CMLS, Cell. Mol. Life Sci. 60 (2003) 2084–2099
1420-682X/03/102084-16
DOI 10.1007/s00018-003-3062-3
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CMLS Cellular and Molecular Life Sciences

Review

Implication of phosphoinositide phosphatases in genetic diseases: the case of myotubularin

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Received 14 February 2003; received after revision 10 April 2003; accepted 14 April 2003

Abstract. Phosphoinositides play a central role in the control of major eukaryotic cell signaling mechanisms. Accordingly, the list of phosphoinositide-metabolizing enzymes implicated in human diseases has considerably increased these last years. Here we will focus on myotubularin, the protein mutated in the X-linked myotubular myopathy (XLMTM) and the founding member of a family of 13 related proteins. Recent data demonstrate that myotubularin and several other members of the family are potent lipid phosphatases showing a marked specificity for phosphatidylinositol 3-phosphate [PtdIns(3)P]. This finding has raised considerable interest as PtdIns(3)P is implicated in vesicular trafficking and sorting through its binding to specific protein domains. The structure of myotubularin, the molecular mechanisms of its function and its implication in the etiology of XLMTM will be discussed, as well as the potential function and role of the other members of the family.

Key words. Myotubularin; myotubular myopathy; Charcot-Marie-Tooth disease; phosphoinositides; phosphoinositide phosphatase.

Introduction

Myotubular myopathy is an X-linked recessive disorder (XLMTM, OMIM 310400) affecting 1 out of 50,000 newborn males. The disease is characterized by muscle weakness and hypotonia. In the most severe cases, it results in death in the first weeks of life because of respiratory failure. The first cases were described by van Wijngaarden et al. in the late sixties [1], and histological analysis of patients' muscles showed the presence of an abnormally high number of muscle fibers with central nuclei. This is a characteristic of fetal myotubes that is also found in other centronuclear myopathies [2]. This hallmark and the persistence of fetal proteins suggested that an arrest in the muscle fiber maturation process was responsible for the disease. However, recent analysis of myotubularin knockout mice does not fully support this hypothesis and suggests a role for myotubularin in the maintenance of muscular fiber organization [3]. The definitive diagnosis for myotubular myopathy is based on the genetic analysis of the MTM1 gene, which was found to be mutated in the X-linked recessive form of the disease (XLMTM) [4]. Today, more than 192 disease-associated mutations affecting the MTM1 gene have been reported in 328 unrelated families [5]. The mutations are spread all along the gene and, in most cases, lead to the inactivation or absence of myotubularin, the MTM1 gene product. Myotubularin is a 66-kDa protein ubiquitously expressed and initially described as a tyrosine phos-

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phatase/dual-specificity phosphatase based on the presence of the consensus sequence in the active phosphatase site found in the members of this family. However, several laboratories demonstrated recently that the preferred substrate for myotubularin was not a protein but a lipid, the phosphatidylinositol 3-phosphate [PtdIns(3)P] [6, 7], thus classifying myotubularin as a phosphoinositide (PI) phosphatase. This protein appears, therefore, as one of the many regulators of the highly active PI metabolism. PIs are minor components of the membrane, representing less than 10% of the total membrane phospholipids. The PI family is composed of eight members with different phosphorylation status of their inositol ring, and interconversion of PIs is tightly regulated by the action of specific kinases and phosphatases [8]. PIs play an essential regulatory role in several signal transduction pathways controlling key cellular functions, and most of them are now considered as second messengers. They can specifically interact with functional protein domains, such as PH (pleckstrin homology), PX (phox homology) or FYVE (Fab1, Yotb, Vac1 and EEA1) domains, thus allowing the targeting of signaling proteins to specific locations in the cell [9]. In the context of XLMTM, the challenge is now to find the link between the function of myotubularin as a regulator of PI metabolism and its implication in the pathogenesis of this genetic muscle disorder.

The aim of this review is to summarize our knowledge of myotubularin, from the gene to the protein. We will first give an overview of the phenotype and the genotype associated with this genetic disorder and then focus on the protein, particularly highlighting its domain structure and its cellular function as a PI phosphatase. Finally, we will present the members of the myotubularin family, also called myotubularin-related proteins (MTMRs), and discuss their function and diversity.

Myotubular myopathy: a genetic muscle disease

Phenotype of the disease

Congenital myopathies are a group of muscular disorders resulting from abnormalities in the muscle fiber development process. Among this group, myotubular myopathy is characterized by the presence of 10–90% of small, rounded muscular fibers with centrally located nuclei, a general feature of fetal myotubes. The nuclei are surrounded by aggregated mitochondria and other organelles and are devoid of contractile myofibrils. Normal mature myofibers have characteristic peripherally settled nuclei. These histological observations have led to the suggestion of a failure of maturation in the onset of the disease [2]. Immunohistochemical studies have shown a high expression of prenatal myosin heavy chain [10] in the mature muscle fibers from myotubular myopathy patients. Nevertheless, some levels of fetal myosin can still be found in normal fibers up to 1 month [11]. The observations of the persistence of vimentin and desmin have been described [12], although the study of other cases conclude for a low increase in desmin and vimentin [13]. N-CAM is present only at neuromuscular junctions in the normal mature or regenerating fibers, and high expression of this cell adhesion molecule is found in XLMTM patients [13, 14]. An increase in expression of laminin $\alpha5$ chain, utrophin and human lymphocyte antigen (HLA)-1 have also been documented by Helliwell et al. [13]. Although there is not a complete consensus on the markers for myotubular myopathy, these different histological studies have supported the hypothesis of a default in fiber maturation or innervation.

Centronuclear myopathies are related disorders with histopathological features milder than XLMTM. The autosomal recessive (OMIM 255200) and dominant forms (OMIM 160150) have a later onset and a respective intermediate-to-mild severity in the course of the disease compared with the X-linked recessive form [15]. The affected patients have limb and facial muscle weakness with a variable evolution of the disease. Recently, the case of a boy with a mild centronuclear myopathy with a point mutation in the MYF6 gene was described [16]. MYF6 is implicated in myotube differentiation [17] and is located on locus 12q21.

The X-linked recessive form, XLMTM, is the most severe congenital myopathy and affects 1 in 50,000 males. It results from mutations in the MTM1 gene positioned at locus Xq28. The affected males present severe respiratory distress with an incapacity to establish spontaneous respiration at birth and usually die in their first months of life from respiratory failure. The prenatal history reports infrequent or weak fetal movements and polyhydramnios due to reduced swallowing of the amniotic fluid. The carrying heterozygous females usually show no clinical symptoms, although cases of stillbirth and miscarriage are reported. Nonetheless, recent reports describe the cases of heterozygous females developing, around age 5, limb girdle, then facial muscle weakness and scoliosis [18–20]. The three cases involve unrelated point mutations in the MTM1 gene, therefore showing the involvement of the X-linked and not the familial autosomal recessive or dominant form of myotubular myopathy. In addition, a case of severe X-linked myotubular myopathy was found in a newborn girl. Clinical and histological patterns were identical to the ones presented by the most affected male and are the result of a skewed X-inactivation pattern favoring the activation of the XLMTM mutated gene [21].

Despite the severity of the X-linked myotubular myopathy, long-term survivors have been described. In a North American study, 74% of the patients survived their first year, with 20% of them able to improve to some independent respiration and movements. The muscle disorder

seems to be nonprogressive for those survivors, who do, however, develop medical complications of nonmuscle organs [22]. These include pyloric stenosis, spherocytosis, gallstones, kidney stones, a vitamin K-responsive diathesis, higher stature and advanced bone age. Liver dysfunction was also described, leading in some cases to fatal hepatic peliosis. This study strongly suggests that the function of MTM1 gene is not restricted to the developing muscle cells, as supported by the ubiquitous expression of the protein (see below).

Genotype of the disease

Different studies of XLMTM cases have helped to place the responsible gene at locus Xq28 by linkage analysis [23–25]. The position was later refined by the characterization of a deletion of Xq27-q28 in a female patient with mild signs of the disease [26]. Further studies involving different research groups narrowed the location of the responsible gene [27, 28], which was then isolated by a positional cloning strategy [4]. The MTM1 gene is composed of 15 exons, exon 1 being untranslated. Northern analysis has shown the presence of a 3.9 kb transcript in all tissues. In muscle and testis an additional alternative transcript of 2.4 kb was detected and resulted from the different use of polyadenylation signals. The significance of the additional transcript in those two tissues remains elusive.

While positioning the MTM1 gene, a related gene, MTMR1, with 59% identity to MTM1 was found 20 kb from MTM1 on Xq28 [4]. MTMR1 results from an intrachromosomal duplication of the MTM1 gene and is transcribed in the same direction [29].

The vast majority of the XLMTM patients screened have a mutation in the MTM1 gene. Laporte et al. have provided an extensive list of the 133 different disease-associated mutations in MTM1 that were reported in 198 unrelated families [30]. Recently, two more studies reported new additional mutations in the MTM1 gene, increasing the number to 192 different mutations linked to XLMTM in 328 unrelated families [5, 31]. The mutations are distributed all along the gene, although more are concentrated in exon 4 and in decreasing order in exons 12, 3, 8, 9 and 11. The presence of hotspots has not been found. The most recurrent mutation is a point mutation leading to a small insertion of three amino acids in frame at the beginning of exon 12 between two highly conserved domains in myotubularin, the PTP (phosphotyrosine phosphatase) domain and SID (SET interaction domain) (see next section). The majority of the point mutations gives rise to a truncated protein, but about 26% are missense mutations and are concentrated between exons 8 and 12 [30]. The authors found that most of the truncating mutations are associated with a severe form of myotubular myopathy. Conversely, missense mutations are often associated with a milder form of the disease. This observation was confirmed by the study of Herman et al. reporting a mild phenotype associated with three missense mutations (R69C, P179S and R241C) [31]. More studies were carried out to find whether there was a relationship between the genotype and the phenotype of the disease [32]. The results of these studies, headed by the European Neuromuscular Center (ENMC) lead to the same conclusion, although several nontruncating mutations are linked to severe form of XLMTM. This highlight the limitation of the use of mutational analysis to diagnose the severity of the disease and should prompt the use of other methods, such as detection of the presence of the protein [33].

Myotubular myopathy: the knockout mouse model

In order to elucidate the physiological mechanisms of X-linked myotubular myopathy, a mouse model invalidated for the MTM1 gene was recently generated by homologous recombination [3]. MTM1 (–/–) male mice are viable, but there is likely neonatal death occurring as (–/–) males represent only 15% of the litter compared with the 25% expected. No obvious phenotype is observed in heterozygous females. The onset of the disease in MTM1 (–/–) males starts at ~ 4–5 weeks with a decrease in muscular hind limb strength and becomes slowly progressive, reaching the muscular forelimb at ~ 5–7 weeks. Total hind limb paralysis, accompanied by reduced muscular mass and respiratory difficulties usually leads to death around the 8th week. Although reproducing the phenotype of the myopathy, one can note a difference in the development of the disease in the mouse model compared with XLMTM patients. In humans, the onset of the disease appears in the neonatal period with a severe phenotype already at birth but no progression in the survivors [22]. The mouse model, on the contrary, develops a later (4–5 weeks postnatal) and very progressive muscle disease. At present there is no explanation for this discrepancy. Of the different tissues tested, only the skeletal muscles present obvious abnormalities, with hypotrophy and the presence of central nuclei, reproducing the phenotype observed in humans. Type 1 (or slow) fibers seem to be more affected by hypotrophy than type II (or fast) fibers in the early stages of the disease. This has already been observed in XLMTM patients but is not a characteristic of the disease, as it also occurs in other congenital myopathies [13, 34]. XLMTM is thought to result from an arrest in muscle development, but the results obtained on the MTM1 (–/–) mice show no difference in the presence of fetal proteins, such as myosin heavy chain, desmin and vimentin. This suggests that myogenesis is probably not impaired in the knockout mice. Increased apoptosis or necrosis in MTM1 (–/–) muscle fibers was also ruled out. In a previous in vitro study using a nerve-

muscle coculture system, Dorchies et al. concluded that XLMTM muscles show normal differentiation and innervation patterns [35]. At that time, the authors proposed that myogenesis was not impaired and that deficient motor neurons could be involved in the development of the disease. A possible role of the nervous system in the pathogenesis of XLMTM was analyzed in tissue-specific conditional knockout mice lines [3]. The skeletal muscle-specific invalidation of MTM1 gene reproduced the phenotype of the general MTM1 knockout mice, including growth defects and progressive muscle myopathy with central nuclei in the muscle fibers. Analysis of neuronal-specific MTM1-invalidated mice presented no obvious phenotype, and histological analysis showed normal muscle fibers, excluding the involvement of myotubularin-deficient neuron innervation in the development of this muscle disease.

Based on the knockout mouse phenotype and histological analysis, the concept of defects in maturation or innervation of the muscular fibers in XLMTM patients should be reevaluated. In MTM1 knockout mice, major disorganization of the muscle fibers is observed, suggesting that maintenance of muscle fiber structure is affected [3]. More studies are now necessary to find the role of myotubularin, the protein product of the MTM1 gene, in muscle fiber organization or survival and to understand the relationship between loss of activity of myotubularin and muscle disease.

Myotubularin: the product of the MTM1 gene

The MTM1 gene codes for a 603-amino acids protein named myotubularin that has a predicted molecular weight of 66 kDa and a migration in SDS-polyacrylamide gel electrophoresis (PAGE) around 70 kDa. It is ubiquitously expressed, although in low amount, as shown by the necessity to concentrate the protein by immunoprecipitation to detect it in different patient cell lines or in mouse tissues [33, 36]. The level of protein expression is low in brain and adrenal gland and is the highest in muscle and heart, with the latter tissues showing a protein doublet at a higher migration resolution. The presence of the muscle protein doublet isoforms seems to be specific to the adult muscle, as they are not detected in myotubes and differentiated myoblasts. Intestine and kidney have a shorter protein isoform, although no difference is detected in the size messenger RNA (mRNA) size. Detection of myotubularin along the maturation of C2C12 myoblasts to myotubes shows that the mRNA and protein expression increases with maturation [3, 37], implicating possible regulations during myogenesis. The presence of a PEST domain in myotubularin (see below) could suggest enhanced sensitivity to degradation. However, transfected myotubularin shows a half-life of 4–5 h, which is not particularly short [36]. The reasons for the low amount of myotubularin in tissues and the impact of mutations on the stability of the protein are still unknown.

Domain structure of myotubularin

Analysis of the myotubularin protein structure has shown the presence of several conserved domains (fig. 1) and key residues. Moreover, database sequences comparisons has defined a family of related proteins, the myotubularin-related proteins, or MTMRs, which will be documented at the end of this review.

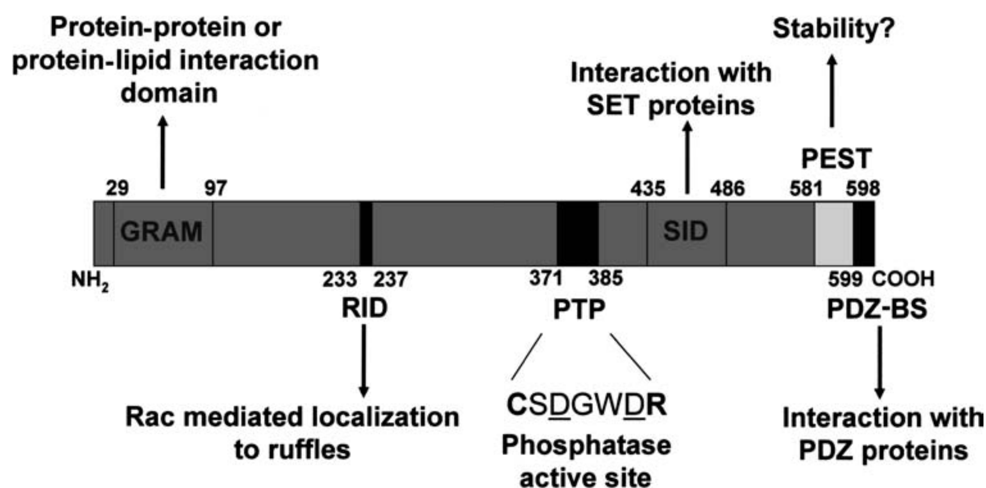

Figure 1. Domain structure of myotubularin. The domain abbreviations are: GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularin common domain); RID (Rac-1 induced localization to membrane ruffles domain); SID (SET interaction domain); PTP (protein tyrosine phosphatase signature domain); PDZ-BS (PDZ binding site). Numbers correspond to the first and last amino acids of the various domains.

PTP/DSP domain

Originally, myotubularin was classified as a member of the phosphotyrosine phosphatase (PTP)/dual-specificity phosphatases (DSPs), a group of phosphatases able to dephosphorylate phosphotyrosine and phosphoserine/threonine residues. This was based on the sequence H**C**SDGWD**R**TXE found in myotubularin and conserved in the MTMRs [4], which fits with the **C**X_5**R** signature of the PTPs. Indeed, myotubularin exhibits phosphatase activity toward the synthetic substrate *p*-nitrophenyl phosphate (*p*NPP) that is totally inhibited in the myotubularin mutant with the mutated cysteine to serine in the PTP catalytic site (C375S) [38]. This activity is also inhibited by the addition of sodium orthovanadate, a tyrosine phosphatase inhibitor. But as reported for the tumor suppressor PTEN (phosphatase and tensin homolog), the phosphatase is not very efficient toward phosphotyrosine and phosphoserine residues [37].

Myotubularin has high similarity in its PTP domain to two lipid phosphatases: PTEN [39] and the yeast Sac1p (suppressor of actin mutation) [40]. PTEN is a lipid phosphatase that preferentially dephosphorylates phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3] specifically on the 3 position of the inositol ring, thereby leading to the production of phosphatidylinositol 4,5-biphosphate [PtdIns(4,5)P_2]. The CX_5R signature in the PTP domain of PTEN (**C**KAGKG**R**) has two basic lysine residues, K125 and K128, that in cooperation with an upstream histidine (H93) are crucial for phosphatase substrate recognition. Solving the crystal structure of PTEN complexed to L(+)-tartrate was used to propose a model for binding 1,3,4,5-tetrakisphosphate, the soluble analog of PtdIns(3,4,5)P_3. It demonstrated charge interactions between the basic residues K125 and K128/H93 and the phosphorylated positions 1 and 5 of the inositol ring, respectively [41]. The two main residues for the enzymatic activity of PTPs are the cysteine and arginine of the CX_5R domain, but a third residue, an aspartate located on a flexible loop at ~ 30 nucleotides upstream, is also necessary for substrate release [42]. A series of systematic point mutations in myotubularin has demonstrated the role of D278 as the substrate trapping aspartate [6]. In the case of myotubularin, the distance between aspartate D278 and the catalytic core cysteine C375 is increased from the classical 30 nucleotides found in the PTPs to 100 nucleotides.

A look at the conserved CX_5R signature in the yeast Sac1p protein (**C**MDCLD**R**) showed the presence of two aspartate residues in place of the lysines in PTEN and a striking homology with myotubularin PTP domain (**C**SDGWD**R**). The Sac1p family of inositol 5-phosphatases has several members in *Saccharomyces cerevisiae* – Sac1p, Fig4p, Inp52p, Inp53p – and in humans – synaptojanin I and II – and has been shown to exhibit PI phosphatase activity [40].

The PI 4-phosphatase family also bears the CX_5R consensus sequence. Type I and II 4-phosphatases preferentially hydrolyze PtdIns(3,4)P_2, leading to the generation of PtdIns(3)P [43].

Based on these comparisons, it was tempting to speculate that myotubularin could be a more efficient lipid phosphatase than a protein phosphatase. Recently two different laboratories have demonstrated that myotubularin is indeed a PtdIns(3)P-phosphatase [6, 7]. Mutation of the cysteine residue from the CX_5R signature (C375S) totally abolishes its lipid phosphatase activity. The consequences of myotubularin as a lipid phosphatase will be developed in the next section.

SID

Located 12 amino acids (aa) downstream from the catalytic core phosphatase domain of myotubularin, the SID (SET interaction domain, aa 435–486) has not been found in any other proteins from the databases but the members of the myotubularin family, in which it shows a remarkably high degree of conservation. Based on the protein sequence deduced from the complementary DNA (cDNA) sequence, the predicted structure of the SID is a double amphipathic helix. The SID was first described in MTMR5, also named SBF1, a member of the myotubularin family [44] that was found in a two-hybrid screen with the SET protein Hrx. SET stands for su(var) 3-9, Enhancer-of-Zeste and Trithorax, which are the *Drosophila* proteins in which the SET domain was first described. Hrx is the human homolog of *Drosophila* Trithorax and is a protooncoprotein, also designated ALL-1, Htrx or Mll, that is frequently mutated in human leukemia [45]. Myotubularin, like MTMR5/SBF1, was shown to interact with Hrx SET domain in vitro through its SID.

However, the physiological role of the SID of myotubularin and its family members remains elusive. SET proteins are nuclear proteins implicated in the regulation of chromatin structure and reorganization, and myotubularin is a cytoplasmic protein [36, 46]. It is therefore difficult to emphasize an in vivo role for the myotubularin SID as an interaction domain with nuclear proteins like the SET proteins. However, the possibility for cytoplasmic proteins to interact with SET domains was suggested in a study where SET domain proteins were found to localize in nonnuclear structures such as gap junctions [47]. Whether this is the case for the myotubularin family of proteins remains to be demonstrated, as does the identification of the real physiological partners of the myotubularin family SIDs.

Gram domain

The GRAM domain was found in myotubularin and MTMR1 after iterative blast searches for homology with

the N-terminal extension of a glucosyltransferase from *Dictyostelium discoideum* [48]. The name of this domain derives from the first letter of the proteins found in the blast search: glucosyltransferases, Rab-like GTPase activators and myotubularin, MTMR1 and MTMR5/SBF1 from the myotubularin family. The predicted structure shows four β-strand sheets and an α helix with a highly conserved glycine residue in the fourth β sheet. The GRAM domain is not found in all the Rab-like GTPase activators and is certainly not essential for GTPase activation. The authors propose that the GRAM domain could be a protein-protein or protein-lipid interaction domain with a function in membrane-associated processes. In myotubularin, the GRAM domain is at the N-terminal end (aa 29–97), where several residues were found mutated in XLMTM patients, with some being missense mutations [30, 31]. Binding partners to the GRAM domain of myotubularin have not been described yet, and the role of this domain is still unknown.

RID

RID stands for Rac1-induced localization domain and was described in myotubularin by Laporte et al. [36] as the smallest domain necessary for colocalization of myotubularin to Rac1-induced ruffles in Cos cells cotransfected with an activated Rac1 construct (RacV12) and myotubularin. Rac1 is a small G protein from the Rho family of GTPases that is able to induce actin-rich lamellipodia formation and also to regulate some lipid kinases, such as the PtdIns 3-kinase [49]. Using deleted or mutated myotubularin constructs, this domain was mapped to aa 233–237 [36]. This observation suggests that cytoplasmic myotubularin could be relocalized to plasma membrane ruffles upon Rac activation through interaction with a still unknown protein. It is noteworthy that the region encompassing the RID is highly conserved in the myotubularin family.

PDZ-BS domain

A PDZ binding site (PDZ-BS) domain was identified at the very C-terminal end of MTMR1 in a two-hybrid screen using TIP-15, a double PDZ-containing protein, along with other PDZ-BS proteins [50]. PDZ domains, which name comes from the first letter of three proteins (PDS-95, a postsynaptic protein, Discs-large, a *Drosophila* tumor suppressor and ZO-1, a tight junction protein), were shown to interact with the C-terminal end of partner proteins bearing the consensus motif S/T-X-V-COOH [51]. The majority of the PDZ proteins are located at the plasma membrane, with some associated with the actin cytoskeleton and many of them implicated in signal transduction mechanisms [52]. The interaction between MTMR1 PDZ-BS and TIP-15 PDZ was confirmed by coimmunoprecipitation. The sequence in MTMR1 PDZ-BS fits the consensus VH**T**S**V**-COOH, like the one in MTMR2, another member of the myotubularin family (Q**T**V**V**-COOH). Although not bearing the conserved valine residue, the myotubularin C-terminal sequence Q**T**H**F**-COOH is likely to be a PDZ-BS also, as it has been shown that a phenylalanine residue could be observed in place of a valine in PDZ-BS interacting with a particular PDZ protein [53]. PDZ protein partners of myotubularin or other members of the family have not yet been found but could be potential regulators of these enzymes by targeting their localization to specific sites.

PEST domain

PEST domains have been implicated in protein stability, as they are highly subjected to proteolysis [54] and degradation through the ubiquitination pathway. A PEST domain was described in myotubularin around aa 581–598 contiguous to the PDZ-BS [36]. This could explain the low abundance of the protein in tissues, as it is undetectable by direct Western blotting and the protein is unstable under sequence changes [33]. However, the turnover of the transfected myotubularin protein is not particularly high (4–5 h).

Subcellular localization of myotubularin

Because of its low abundance in all tissues and cell lines tested, immunolocalization of endogenous myotubularin has not been possible to perform. The localization studies described below refer to cell lines overexpressing this lipid phosphatase, and one should therefore keep in mind that they may not reflect the distribution pattern of the endogenous protein.

Although reported once as located in the nucleus [44], numerous reports have confirmed by immunocytochemistry a cytoplasmic localization of transfected myotubularin, either GFP-tagged [7], flag-tagged or untagged [6, 36]. Cytoplasmic myotubularin appears as a dense network but does not colocalize with endogenous markers of the cytoskeleton like actin, tubulin and keratin do [36, 46]. Desmin, whose immunohistochemical profile has been shown to be modified in muscle fiber samples from XLMTM patients, does not colocalize with myotubularin [12]. Vimentin, still presents in XLMTM patient fibers, may partially colocalize with myotubularin, although confocal microscopy experiments did not reveal a clear colocalization. Immunoprecipitations of myotubularin after classical fractionation studies on different cell lines have confirmed a cytoplasmic localization of the protein with no link to polymerized actin or the intermediate filament network [36]. Myotubularin remains located in the cytoplasm during the maturation process of C2C12 myoblasts into myotubes. The phosphatase-dead myotubularin mu-

tant (C375S) localization profile is identical to the wild-type protein, suggesting that its localization is independent of its phosphatase activity. Myotubularin also labels plasma membrane and plasma membrane extensions such as ruffles or filopodia, with increased numbers of the latter in cells with high levels of transfected myotubularin [36]. Interestingly, the substrate-trapping myotubularin mutant D278A shows an exclusive localization at plasma membrane extensions, and electron microscopy further pinpointed the localization to membrane microvilli with no contact with the substratum [6]. When plasma membrane remodeling was induced by overexpression of a constitutively active Rac1 construct (Rac1 V12), myotubularin was strongly relocalized to the ruffles [36, 46]. This phenomenon is independent of phosphatase activity, as the phosphatase-dead and substrate-trapping myotubularin mutants show the same relocalization. As mentioned above, localization to Rac1-induced ruffles is dependent on the RID.

Laporte et al. analyzed the impact of a set of deletions on the localization of myotubularin [36]. All of them show a pattern identical to wild-type myotubularin, but a C-terminal construct (aa 336–663) missing the RID and GRAM domain presented a cytoplasmic and nuclear dot-like pattern. In the nucleus, the C-terminal construct did not colocalize with heterochromatin or PML (promyelocytic leukemia bodies, a nonheterochromatin nuclear marker). Deletion of the SID in this construct led to an exclusively cytoplasmic dot pattern. These results suggest that the myotubularin SID could drive a nuclear localization, although it does not have any known nuclear localization signal. Moreover, the SID is not able to target the full-length wild-type myotubularin. This discrepancy may be explained by the inaccessibility of this domain, which might be buried in the protein. Finally, neither wild-type myotubularin nor deletion mutants label intravesicular compartments.

Myotubularin: from a dual-specificity phosphatase to a lipid phosphatase

Myotubularin, a PtdIns(3)P-phosphatase

Sequence homology searches first classified myotubularin among the phosphotyrosine phosphatases, and it was shown in vitro that myotubularin was able to dephosphorylate the synthetic substrate *p*NPP, but the activity was quite weak [38]. The putative tyrosine phosphatase activity was also studied in vivo in the yeast *Schizosaccharomyces pombe* [6]. Compared with other known phosphotyrosine phosphatases, myotubularin did not induce any reduction in phosphorylation patterns, confirming a weak or even nonexistent tyrosine dephosphorylation activity in vivo. However, overexpressed myotubularin induced a remarkable phenotype in *S. pombe*: reduced growth rate and the presence of large vacuoles. An identical phenotype had been observed in *S. pombe* deleted for the gene coding for VPS34, (vacuolar protein sorting 34), a PtdIns 3-kinase that specifically phosphorylates phosphatidylinositol (PtdIns) to PtdIns(3)P [55]. This observation and the fact that the PTP consensus sequence of myotubularin CX_5R was highly homologous to the yeast *S. cerevisiae* sac1p, a PI phosphatase, led two different groups to look for a possible role of myotubularin as a lipid phosphatase [6, 7]. In *S. pombe* overexpressing VPS34, the invalidation of the myotubularin homolog gene (SpMTM) induced a dramatic accumulation of PtdIns(3)P. Myotubularin activity toward different lipid substrates was analyzed in vitro after overexpression in *S. pombe* and immunoprecipitation. The results showed specificity for PtdIns(3)P [6]. Using an *Escherichia coli* recombinant myotubularin fused to GST (glutathione *S*-transferase), Taylor et al. also demonstrated that myotubularin had a high specific activity toward PtdIns(3)P in vitro when tested together with a panel of different PIs [7]. They further confirmed that PtdIns(3)P is likely to be the physiological substrate of myotubularin by overexpression of myotubularin in HEK293 cells or in the yeast *S. cerevisiae* and by analyzing the intracellular level of PIs. Mutation of cysteine C375 from the active PTP consensus site or the D278 mutation in the substrate-trapping mutant abrogated myotubularin activity toward PtdIns(3)P. Other members of the myotubularin-related protein family also display PtdIns(3)P-phosphatase activity [46, 56]. However, recent reports suggest that MTMR2 and MTMR3 are also very active PtdIns(3,5)P_2 3-phosphatases [37, 57], as discussed later in this review.

These different observations demonstrate that myotubularin regulates PtdIns(3)P levels by direct interaction and hydrolysis. However, one cannot rule out a possible control of the PtdIns(3)P level by myotubularin through a regulation of VPS34. Indeed, Blondeau et al. have observed that the myotubularin substrate-trapping mutant D278 was able to coimmunoprecipitate PtdIns 3-kinase activity from a *S. pombe* extract [6]. This activity was not measurable when wild-type myotubularin or inactive C375 mutants were used, consistent with a transient interaction phosphatase-substrate. In yeast, VPS34 is phosphorylated and activated by the serine/threonine kinase VPS15 (vacuolar protein sorting 15), and human PtdIns 3-kinase is also autophosphorylated on serine/threonine residues. Although we cannot rule out the possible regulation of a PtdIns 3-kinase by myotubularin, a putative role for this enzyme as a serine/threonine phosphatase remains to be demonstrated.

The place of myotubularin in PI metabolism

The eight members of the PI family are subjected to a highly active metabolism regulated by a set of specific ki-

nases and phosphatases (fig. 2). They are considered as key spatio-temporal regulators of fundamental signaling mechanisms implicated in the control of cellular functions [8, 9]. By directly interacting with specific protein modules, these minor constituents of cell membranes are implicated in the control of cell proliferation and death, actin cytoskeleton organization and cell motility as well as vesicular sorting and trafficking [9]. In mammalian cells, PtdIns(3)P represents about 1–2% of the total phosphoinositides and 5% of the monophosphorylated PIs and originates mainly from the phosphorylation of PtdIns by a type III PtdIns 3-kinase. In eukaryotic cells, PtdIns(3)P is constitutively present, and its level does not change significantly upon physiological stimulation [58] except in human blood platelets, where PtdIns(3)P level increases upon integrin engagement [59]. Whether such an increase is restricted to the platelet model or can also be found in other cells is still unknown, probably because of the technical difficulties in measuring such discrete variations.

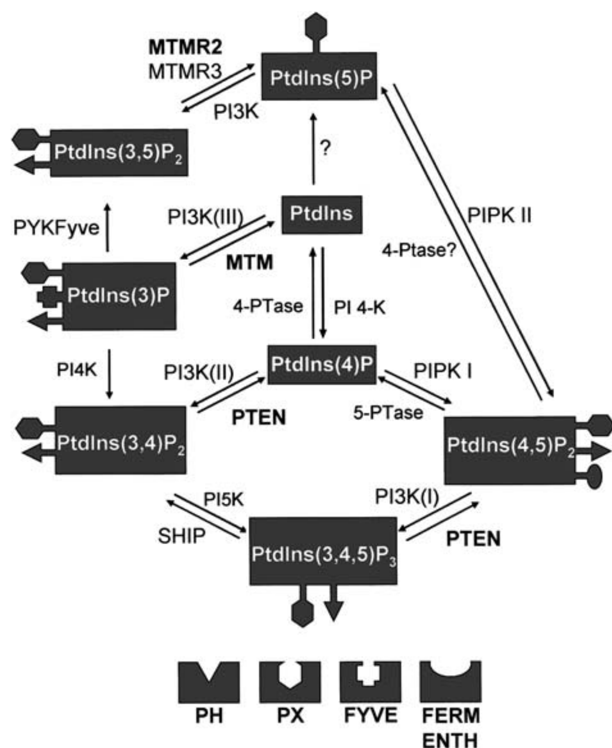

Figure 2. Phosphoinositide metabolism and protein-interacting domains. The main pathways of PI interconversions implicating the various kinases and phosphatases are presented. The phosphatases directly implicated in human disease are in bold. 4-Ptase (inositol 4-phosphatase); 5-Ptase (inositol 5-phosphatase); SHIP (SH2 domain containing inositol 5-phosphatase); PTEN (phosphatase and tensin homolog); MTM (myotubularin). The potential binding site of each PI is indicated schematically. PX (phox) domain; PH (pleckstrin homology) domain; FYVE (Fab1, Yotb, Vac1 and EEA1); FERM (band 4.1, ezrin, radixin, moesin) domain; ENTH (epsin NH_2-terminal homology) domain.

Myotubularin is actually the first PtdIns(3)P-specific phosphatase to be clearly identified so far. Other known PI-phosphatases acting on position 3 of the inositol ring, such as PTEN, are primarily directed toward PtdIns(3,4,5)P_3 [60]. Overexpression of human myotubularin or its D278A substrate-trapping inactive mutant in yeast has a dramatic impact on PtdIns(3)P level [6, 7]. This is not the case in mammalian cells, where the change in PtdIns(3)P level upon overexpression of myotubularin never exceeds 25% [7]. This discrete effect suggests that a negative regulator of myotubularin is expressed in mammalian cells and/or that myotubularin utilizes only a specific pool of PtdIns(3)P which function and precise location remain obscure. Because two other members of the myotubularin familly, MTMR2 and MTMR3, can hydrolyze PtdIns(3,5)P_2 in vitro and in yeast [37, 57], it will be important to determine whether myotubularin can also transform PtdIns(3,5)P_2 into PtdIns(5)P. The role of PtdIns(3,5)P_2 is poorly known, but as developed below, it may be involved in the control of vesicular trafficking. The role of PtdIns(5)P is still unknown, but recent reports suggest that its level can change upon human platelet stimulation [61] or during bacterial invasion of eukaryotic cells [62].

PtdIns(3)P and PtdIns(3,5)P_2 in vesicular trafficking

PtdIns(3)P plays a major role in vesicular trafficking, and the elucidation of its role comes from the work on VPS34 in *S. cerevisiae*. VPS34 was first shown to be required for vacuolar protein-sorting mechanisms [63] and was later identified as a PtdIns 3-kinase [64]. In mammalian cells, the role of PtdIns(3)P in vesicular trafficking was more difficult to prove unambiguously as, unlike yeast, mammalian cells have several isoforms of PtdIns 3-kinase that can phosphorylate position 3 of the inositol ring on substrates other than PtdIns. The discovery that an endosomal protein, EEA1 (early endosomal antigen-1), necessary for endosome fusion is able to specifically bind to PtdIns(3)P linked this lipid to the vesicular trafficking pathways [65, 66]. The domain of EEA1 responsible for the binding to PtdIns(3)P is named the FYVE domain [67, 68]. The FYVE domain is a RING-type zinc-finger domain of ~ 60–80 aa and is composed of two-stranded β sheets with eight cysteine residues coordinating two Zn^{2+} ions. X-ray crystallographic studies and nuclear magnetic resonance (NMR) studies helped to define the interaction of the consensus sequence (R/K)(R/K)HHCR located in the α helix at the end of the FYVE domain with PtdIns(3)P [66, 69–71]. In cooperation with proteins such as the GTPase rab5, PtdIns(3)P is involved in the targeting of EEA1 to its subcellular localization, the early endosome [72]. Studies using GFP-FYVE or GFP-2XFYVE probes, specifically binding to PtdIns(3)P with high affinity, indicated that a major pool of PtdIns(3)P is located on endosomes [73].

Another study in *S. cerevisiae* has implicated PtdIns(3,5)P_2 in the maintenance of vacuole integrity [74]. Indeed, the yeast protein Fab1p is a PtdIns(3)P 5-kinase that transforms PtdIns(3)P into PtdIns(3,5)P_2 and which function appears to be necessary to maintain a normal vacuole morphology. PtdIns(3,5)P_2 is therefore postulated to be involved in maintaining vacuole membrane recycling and turnover. Although no specific targets of PtdIns(3,5)P_2 have been discovered to date, evidence is accumulating that this lipid plays important functions in the cell.

Recently the PX (phox) domain has been shown to bind PtdIns(3)P [75–77]. The PX homology domain is ~120 residues and takes its name from its presence in the p40^{phox} and p47^{phox} proteins, two subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system [78]. Sequence homology searches have increased the number of PX-domain-containing proteins to 54 in *Homo sapiens*. This regroups proteins with diverse functions like the phosphatidylcholine-specific phospholipase D [79], vam7 (a t-SNARE protein in yeast [80]), the serine/threonine kinase CISK [81], RGS-PX1 (a GAP for Gαs [82]) and the sorting nexins (a family of proteins implicated in receptor degradation [83]). The structure of the PX domain was solved by NMR and consists of three β strands, four α helices and a characteristic proline-rich loop, which has been shown to bind an SH3 motif between the two α helices [84]. Several sets of basic residues are implicated in the interaction with PtdIns(3)P [75]. This structure is unique among the PI binding sites described so far. In the yeast *S. cerevisiae*, all the PX domains described show a strict specificity for PtdIns(3)P but with different binding affinities, suggesting that interactions of the PX-bearing proteins with the membranes require additional protein-protein interactions in vivo [85]. In humans, some experiments suggest that the PX domain does not show such a strict PtdIns specificity. PX domains of p40^{phox}, vam7 and SNX3 bind to Ptd Ins(3)P [75–77]. In the case of SNX3, the binding of its PX domain to PtdIns(3)P is essential for regulation of its endosomal functions [77]. In vitro, the PX domain of P47^{phox} seems to bind preferentially PtdIns(3,4)P_2 [86], while the PX domain of CISK and SNX1 bind Ptd Ins(3,5)P_2 and PtdIns(3,4,5)P_3 [87, 88], and the PX domain of type II PI 3-kinase binds to PtdIns(4,5)P_2 [89].

Altogether, these recent results suggest that PtdIns(3)P, and possibly PtdIns(3,5)P_2, actively participates in the subcellular targeting of FYVE- or PX-domain-containing proteins involved in trafficking.

Myotubularin as a regulator of vesicular trafficking

A role for myotubularin in the regulation of the specific pool of endosomal PtdIns(3)P was proposed by Kim et al. [37]. In this study, the authors show that myotubularin, overexpressed as a green fluorescent protein (GFP)-tagged protein in Cos cells, is able to dephosphorylate a pool of endosomal PtdIns(3)P. This was demonstrated by the disappearance in the GFP-myotubularin-transfected cells of the punctuate endosomal staining pattern labeled by a biotinylated GST-2XFYVE probe [73]. The phosphatase activity caused the disappearance, as the GFP-myotubularin C375S inactive mutant had no effect on the staining pattern. In this study, GFP-myotubularin shows a cytoplasmic labeling and does not colocalize with the late endosomes. Myotubularin is also observed in plasma membrane projections, as previously described [6]. In a recent study, Laporte et al. did not observe the effect of myotubularin on the disruption of the endosomal staining by a 2X-FYVE probe [36]. A possible reason for this discrepancy is that in their study both myotubularin and the 2X-FYVE probe were cotransfected and could therefore compete for an available PtdIns(3)P pool. In the study of Kim et al. [37] cells were first transfected with myotubularin, and the pool of PtdIns(3)P was then labeled after fixation and permeabilization with the biotinylated GST-2XFYVE probe. Under these conditions, the probe was able to bind to PtdIns(3)P after the action of the phosphatase, thus avoiding any competition between the two proteins.

In conclusion, myotubularin, although mainly cytoplasmic, can affect a pool of PtdIns(3)P located in endosomal vesicles, suggesting that this lipid phosphatase can play a role in vesicular trafficking events. More studies are now necessary to determine the physiological downstream effectors of myotubularin.

The myotubularin family

Homology searches for myotubularin proteins in the databanks have led to the definition of a large and conserved family of proteins, the myotubularin myopathy-related proteins (MTMR) (table 1). MTMR proteins are found in various species (plant, yeast, worms, flies, mice) but not in bacteria. In *H. sapiens*, the family now has 14 members classified in four groups, based on structural identity in the SID and PTP domain.

The MTM group

In addition to myotubularin, two members compose this group in humans: MTMR1 and MTMR2, together with one gene from *Caenorhabditis elegans* and one gene from *Drosophila*. One striking feature of the three human proteins is that they are involved in three different neuromuscular disorders.

MTMR1 results from duplication of the MTM1 gene and is located 30 kb downstream from MTM1 on Xq28. The MTMR1 protein shows a 59% amino acid identity with

Table 1. Myotubularin and myotubularin related proteins.

Name	Chromosome location	Disease/physio-pathology	Phosphatase or dead-phosphatase site	Known lipid substrates	Major localization	Tissue expression
MTM1/ (myotubularin)	Xq28	myotubular myopathy	HCSDGWDR	PI(3)P	cytosol + plasma mb.	ubiquitous*
MTMR1	Xq28	aberrant spliced form in cDM1	HCSDGWDR	PI(3)P	cytosol + plasma mb.	ubiquitous*
MTMR2	11q22	Charcot-Marie-Tooth 4B1	HCSDGWDR	PI(3,5)P2; PI(3)P	cytosol + plasma mb. conc. around nucleus	ubiquitous*
MTMR3	22q12		HCSDGWDR	PI(3,5)P2; PI(3)P	cytosol + plasma mb.	ubiquitous
MTMR4	17q21		HCSDGWDR	PI(3)P	ND	
MTMR6	13q12		HCSDGWDR	PI(3)P	ND	ubiquitous
MTMR7	8p22		HCSDGWDR	ND	ND	brain
MTMR8	Xq11.2		HCSDGWDR	ND	ND	ubiquitous
MTMR5/ (SBF1)	22q13.3	spermatogenesis defect	GLEDGWDS		cytosol	> in testis
MTMR9/ (LIPSTIX)	8p23.1		HGTEGTDS		cytosol+punctuate	
MTMR10	15q13.3		QEEEGRDL		ND	ND
MTMR11/ (3-PAP)	5		LEENASDL		ND	ubiquitous
MTMR12/ (CRAα/β)	1q21.3					
MTMR13/ (SBF2)	11p15	Charcot-Marie-Tooth 4B2			ND	> brain

The name under which some MTMR members are also known are in parentheses. *Note that the expression of some of these members is tightly regulated during cell differentiation. ND, not determined.

myotubularin. Although it is highly homologous to myotubularin and is located at the same locus, no mutations in MTMR1 have ever been found in patients with myotubular myopathy [90]. This suggests that MTMR1 is not likely to be a mutation target in XLMTM disorder. Like myotubularin, MTMR1 presents a ubiquitous expression and a PtdIns(3)P phosphatase activity [38, 56], but its role seems not to be redundant as MTMR1 is not able to compensate in vivo for the loss of myotubularin activity in XLMTM patients. MTMR1 has six mRNA isoforms that result from alternative splicing. One of these isoforms, called isoform C, is specifically expressed in muscle during myogenesis and is the major form found in adult skeletal muscles [56]. It has a comparable localization with MTMR1 and myotubularin and exhibits an in vitro PtdIns(3)P phosphatase activity. The expression of this MTMR1C isoform is greatly reduced and replaced by an aberrant isoform in skeletal muscle cells from cDM1 (congenital myotonic dystrophy) patients. Congenital myotonic dystrophy (OMIM 160900) is one of the forms of the autosomal dominant neuromuscular disorder that shares some pathological features with XLMTM [91]. The mutated gene responsible for the disease is located at locus 19q13 and codes for the myotonin protein kinase (MPK), a neuromuscular junction protein implicated in myogenesis. It was shown that DM1 patients have an abnormally increased repetition of a CTG triplet in the 3′-untranslated region of the MPK gene and that the length of the repeat is correlated to the severity of the disease [92]. Moreover, the level of CUG-BP, a protein binding to these repeats, is increased and consequently binds to other CUG repeats, thereby transregulating the alternative splicing of other genes. This was demonstrated for the cardiac troponin T and the insulin receptor genes in skeletal muscle and for the microtubule-associated protein tau gene in brain. These genes have thus been implicated in the pathology of DM1 [93–95]. MTMR1 represents another target for aberrant splicing in DM1, and more studies are now necessary to decipher the role of MTMR1 in myogenesis and its implication in myotonic dystrophy together with the other proteins implicated.

The myotubularin-related protein MTMR2 is 65% identical to MTM1. The gene coding for MTMR2, mapped on chromosome 11q22, was found mutated in the autosomal recessive demyelinating neuropathy, Charcot-Marie-Tooth type 4B disease CMT4B1 (OMIM 601382) [96]. This disease, affecting the peripheral nervous system, is characterized by focally folded myelin sheaths. There are several types of Charcot-Marie-Tooth disease, some of the demyelinating type, others of the axonal type. At present, mutations in more than seven different genes, impli-

cating proteins of very diverse functions, have been found in Charcot-Marie-Tooth disease. The mutations in MTMR2 described to date are all along the gene, and some of them affect key conserved domains like the SID or the lipid phosphatase domain, giving rise to truncated or inactive proteins [96–100]. Like myotubularin, MTMR2 is a potent PtdIns(3)P phosphatase and is localized in the cytoplasm, although it is more concentrated around the nucleus and in the plasma membrane [37, 46]. Several observations can explain the lack of complementation of these two ubiquitously expressed proteins that have similar activities but are involved in two unrelated diseases. Myotubularin and MTMR2 present an inversed expression pattern during myogenesis. MTMR2 is expressed in the C2C12 myoblast cells and decreases during the differentiation process [37]. Moreover, it has been suggested that these two lipid phosphatases could affect different pools of PtdIns(3)P as, in contrast to myotubularin, MTMR2 is unable to provoke a disruption of early endosomal staining [37]. When MTMR2 is overexpressed in the yeast *S. pombe* invalidated for endogenous myotubularin and overexpressing VPS34, the phenotype of the yeast is characterized by smaller and more numerous vacuoles compared with yeast overexpressing myotubularin [46]. These observations could explain how these two structurally and functionally redundant proteins are involved in different pathologies. Recently, the characterization of the mouse MTMR2 homolog, mMTMR2, was described [99, 101]. In situ studies showed that mMTMR2 is more abundant in the peripheral nervous system, mainly the dorsal root ganglia and the spinal roots [101]. Interestingly, Berger et al. [99] demonstrated that among various PIs, mMTMR2 was able to dephosphorylate PtdIns(3)P but also PtdIns(3,5)P_2 with an even higher efficiency than PtdIns(3)P in vitro at neutral pH. Accordingly, recombinant mMTMR2 led to the generation of PtdIns(5)P in vitro [99].

The MTMR3 group

Two human proteins, MTMR3 and MTMR4, and homolog in *Drosophila* and *C. elegans* compose this group of myotubularin-related members. In addition to the conserved SID and PTP domain, they present at their C-terminus end a FYVE domain and were previously named FYVE-DSP1 [102] and FYVE-DSP2 [103]. MTMR3 and MTMR4 were located at locus 22q12 and locus 17q21, respectively, and present a similar intron/exon organization [103]. They have not been found mutated in a genetic disease so far. They share a 47% amino acid identity and present in vitro a specificity toward PtdIns(3)P compared with the other monophosphoinositides [46, 103]. However, when assayed in vitro against the whole panel of PIs, recombinant MTMR3 also showed a strong activity toward PtdIns(3,5)P_2, comparable to PtdIns(3)P. Accordingly, high-pressure liquid chromatography (HPLC) analysis of PIs demonstrated that PtdIns(5)P was increased in yeast *S. cerevisiae* overexpressing MTMR3 [57]. The phenotype of these yeast showed aberrant, enlarged vesicles that were proposed to be autophagosomes [6, 57]. This phenotype is similar to the one observed in a Fab1p-invalidated strain, which showed the presence of large autophagic vacuoles. Fab1p and its human ortholog, PYKfyve [104], are lipid kinases that specifically phosphorylate PtdIns(3)P to produce PtdIns(3,5)P_2. So MTMR3 is likely to be involved in intracellular trafficking by its effects on these two PIs. MTMR3 has a FYVE domain at its C-terminal end, but localization of overexpressed MTMR3 in mammalian cells is cytoplasmic with some plasma membrane labeling but with no labeling of internal vesicles like EEA1-containing vesicles. Whether PtdIns(3)P can bind to the FYVE domain of MTMR3 or MTMR4 has never been tested in vitro. In vivo, the FYVE domain of MTMR3 is apparently not able to drive the localization of the protein to internal membranes. With respect to its plasma membrane association, it is also possible that this FYVE domain specifically recognizes a discrete pool of PtdIns(3)P located in the plasma membrane that would target, in association with a protein partner, the localization of MTMR3 to those membranes.

The MTMR6 group

MTMR6, MTMR7 and MTMR8 compose this third group of myotubularin-related proteins in humans and have been assigned to locus 13q12, 8p22 [38] and Xq11.2, respectively. MTMR6 is ubiquitously expressed, and only MTMR7 has a specific brain expression [38]. The three proteins possess the conserved characteristic domains of the myotubularin family, that is, a phosphatase domain with a CX_5R motif identical to myotubularin and a SID domain. Accordingly, MTMR6 was shown to specifically dephosphorylate PtdIns(3)P in vitro [37]. More studies should help characterize the physiological function of the members of this group.

The nonactive phosphatase group

The common feature of this group is the presence of an inactive phosphatase catalytic site, and some of the members were proposed to act as antiphosphatase (i.e., binding the substrate and consequently protecting it from the active lipid phosphatase). Six proteins compose this group in humans: MTMR5/SBF1, MTMR9/LIPSTYX, MTMR10, MTMR11/3-PAP adaptor, MTMR12/CRAα/β and MTMR13/SBF2. Homologs are also found in flies and worms, suggesting that antiphosphatases play an important physiological role. MTMR5/SBF1 is the most studied member of this group, and instead of the CX_5R

phosphatase active site of myotubularin, CSDGWDR has the LEDGWDI sequence, leading to an inactive phosphatase site. A PH domain (pleckstrin homology) is located at the C-terminus end of MTMR5/SBF1 that was shown to bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [105]. An MTMR5/SBF1 clone lacking a part of the 5′-translated region was found during a two-hybrid screen with the SET-domain-containing protein Hrx, the human homolog of *Drosophila* trithorax [44]. The domain of MTMR5/SBF1 interacting with the SET protein was defined as the SID. This truncated N-terminus version of MTMR5/SBF1 was shown to localize in the nucleus and induce an oncogenic transformation of NIH 3T3 fibroblasts, suggesting that the SID from the myotubularin family could link the epigenic regulatory machinery and the signaling pathways implicated in cellular growth and differentiation [44]. This transformation activity was abrogated by restoration of an active phosphatase site by mutation. The antiphosphatase was also shown to induce the growth of B cell precursors in vitro through its SID [106] and to arrest the differentiation of C2C12 myoblasts in vitro. The truncated MTMR5/SBF1 was also reported to specifically phosphorylate the SET protein SUV39H1 and reverse its suppressive effects on growth and its repressive effects on transcription [107]. However, the full-length MTMR5/SBF1 was cloned later and does not show any proliferative effects. These results suggested that the N-terminus missing part controls the growth properties of the molecule [108]. A GEF (guanine exchange factor) homology domain or dDENN (differentially expressed in normal and neoplastic cells) domain has been assigned to the N-terminal end of MTMR5/SBF1, which drives the localization of the antiphosphatase to the cytoplasm. Using MTMR5/SBF1 knockout mice, it was shown that the antiphosphatase plays an essential role in spermatogenesis [109]. A role for the rat homolog of myotubularin in spermatogenesis has also been suggested [110].

The characterization of MTMR9, MTMR10 and MTMR12 has still to be done. MTMR9/LIPSTYX was named after its homology in the CX_5R motif (a mutated cysteine to glycine residue) found in STYX, a phosphoserine/threonine/tyrosine interaction protein described as an antiphosphatase [111]. Overexpressed MTMR9 is localized in the cytoplasm with some highly stained spots that have not yet been characterized by colocalization with known markers [46].

MTMR11/3-PAP is an interesting member of the nonactive phosphatase group. It has a phosphatase inactive CX_5R and a SID and was originally cloned as the human homolog of the adapter subunit of a rat heterodimeric PtdIns(3)P phosphatase, which is only partly characterized [112]. In rat, the heterodimer is composed of a 65-kDa catalytic subunit and a 78-kDa adapter subunit [113]. MTMR11/3-PAP has no intrinsic PtdIns(3)P activity but can coimmunoprecipitate a PtdIns(3)P-phosphatase activity directed toward PtdIns(3)P and PtdIns(3,4)P_2. The identity of the putative catalytic subunit and of the domains involved in the interaction should shed some light on the function of this member of the family.

MTMR13/SBF2 is the latest nonactive phosphatase member characterized so far. It has a 59% amino acid identity with MTMR5/SBF1 and has the cysteine from its phosphatase site replaced by a leucine, which predicts an abolished lipid phosphatase activity. The gene for SBF2 was located to chromosome 11p15 by linkage analysis and found mutated in cases of autosomal recessive Charcot-Marie-Tooth disease CMT4B2 with focally folded myelin (OMIM 604563) associated with early-onset glaucoma [114, 115]. This is the first report of an inactive phosphatase mutated in a human genetic disease. It is striking to note that the nonactive MTMR13/SBF2 and the lipid phosphatase active MTMR2 are both implicated in two forms of CMT4B. A hypothesis could be that both proteins act on the same signaling pathway. MTMR13/SBF2 may therefore play the role of adaptor or regulator of the active phosphatase, as proposed for the MTMR11/3-PAP by Nandurkar et al [112].

Conclusion and perspectives

Since the cloning of the MTM1 gene in 1996, great progress has been made in deciphering the function of myotubularin. The elucidation of its PtdIns(3)P phosphatase activity has opened new research directions by linking myotubularin to PI metabolism. Myotubularin is one more example of the essential role of PIs in cellular regulation. However, although the PtdIns(3)P phosphatase activity of myotubularin suggests the implication of this lipid in the etiology of myotubular myopathy, many questions remain.

For instance, one important point will be to define the in vivo regulation of myotubularin phosphatase activity. There is no report yet of proteins interacting directly with myotubularin despite the fact that several protein-interacting domains have been found on this lipid phosphatase. This is important given the fact that myotubularin is a very potent PtIns(3)P phosphatase in vitro, and its activity is likely to be tightly regulated in vivo through interaction(s) with partner(s). Candidates could be PDZ-containing proteins that are known to be part of multiprotein complexes at the plasma membrane or an adaptor like the 3-PAP phosphatase, a member itself of the myotubularin family with no catalytic activity. Moreover, myotubularin is mainly cytoplasmic and must therefore be directed to membranes containing its substrate. Myotubularin has no direct lipid-binding domain that could target the lipid phosphatase to membranes, suggesting

that the cellular localization of the phosphatase is instead directed by interaction with a protein. Moreover, one can question the strict specificity of myotubularin toward PtdIns(3)P. Indeed, two members of the myotubularin family, MTMR2 and MTMR3, have been shown to hydrolyze PtdIns(3,5)P_2 in addition to PtdIns(3)P. Although this point remains controversial because Kim et al. [37] have not measured such an activity, it will be important to establish whether myotubularin can transform PtdIns(3,5)P_2 into PtdIns5(P), a newly described PI.

Another question to be addressed is the discrepancy between the localization of myotubularin – cytosol and plasma membrane – and the localization of its substrate PtdIns(3)P, which is mainly located on vesicular membranes. Is myotubularin targeted to intracellular vesicles in response to a specific stimulus to hydrolyze PtdIns(3)P, or could myotubularin also affect a discrete pool of PtdIns(3)P in the plasma membrane? Results obtained in yeast suggest a role for myotubularin in intracellular traffic regulation. However, although most of the regulators of vesicular trafficking in yeast have their equivalent in higher eukaryotic cells, the role of myotubularin in the regulation of membrane traffic in mammalian cells remains to be clearly demonstrated. Resolution of this question is essential to establish a clear link between the loss of enzyme activity, its consequence on the levels of PIs and manifestation of genetic disease. In that aspect, the myotubularin knockout mouse should prove to be a useful tool. Moreover, rescue experiments of myotubularin knockout using different myotubularin family members could indicate to what extend redundancy exists in the family. A better understanding of myotubularin function and regulation should help the development of new diagnostic tests and gene therapy assays in the context of myotubular myopathy. Finally, in addition to myotubularin and MTMR2, the high level of structure identity and the conserved substrate specificity in the myotubularin family from yeast to human suggest the implication of other members in human diseases.

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