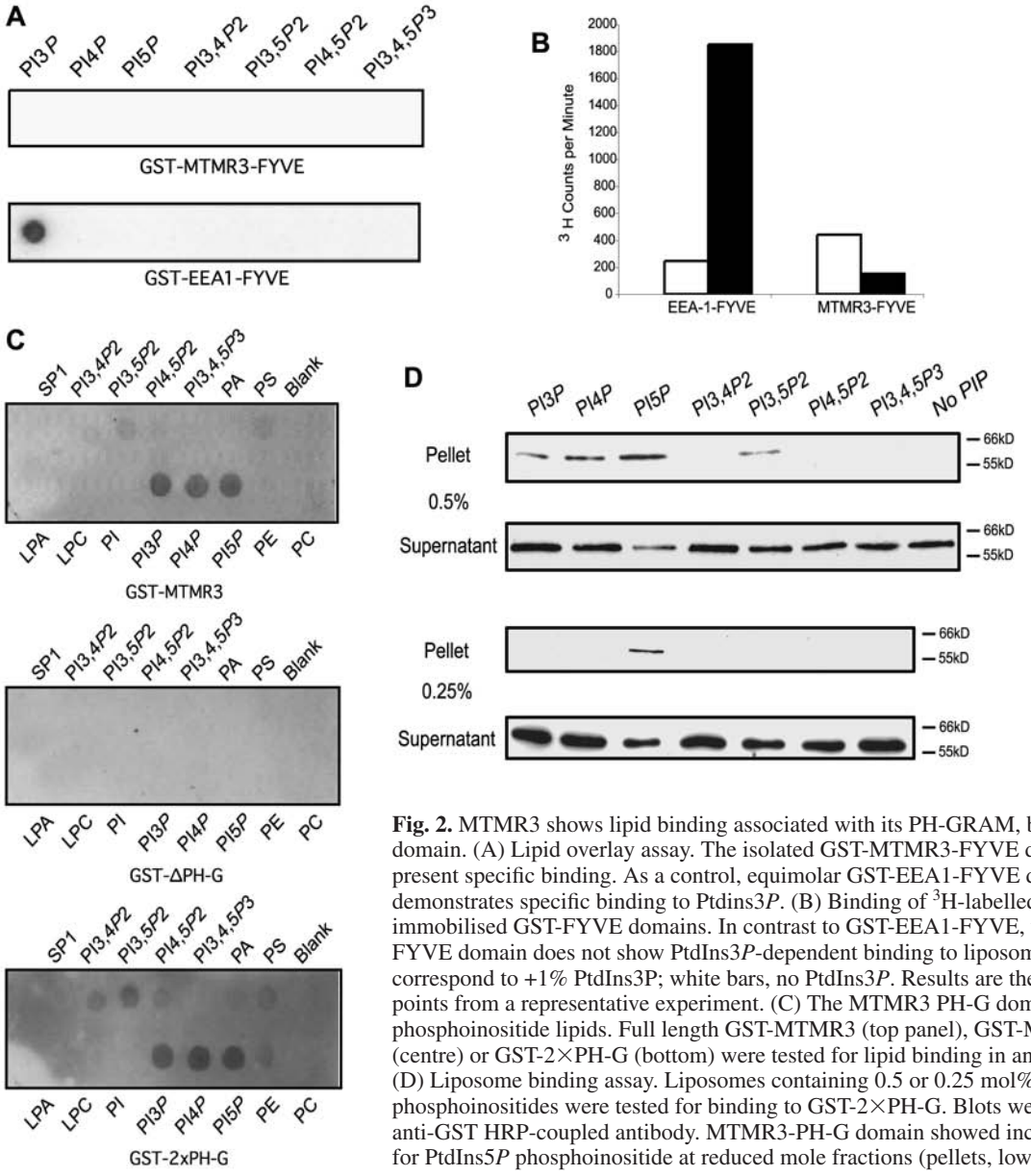

Fig. 2. MTMR3 shows lipid binding associated with its PH-GRAM, but not its FYVE domain. (A) Lipid overlay assay. The isolated GST-MTMR3-FYVE domain does not present specific binding. As a control, equimolar GST-EEA1-FYVE domain demonstrates specific binding to Ptdins3*P*. (B) Binding of ^{3}H-labelled liposomes to immobilised GST-FYVE domains. In contrast to GST-EEA1-FYVE, the GST-MTMR3-FYVE domain does not show PtdIns3*P*-dependent binding to liposomes. Black bars correspond to +1% PtdIns3P; white bars, no PtdIns3*P*. Results are the mean of duplicate points from a representative experiment. (C) The MTMR3 PH-G domain binds phosphoinositide lipids. Full length GST-MTMR3 (top panel), GST-MTMR3 (ΔPH-G) (centre) or GST-2×PH-G (bottom) were tested for lipid binding in an overlay assay. (D) Liposome binding assay. Liposomes containing 0.5 or 0.25 mol% of the indicated phosphoinositides were tested for binding to GST-2×PH-G. Blots were developed using anti-GST HRP-coupled antibody. MTMR3-PH-G domain showed increased selectivity for PtdIns5*P* phosphoinositide at reduced mole fractions (pellets, lower panels).

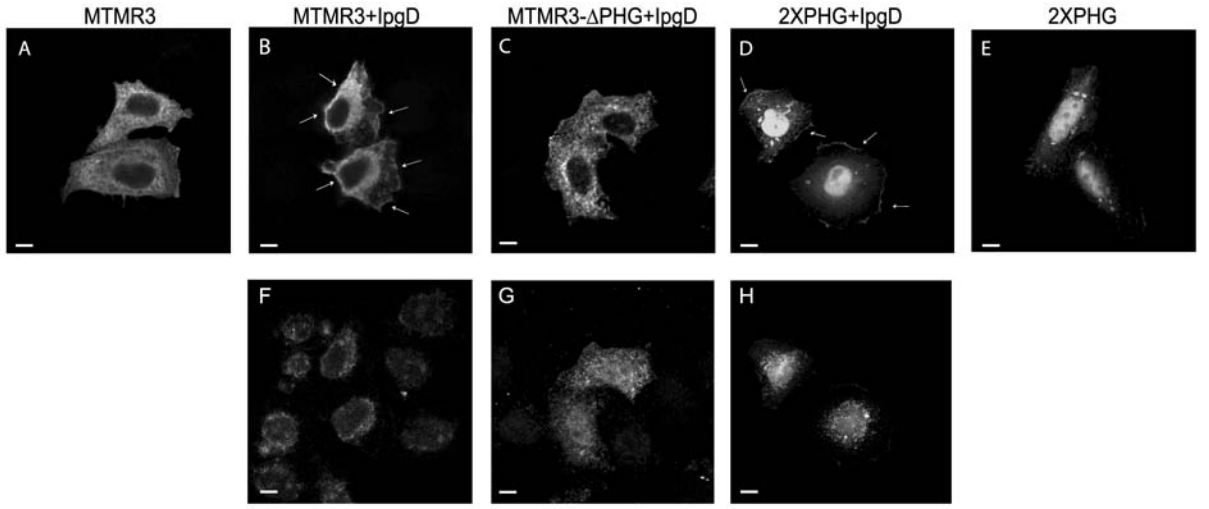

Fig. 3. PH-G domain of MTMR3 binds PtdIns5*P*. HeLa cells were transfected with HA-MTMR3 (A and B), HA-MTMR3-ΔPH-G (C) or RFP-2×PH-G (D,E). In addition, some cells were cotransfected with EGFP-IpgD phosphatase (B-D), EGFP-IpgD staining is shown for each in the corresponding lower panel (F-H). IpgD expression results in MTMR3 translocation to the plasma membrane (B). Deletion of the PH-G domain (ΔPH-G) from MTMR3 abolishes translocation (C), whereas RFP-2×PH-G redistributes similarly to wild-type protein (D). Bar, 10 μm.

activity was strongest for the allosteric regulator PtdIns5*P* as well as the other monophosphoinositides PtdIns3*P* and Ptdins4*P* (Fig. 2C). Full-length MTMR3 protein showed similar binding activity as the isolated PH-G domain using the same lipid overlay, whereas deletion of the PH-G domain abrogated lipid binding. When a liposome binding assay was used to test for lipid interactions we found phosphoinositide-dependent association of the PH-G domain that exhibited increasing specificity for PtdIns5*P* as the phosphoinositide concentration was reduced (Fig. 2D, pellets).

The bacterial PtdIns 4-phosphatase IpgD cleaves the relatively abundant phosphoinositide PtdIns(4,5)P_2, which is principally located at the plasma membrane (Niebuhr et al., 2002). Cellular expression of IpgD therefore leads to generation of a pool of PtdIns5*P* at the plasma membrane, which could act to translocate proteins with lipid binding domains which have specificity for PtdIns5*P* over PtdIns(4,5)P_2. This strategy has recently been used to confirm a proposed PtdIns5*P* binding of the PHD domain of Ing2, a regulator of chromatin organisation (Gozani et al., 2003). Coexpression of EGFP-IpgD and HA-MTMR3 led to a marked translocation of HA-MTMR3 to the plasma membrane consistent with binding to PtdIns5*P*. An MTMR3 construct lacking the PH-G domain, HA-MTMR3 (ΔPH-G), shows a similar subcellular distribution to wild-type MTMR3, but does not undergo plasma membrane translocation when expressed together with IpgD (Fig. 3). The tandem PH-G-domain appended to red fluorescent protein (RFP) recapitulated IpgD-dependent translocation to the plasma membrane.

The PH-G domain but not the FYVE domain is required for enzymatic activity of MTMR3

GST-MTMR3 and GST-MTMR3 (ΔPH-G) were expressed in, and purified from Sf9 cells with similar yields and solubility properties. We observed a complete loss of activity of the enzyme lacking an intact PH-G domain (Fig. 4A, black bars), consistent with previous results showing reduction of myotubularin activity associated with point mutations within this domain (Schaletzky et al., 2003). His_6-MTMR3 and a point mutant His_6-MTMR3 (C1174S) were also purified from Sf9 cells. The C1174S mutation will destabilise the FYVE domain structure owing to a failure to fully coordinate one of two zinc atoms within this domain (Stenmark et al., 2002). No

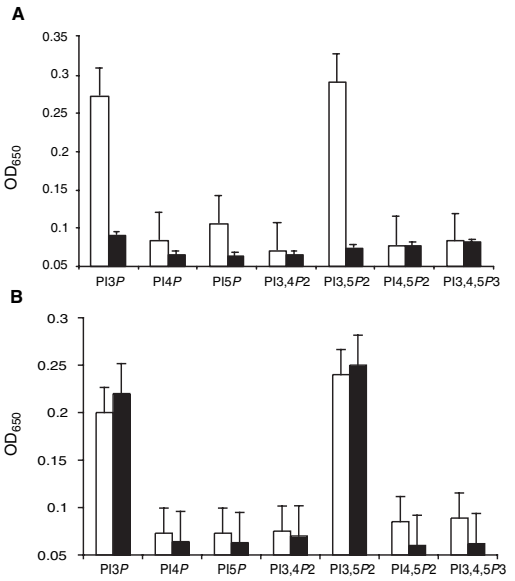

Fig. 4. Requirement of MTMR3 domains for enzymatic activity. (A) Deletion of PH-G domain abolishes MTMR3 phosphatase activity. GST-MTMR3 (white bars) and GST-MTMR3 (ΔPH-G) (black bars) were tested for lipid phosphatase activity using a colorimetric assay as described in Materials and Methods. (B) The FYVE domain is not essential for MTMR3 activity. The FYVE domain mutant (His_6-MTMR3 (C1174S); black bars) shows similar phosphatase activity towards PtdIns3*P* and PtdIns3,5P_2 compared with wild-type His_6-MTMR3 (white bars). Graphs show the mean±s.d. of 4 separate experiments.

difference in enzyme activity against either phosphoinositide substrate could be detected when comparing this mutant to the wild-type enzyme (Fig. 4B). Similarly, no difference in activity could be detected between HA-MTMR3 and HA-MTMR3 (C1174S) following immunoprecipitation from transfected HeLa cells (data not shown).

Role of FYVE domain and PH-G domain in subcellular localisation of MTMR3

So far we have been unable to generate specific antibodies that recognise endogenous MTMR3 by immunofluorescence. Overexpressed, wild-type MTMR3 partitions between cytosolic and particulate fractions. It is associated with a reticular network, which shows significant overlap with the endoplasmic reticulum marker calnexin (Walker et al., 2001). HA-MTMR3 (ΔPH-G) and HA-MTMR3 (C1174S) show similar immunofluorescence labelling patterns to wild-type HA-MTMR3 (compare Fig. 5A,B with Fig. 3A and Fig. 6A).

Expression of HA-MTMR3 (C413S), bearing a mutation at the catalytic cysteine, results in a characteristic punctate staining (Fig. 5C), which electron microscopy reveals to be vacuoles resembling autophagosomes (Walker et al., 2001); organelles that are classically induced by nutrient deprivation and serve to degrade cellular components (Yoshimori, 2004). These vacuoles contain HA-MTMR3 (C413S), associated with internal membranes and cytosol. They are enclosed by a limiting membrane, which does not label with HA antibodies (Walker et al., 2001). This feature is peculiar to this C413S point mutant rather than a general consequence of expressing inactive enzyme. The other inactive mutants HA-MTMR3 (ΔPH-G) (Fig. 5A) or HA-MTMR3 (D316A) (not shown) display similar labelling patterns to the wild-type enzyme (Figs 3, 6). We have examined the effect of combining this C413S mutation with the C1174S and/or ΔPH-G mutation. HA-MTMR3 (C413/1174S) appears by immunofluorescence to be intermediate between the wild type and the C413S point mutant, showing both prominent punctate and reticular staining (Fig. 5D). Surprisingly the HA-MTMR3 (ΔPH-G/C413S) mutant showed a completely distinct distribution to a perinuclear compartment with ribbon-like features (Fig. 5E). We confirmed this to be the Golgi complex through striking colocalisation with the Golgi marker GRASP-55 (Barr et al., 1997) (Fig. 5F). Introduction of a point mutation in the FYVE domain to generate the triple mutant ΔPH-G/C413S/C1174S did not alter this Golgi localisation (Fig. 5G,H). We noticed that the HA-MTMR3 (C413S) single mutant also showed Golgi localisation in some cells that expressed relatively low levels of protein (data not shown). Higher expressing cells showed the vacuolar phenotype and notably disrupted the Golgi as judged by dispersal of GRASP55 staining (Fig. 6C,D). In wild-type HA-MTMR3-expressing cells, the Golgi complex appears intact, independent of the protein expression level (Fig. 6A,B).

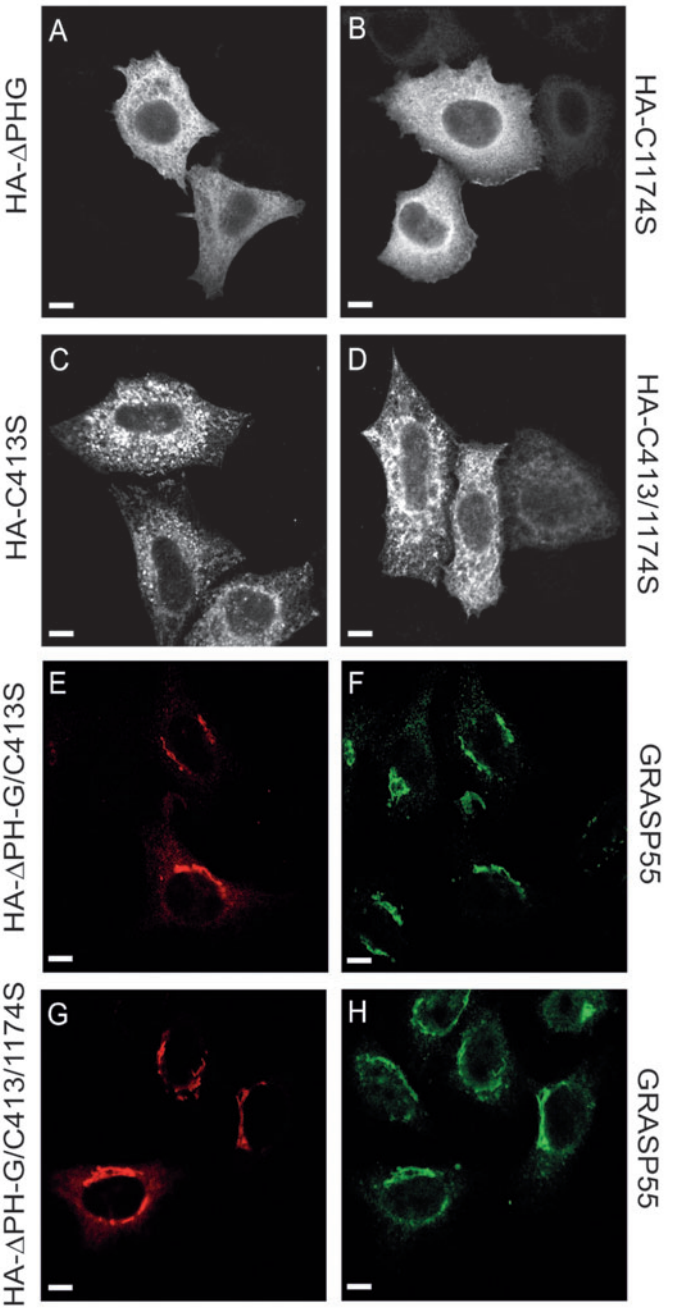

Fig. 5. Mutations in MTMR3 alter subcellular localisation. HeLa cells were transfected with HA-MTMR3 constructs and stained with anti-HA antibodies (A-E,G) or GRASP55 antibodies (F,H). Images of HA-MTMR3 constructs, ΔPH-G (A), C1174S (B), C413S (C), C413/1174S (D), ΔPH-G/C413S (E,F) and ΔPH-G/C413/1174S (G,H) were obtained by confocal microscopy. Bar, 10 μm.

Discussion

The human genome contains 27 identifiable proteins with FYVE domains (Stenmark et al., 2002). The majority of the FYVE domains that have been characterised so far specifically bind to PtdIns3*P*. This interaction frequently contributes to the localisation of proteins to endosomes where PtdIns3*P* is concentrated (Gillooly et al., 2000). The second lipid substrate of myotubularins, PtdIns(3,5)P_2, also plays a functional role on the endocytic pathway (Hicke, 2003). The MTMR3 FYVE domain has been conserved in multicellular organisms over a significant evolutionary period; the amino acid sequence indicates that it is a canonical FYVE domain (Stenmark et al., 2002). Our data shows that the FYVE domain does not

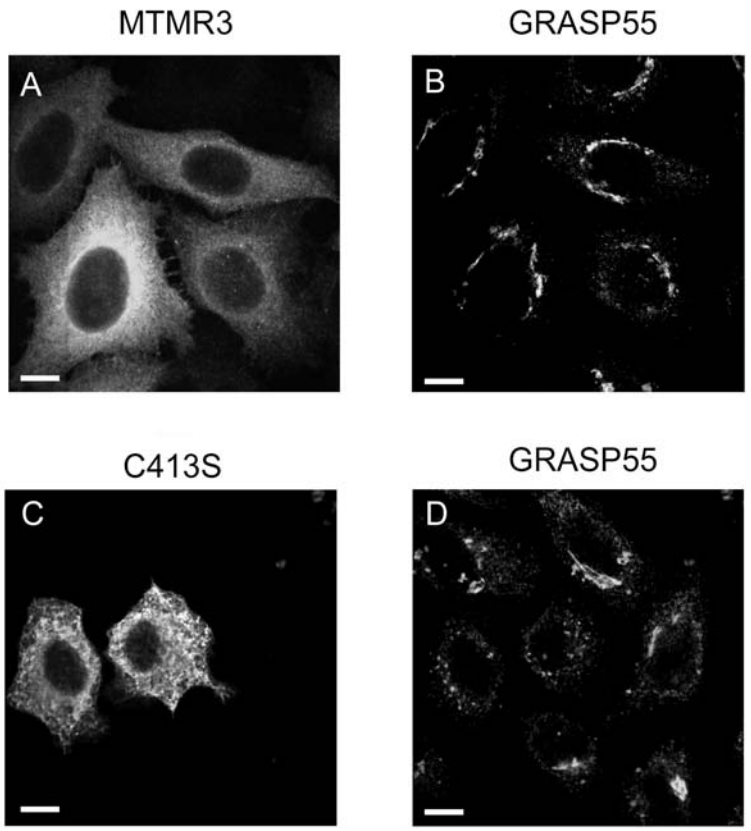

Fig. 6. Golgi organisation is lost in cells expressing HA-MTMR3 (C413S). HeLa cells were transfected with wild-type HA-MTMR3 (A,B) or HA-MTMR3 (C413S) (C,D) and stained with anti-HA antibodies (A,C) and GRASP55 antibodies (B,D). Images were then obtained by confocal microscopy. Bar, 10 μm.

influence MTMR3 enzyme activity using an in vitro assay. At face value, we would have expected this domain to confer endosomal localisation upon MTMR3. However, we do not observe any endosomal population of MTMR3 even with phosphatase-inactive mutants (Walker et al., 2001), which can now be accounted for, by our findings that the FYVE domain of MTMR3 is atypical in that it does not bind to PtdIns3*P*. There are other examples of FYVE domain proteins that fail to localise to endosomes, such as DFCP1, which localises to the Golgi (Ridley et al., 2001) or Alfy, which localises to the nuclear envelope (Simonsen et al., 2004). However in these cases the authors have been able to detect PtdIns3*P* binding of the isolated FYVE domains, suggesting that there may be a hierarchy of localisation signals.

All myotubularin family members contain a N-terminal domain, originally proposed to be a GRAM domain (Doerks et al., 2000), but which is now known to overlap with a true PH domain, as revealed by the crystal structure of MTMR2 (Begley et al., 2003). One major challenge is to understand the function of this domain and to account for how mutations in this domain may lead to inherited diseases. Our previous data and those of others have shown that point mutations within this domain can lead to reduced enzymatic activity of MTM1 (Schaletzky et al., 2003). We now show that disruption of this domain through partial deletion inhibits enzyme activity of MTMR3, highlighting its importance to catalytic activity throughout the myotubularin family. Many PH domains are known to bind phosphoinositides with widely varying specificities and affinities and there is wide interest in this property of the PH-G domain of myotubularins. PtdIns5*P* is an allosteric activator of both MTM1 and MTMR3 in vitro and the PH domain is a plausible site of action (Clague et al., 2004; Schaletzky et al., 2003). Although other non-substrate phosphoinositides do not activate myotubularins, available data cannot discount the possibility that the substrate lipids, PtdIns3*P* and PtdIns(3,5)P_2 can also act as allosteric activators (Fig. 2C,D) (Clague et al., 2004; Schaletzky et al., 2003).

An alternative or complementary function for PH-G domain-lipid binding is to mediate recruitment of myotubularins to specific subcellular membranes in a regulated fashion. PH-G domain-dependent translocations of MTMR2 to hypo-osmotic stress-induced vacuoles in COS7 cells and of MTM1 to late endosomal compartments following EGF stimulation of HeLa cells have been observed (Berger et al., 2003; Tsujita et al., 2004). In both cases isolated PH-G domains have been shown to bind phosphoinositides with some specificity, yet there are disparities between methodologies as we also observe. Lipid overlay assays have been performed in each case including the present study. For both MTMR2 (Berger et al., 2003) and MTMR3 (this study), the binding specificity obtained with full-length proteins is similar to that of the isolated PH-G domain. A common feature of all three PH-G domains in overlay assays is binding to PtdIns5*P* and PtdIns(3,5)P_2, product and substrate respectively, as well as potential allosteric regulators. The study of Tsujita and co-workers uses an alternative ELISA assay which indicates specificity of MTM1 for PtdIns(3,5)P_2, and little PtdIns5*P* binding (Tsujita et al., 2004). We in turn have used a liposome-binding assay with which we measured association of the MTMR3-PH-G domain with unilamellar liposomes. This form of binding assay in which phosphoinositide lipids are embedded in a membrane bilayer principally comprised of other naturally occurring lipids is likely to be a better model for cellular membranes. By reducing the mole percentage of phosphoinositide in the liposomes we were able to reveal that PtdIns5*P* is the preferred phosphoinositide binding partner of the MTMR3-PH-G domain.

PtdIns5*P* is a minor component of the cellular mass of phosphoinositide lipids, about which little is known (Rameh et al., 1997). It has been proposed to play a role in the regulation of Akt signalling (Carricaburu et al., 2003) and in regulation of nuclear responses to DNA damage (Gozani et al., 2003). In L6 myotubes and in Jurkat cells, overexpression of MTM1 leads to enhanced PtdIns5*P* production (Tronchere et al., 2004). We have tested whether PtdIns5*P* interaction can in principle drive subcellular localisation of MTMR3. To do this we have used a bacterial phosphatase IpgD to generate PtdIns5*P* from the large reservoir of PtdIns(4,5)P_2 at the plasma membrane (Niebuhr et al., 2002). Following coexpression with EGFP-IpgD, we observed a markedly enhanced association of HA-MTMR3 with the plasma membrane, which is not seen with HA-MTMR3 (ΔPH-G) but

can be recapitulated by an RFP-2×PH-G construct (Fig. 3). This experiment confirms that the PtdIns5*P*-PH-G interaction, which we detect in vitro, can also occur in a more complex cellular environment. At this point, it is unclear whether sufficient PtdIns5*P* concentrations are attained under normal physiological conditions, to drive large-scale translocation of MTMR3. In any case, MTMR3 hydrolysis of PtdIns(3,5)P_2 will generate PtdIns5*P* locally, which could serve both as an allosteric activator and a recruiter of further enzyme molecules.

A specific inactivating mutation of the catalytic cysteine (C413S) in HA-MTMR3 leads to formation of intriguing vacuolar structures, which resemble autophagosomes, as judged by electron microscopy (Walker et al., 2001). Combining this mutation with a FYVE domain mutant (C413/1174S) leads to an apparent reduction in vacuole production and an overall appearance that combines aspects of both single mutants; vacuolar and reticular, as well as cytosolic. Promotion of the C413S-dependent vacuolar phenotype is the first function we have so far been able to attribute to the FYVE domain of MTMR3. A much more striking redistribution is observed when we combine C413S with ΔPH-G, which results in perfect colocalisation with the Golgi marker, GRASP-55 (Shorter et al., 1999) and little cytosolic background. The C413S mutant might act as a substrate trap, similar to catalytically inactive mutants of tyrosine phosphatases (Flint et al., 1997), which could then be stabilised in an alternative configuration that generates a Golgi localisation signal that is greatly enhanced by deletion of the PH-G domain (Clague et al., 2004). Why must the PH-GRAM domain be removed to clearly observe Golgi localisation? Two models can be envisaged: (1) the C413S mutant associates with the Golgi, but then acts to remodel it by generating vacuoles in a PH-G dependent manner; or (2) the PH-G domain masks a Golgi localisation signal generated by the C413S mutation. Our observation that at lower expression levels HA-MTMR3 (C413S) also shows some Golgi localisation, albeit not as tight as the ΔPH-G/C413S points towards the first model. Consistent with this, when HA-MTMR3 (C413S) is expressed at high levels, inducing a vacuolar phenotype, the Golgi complex is effectively lost or highly fragmented (Fig. 6C,D). Hence the PH-G domain is associated with Golgi disruption rather than localisation and only when it is deleted, is the Golgi localisation clear.

It is widely held that the phosphoinositide substrates of MTMR3 are concentrated on the endocytic pathway (Gillooly et al., 2000; Roth, 2004; Tsujita et al., 2004). However, we see no displacement of the PtdIns3*P* binding protein EEA1 from endosomes following high-level expression of HA-MTMR3 (our unpublished results). In fact, all of our data point to MTMR3 association with organelles of the secretory pathway (Walker et al., 2001) (this study). One possibility is that an accessory molecule may be required for endosomal localisation, thus leading to mislocalisation of overexpressed proteins. Alternatively, the phosphoinositide profile of the secretory pathway is richer than previously anticipated, as suggested by recent electron microscopic studies (Watt et al., 2004; Watt et al., 2002). Interestingly, overexpression of MTM1 in muscle cell lines hydrolyses endosomal PtdIns3*P* to the point at which binding of EEA1 is lost, but the cellular mass of PtdIns3*P* is little changed (Chaussade et al., 2003). This suggests that the bulk of this lipid may be distributed elsewhere in the cell at concentrations too low to allow effective recruitment of FYVE-domain probes. Accumulation of particular phosphoinositides may confer or maintain the identity of discrete organelles; one role of myotubularins may be to suppress concentration of substrate lipids at inappropriate compartments, for example PtdIns3*P* on the secretory pathway. A Golgi-localised inositide phosphatase, PLIP, has been characterised, which favours PtdIns5*P* as a substrate in vitro (Merlot et al., 2003), suggesting the presence of this lipid on the Golgi. We propose that the specific association with the Golgi of HA-MTMR3 (ΔPH-G/C413S) (Fig. 5) is an accentuation of an interaction also observed with wild-type MTMR3, which is likely to be more transient. This localisation together with the observation that Golgi disruption is coupled to MTMR3 (C413S)-induced vacuole formation suggests that MTMR3 at the Golgi could regulate processes linked to autophagy.

With this study, we have extended the analysis of the role of the PH-G domain in the myotubularin family proteins to MTMR3. Our data support previous lipid binding studies, which revealed some specificity in binding to phosphoinositide species, but emphasise the preferred binding to PtdIns5*P*. In contrast, the lipid binding properties of the MTMR3 FYVE domain are exceptional amongst this domain family, lacking both detectable PtdIns3*P* binding and the ability to target to endosomes. We find roles for the PH-G domain beyond localisation of the protein, including the regulation of enzyme activity and production of autophagic-like vacuoles.

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