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Phosphatidylinositol-5-Phosphate Activation and Conserved Substrate Specificity of the Myotubularin Phosphatidylinositol 3-Phosphatases

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Summary

Phosphoinositides control many different processes required for normal cellular function [1, 2]. Myotubularins are a family of Phosphatidylinositol 3-phosphate (PtdIns3P) phosphatases identified by the positional cloning of the MTM1 gene in patients suffering from X-linked myotubular myopathy and the MTMR2 gene in patients suffering from the demyelinating neuropathy Charcot-Marie-Tooth disease type 4B [3–9]. MTM1 is a phosphatidylinositol phosphatase with reported specificity toward PtdIns3P [6, 7], while the related proteins MTMR2 and MTMR3 hydrolyze both PtdIns3P and PtdIns(3,5)P2 [10, 11]. We have investigated MTM1 and MTMR6 and find that they use PtdIns(3,5)P2 in addition to PtdIns3P as a substrate in vitro. The product of PtdIns(3,5)P2 hydrolysis, PtdIns5P, causes MTM1 to form a heptameric ring that is 12.5 nm in diameter, and it is a specific allosteric activator of MTM1, MTMR3, and MTMR6. A disease-causing mutation at arginine 69 of MTM1 falling within a putative pleckstrin homology domain reduces the ability of the enzyme to respond to PtdIns5P. We propose that the myotubularin family of enzymes utilize both PtdIns3P and PtdIns(3,5)P2 as substrates, and that PtdIns5P functions in a positive feedback loop controlling their activity. These findings highlight the importance of regulated phosphatase activity for the control of phosphoinositide metabolism.

Results and Discussion

We investigated the phosphatase activities of MTM1 and MTMR6 toward phosphoinositides under defined conditions to determine their substrate specificity (Figures 1 and S1 [the latter is available in the Supplemental Data available with this article online]). Purified MTM1 displayed activity toward PtdIns3*P* and PtdIns(3,5)P_2, but not the other naturally occurring phosphoinositides (Figure 1A). The activity toward PtdIns(3,5)P_2 adds a novel substrate specificity for MTM1 [6] and is consistent with reports that MTMR2 and MTMR3 use PtdIns3*P* and PtdIns(3,5)P_2 as substrates [10, 11]. Purified MTMR6 showed the same substrate specificity as MTM1 (Figure 1C), while the active site mutant protein MTMR6^{C336S} lacked any detectable activity toward either PtdIns3*P* or PtdIns(3,5)P_2. To confirm that MTM1 hydrolyses PtdIns(3,5)P_2, we performed an alternate analysis of activity described previously for MTMR3 [10]. This assay is carried out in living yeast cells and is useful for establishing 3-phosphatase activity against PtdIns(3,5)P_2, since it allows detection of the reaction product PtdIns5*P*, which is not normally detectable in yeast [10, 12, 13]. In control yeast, no PtdIns5*P* was observed, whereas, in MTM1-expressing yeast, a peak of PtdIns5*P* was generated, presumably by 3-phosphatase activity against PtdIns(3,5)P_2 (Figure 1C). In accordance with this interpretation, increased PtdIns5*P* production was seen when cellular levels of PtdIns(3,5)P_2 were increased following osmotic shock (Figure 1C; [10, 12, 13]). Like MTMR2 and MTMR3, MTM1 and MTMR6 therefore utilize both PtdIns3*P* and PtdIns(3,5)P_2 as substrates, suggesting that this is a general property of the myotubularins.

To further investigate the properties of MTM1 and MTMR6, we performed a kinetic analysis of their enzyme activity with PtdIns3*P* and PtdIns(3,5)P_2 as substrates (Figure 2). For this analysis, we assumed that, like other phosphatases, MTM1 and MTMR6 have a two-step reaction mechanism in which the rate-limiting step is transfer of phosphate from the substrate to the active site cysteine, that this phosphoenzyme intermediate is short lived, and that the second substrate water is in great excess [14]. Activity measurements were performed for MTM1 with PtdIns3*P* and PtdIns(3,5)P_2 concentrations between 10 and 100 μM (Figure 2). Plots of initial reaction velocity as a function of substrate concentration should be hyperbolic if the enzyme obeys Michaelis-Menten kinetics [15]; but, in this case, the curves are hyperbolic for neither MTM1 (Figures 2A and 2B) nor MTMR6 (Figure S2). The sigmoidal curves of initial velocity as a function of substrate concentration obtained for MTM1 and MTMR6 are reminiscent of oligomeric enzymes such as pyruvate kinase and isocitrate dehydrogenase in the absence of their allosteric activators and suggest cooperative substrate binding at multiple sites [15]. One key point in the general theoretical treatments of allostery is that the enzyme is assumed to be oligomeric to explain cooperative substrate binding [15]. We therefore examined MTM1 and MTMR6 by gel filtration and found that both enzymes have apparent molecular sizes consistent with a monomeric state in the absence of substrate [16]. It was not practical to perform the analysis in the presence of phosphoinositides, and we could not exclude the possibility that the substrate-induced oligomerization observed for enzymes such as D-amino acid oxidase occurs with myo-

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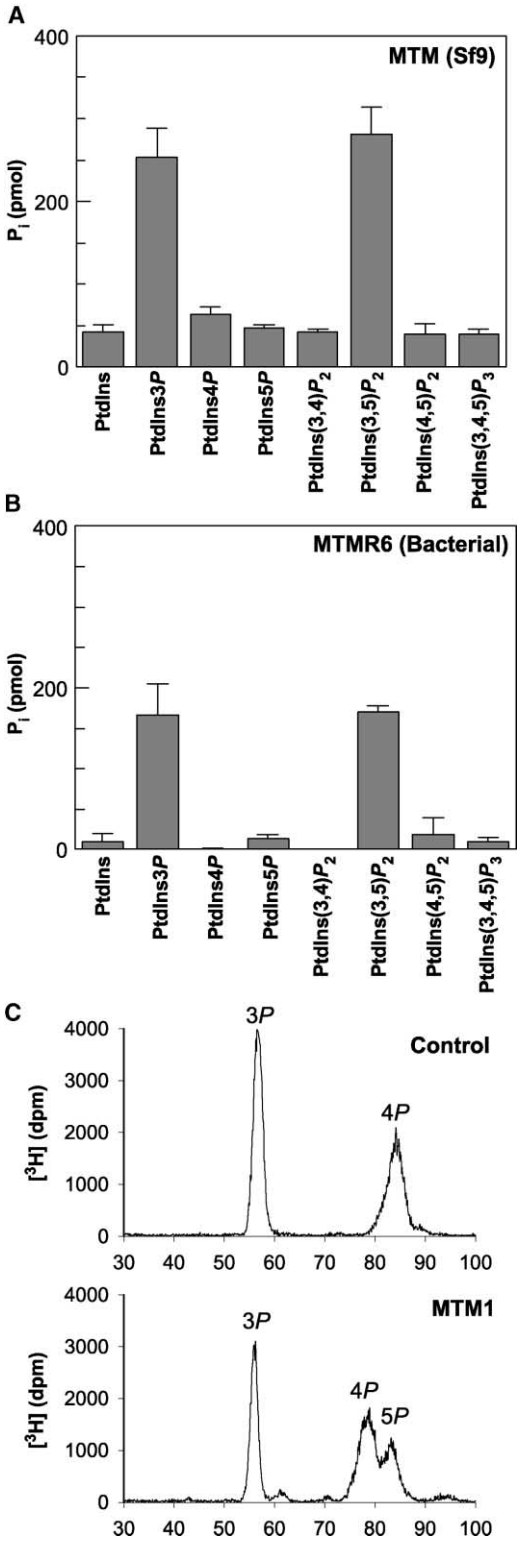

Figure 1. Substrate Specificity of MTM1 and MTMR6 In Vitro

(A) The activity of 30 nM MTM1 expressed in, and purified from, insect cells toward 50 μM phosphatidylinositol and the indicated phosphoinositides was measured for 10 min at 37°C (n = 4).

(B) MTMR6 expressed in, and purified from, bacteria was assayed in the same manner as MTM1 (n = 4).

(C) As described previously [12, 13], cellular phosphoinositides were extracted and analyzed by high-pressure liquid chromatography from yeast strains containing a control plasmid or an MTM1 expression plasmid stimulated with 0.9 M NaCl for 10 min.

tubularins [17]. While unusual, there are a number of monomeric enzymes displaying allosteric regulation [18], such as the ribonucleotide reductase of *Lactobacillus leichmannii* that has a catalytic site where ribonucleotides bind and a discrete regulatory site for nucleotides [19, 20]. To explain the sigmoidal curves of initial velocity as a function of substrate concentration for MTM1, we initially postulated that the substrate, PtdIns3*P*, is a weak allosteric activator capable of binding at a regulatory site on the monomeric enzyme. By analogy with ribonucleotide reductase, we felt it was unlikely that PtdIns3*P* was the physiological activator, but that this could be a related molecule. Non-substrate phosphoinositides were therefore tested for their ability to activate MTM1. The activity of MTM1 toward 50 μM PtdIns3*P* was stimulated by 20 μM PtdIns5*P*, while PtdIns4*P* had no effect, and the other phosphoinositides tested had little or an inhibitory effect (Figure S3A). PtdIns5*P* activates the catalytic activity of MTM1 toward both PtdIns3*P* and PtdIns(3,5)P_2 (Figures 2C and 2D). Additionally, the effect of PtdIns5*P* on PtdIns3*P* hydrolysis by MTM1 was also observed when long chain derivatives of these phosphoinositides were incorporated into liposomes (Figure 2E). Likewise, the activity of MTMR3 toward 50 μM PtdIns3*P* was activated in the presence of 20 μM PtdIns5*P* (Figure 2F), while, under the same conditions, the initial rate of reaction was increased for MTMR6 although the sustained reaction velocity at later time points was similar (Figure S3B). It is important to note that PtdIns5*P* is not a substrate for these enzymes (Figure 1; [10]), and we conclude that it acts as an allosteric activator for MTM1, MTMR3, and possibly MTMR6. When PtdIns(3,5)P_2 is used as the substrate, PtdIns5*P* is generated and could activate the enzyme by a positive feedback mechanism. It is unlikely that such a regulatory mechanism would exist to no effect, and this finding supports the proposition that PtdIns(3,5)P_2 represents a physiologically relevant substrate for myotubularins. In addition to localization to membrane subcompartments such as plasma membrane ruffles [21], myotubularin activity could also be compartmentalized by the availability of PtdIns5*P*. Thus, PtdIns3*P* and PtdIns(3,5)P_2 in membrane domains lacking PtdIns5*P* would be poor substrates for myotubularins.

During our investigations of MTM1, MTMR3, and MTMR6, we noticed that their catalytic activity showed a sigmoidal dependence on enzyme concentration, suggestive of oligomerization under reaction conditions. MTM1 shows a 9-fold increase in initial reaction velocity toward PtdIns3*P* when enzyme concentration is increased from 15 to 30 nM (Figure 3A). To investigate the potential oligomerization of MTM1 under reaction conditions, we exploited the fact that mutations in the catalytic cysteine of phosphatases are thought to abolish catalytic activity without altering their substrate binding properties [14]. First, the activity of 30 nM MTM1 and a mutant form in which the catalytic cysteine was changed to serine were compared, and MTM1^{C375S} was

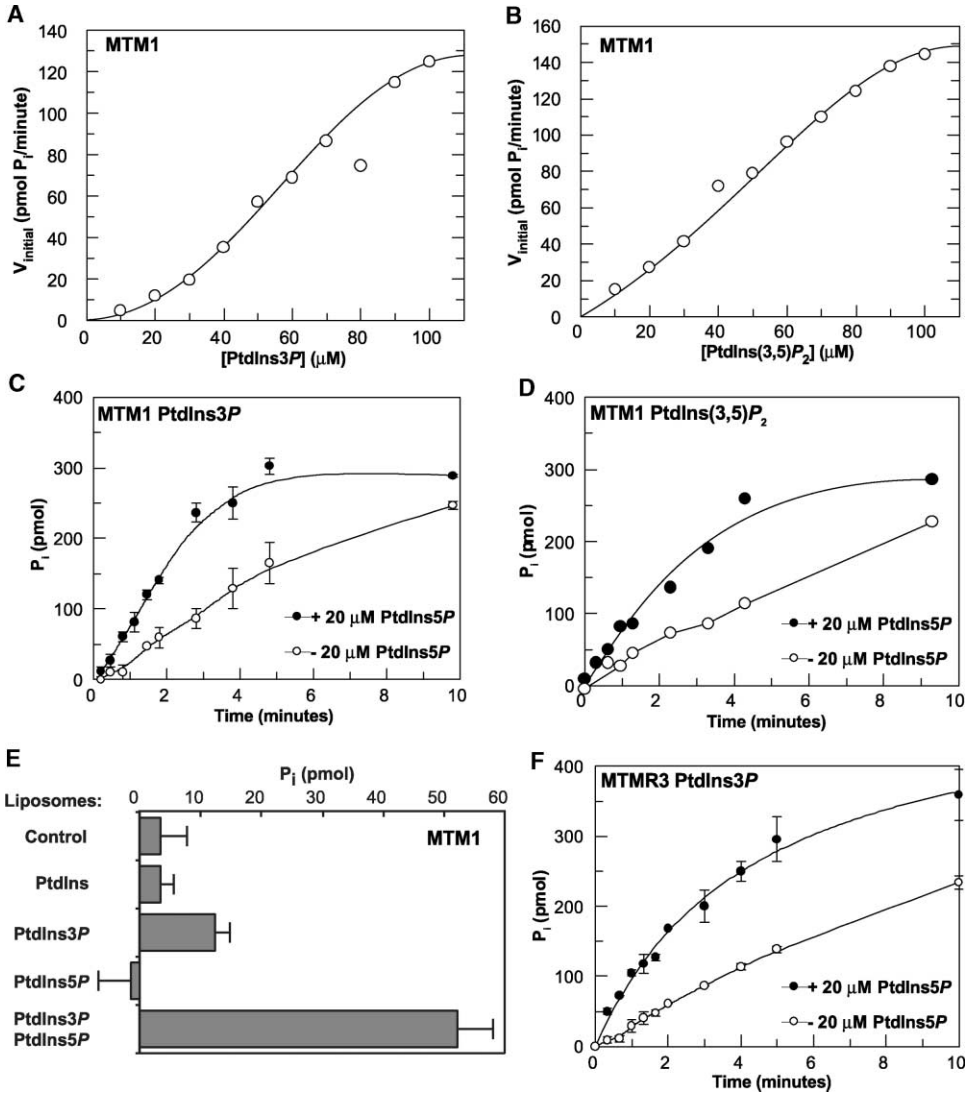

Figure 2. PtdIns5*P* Is an Activator of MTM1 and MTMR3 In Vitro

(A and B) The activity of MTM1 toward the indicated concentrations of PtdIns3*P* and PtdIns(3,5)P_2 was measured. Initial reaction velocities were calculated from these time courses and are plotted against the respective substrate concentration.

(C and D) The activity of MTM1 toward 50 μM PtdIns3*P* and 50 μM PtdIns(3,5)P_2 in the presence and absence of 20 μM PtdIns5*P* was measured (n = 2).

(E) The activity of 15 nM MTM1 was tested toward control liposomes and liposomes containing 10 mol% of the indicated phosphoinositides (n = 4).

(F) The activity of MTMR3 toward 50 μM PtdIns3*P* in the presence and absence of 20 μM PtdIns5*P* was measured (n = 2).

found to lack activity toward PtdIns3*P*, even in the presence of PtdIns5*P* (Figure 3B). To shift the equilibrium from monomeric to oligomeric MTM1, catalytically dead MTM1^{C375S} was titrated into assays containing 18 nM MTM1, a concentration at which MTM1 shows approximately half maximal activity toward PtdIns3*P* and is thus poised for activation (Figure 3A). The addition of 1.8 nM MTM1^{C375S} caused an increase in the activity of MTM1, and this effect was already maximal when equimolar MTM1^{C375S} was used (Figure 3C). When MTM1^{C375S} was replaced by catalytically inactive MTMR6 or bovine serum albumin, these effects were not seen, indicating that this is a specific effect of MTM1 (Figure S4). The simplest explanation for this observation is that MTM1 interacts with MTM1^{C375S} in the presence of substrate and that these oligomeric forms of the enzyme have increased activity compared to monomeric MTM1. To visualize these oligomeric forms of MTM1, we analyzed enzyme reactions by negative stain electron microscopy (Figure 3D). Catalytically inactive MTM1^{C375S} was used for these experiments to trap the substrate and activator bound intermediates, since these are rapidly turned over by the active enzyme. This analysis revealed that, under activated conditions, MTM1 forms a heptameric ring structure that is on average 12.5 nm in diameter, and that these ring structures are essentially absent prior to activation of MTM1 with PtdIns5*P* and substrate (Figure 3D).

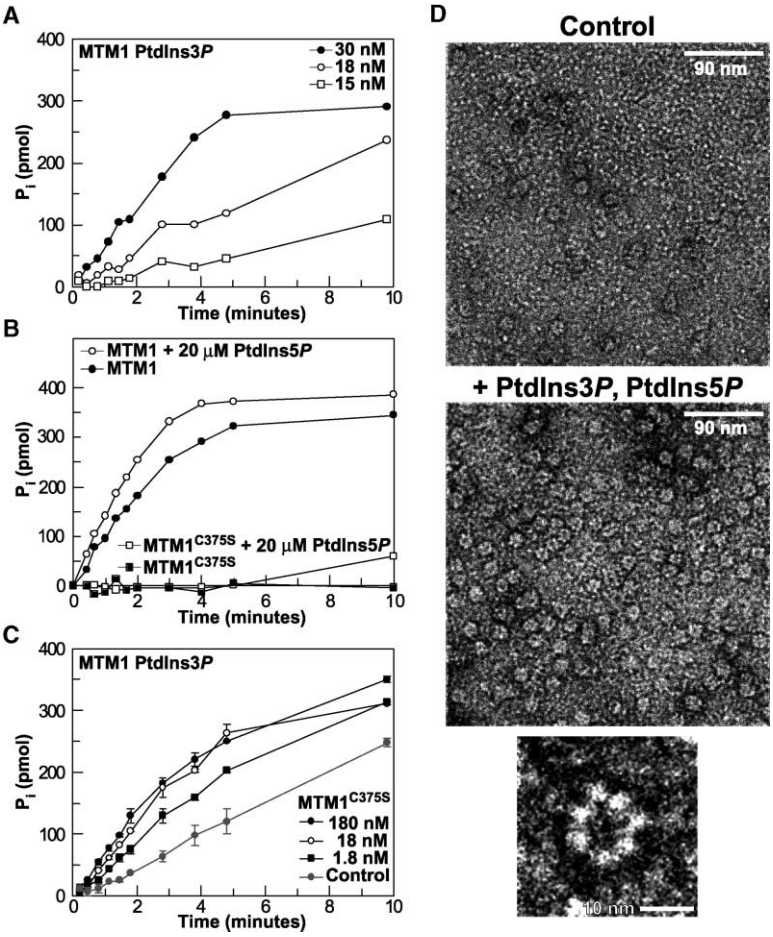

Figure 3. Activation of MTM1 by Oligomerization

(A) MTM1 activity toward 50 μM PtdIns3*P* was determined at the indicated enzyme concentrations.

(B) The activity of 30 nM MTM1 or catalytic site mutant MTM1^{C375S} toward 50 μM PtdIns3*P* was determined in the presence and absence of 20 μM PtdIns5*P*.

(C) Catalytic site mutant MTM1^{C375S} was titrated into assays containing 18 nM MTM1 and 50 μM PtdIns3*P* (n = 2).

(D) Phosphatase assays containing 200 ng/μl MTM1^{C375S} with either buffer (control) or 100 μM PtdIns3*P* and 40 μM PtdIns5*P* were incubated for 5 min at 37°C, then spotted onto copper grids for staining with uranyl acetate and visualization by electron microscopy. Images were taken at 35,000× magnification.

We then examined the sequence of MTM1 for conserved basic residues associated with disease-causing mutations in X-linked myotubular myopathy [22, 23], which, by analogy with pleckstrin homology (PH) domains, could mediate the specific recognition of phosphoinositides [24]. Candidate arginine residues at positions 69, 184, 241, 421, and histidine 469 of MTM1 were mutated to the amino acids found in the disease state, and the conserved arginines at positions 114 and 220 were mutated to alanine. Corresponding mutant proteins were tested for basal and PtdIns5*P*-stimulated activity toward PtdIns3*P* (Figure 4A). Mutations at positions 220, 241, and 469 showed no activity (J.S. and F.A.B, unpublished data), in agreement with previous reports that MTM1^{R241L} is inactive [6, 25]. MTM1^{R184G} and MTM1^{R421Q} could be stimulated by the addition of 20 μM PtdIns5*P*, but they showed only low levels of activity compared to MTM1, even at high enzyme concentration (Figure 4A). MTM1^{R69C} had a lower activity than MTM1 in the presence or absence of 20 μM PtdIns5*P*, conditions at which MTM1 is maximally active, but was more active than the other mutants tested (Figure 4A). If the reduced activity of MTM1^{R69C} reflects a decreased affinity for the allosteric activator, then it should be possible to increase MTM1^{R69C} activity by titrating in PtdIns5*P*. We therefore tested the ability of PtdIns5*P* to activate 30 nM MTM1 and MTM1^{R69C} (Figure 4B). MTM1 is maximally activated by 10–20 μM PtdIns5*P*, while MTM1^{R69C} requires 50 μM PtdIns5*P* to achieve the same level of activity (Figure 4B). This observation is consistent with the idea that MTM1^{R69C} shows a reduced level of activity due to a mutation in its allosteric regulatory site and has a reduced affinity for PtdIns5*P*. The amino-terminal region of myotubularins contains a divergent PH domain [9] that might be a binding site for PtdIns5*P*, since arginine 69 of MTM1 lies within this domain and a basic residue is conserved at this position in other myotubularins. Consistent with this idea, another mutation at a conserved site in this putative PH domain, MTM1^{K114A}, also shows a decreased response to PtdIns5*P* (Figure 4B).

Our results show that myotubularins have a common substrate specificity toward PtdIns3*P* and PtdIns(3,5)P_2 and identify a regulatory function for PtdIns5*P*. We propose a model in which the monomeric myotubularins are essentially catalytically inactive and binding of substrate phosphoinositide and the allosteric activator PtdIns5*P* triggers oligomerization and thus activates these enzymes.

Supplemental Data

Supplemental Data including details of the experimental procedures used, additional data relating to MTM1 and MTMR6 substrate specificity and regulation, and control experiments is available upon request from the authors and at http://images.cellpress.com/supmat/supmatin.htm.

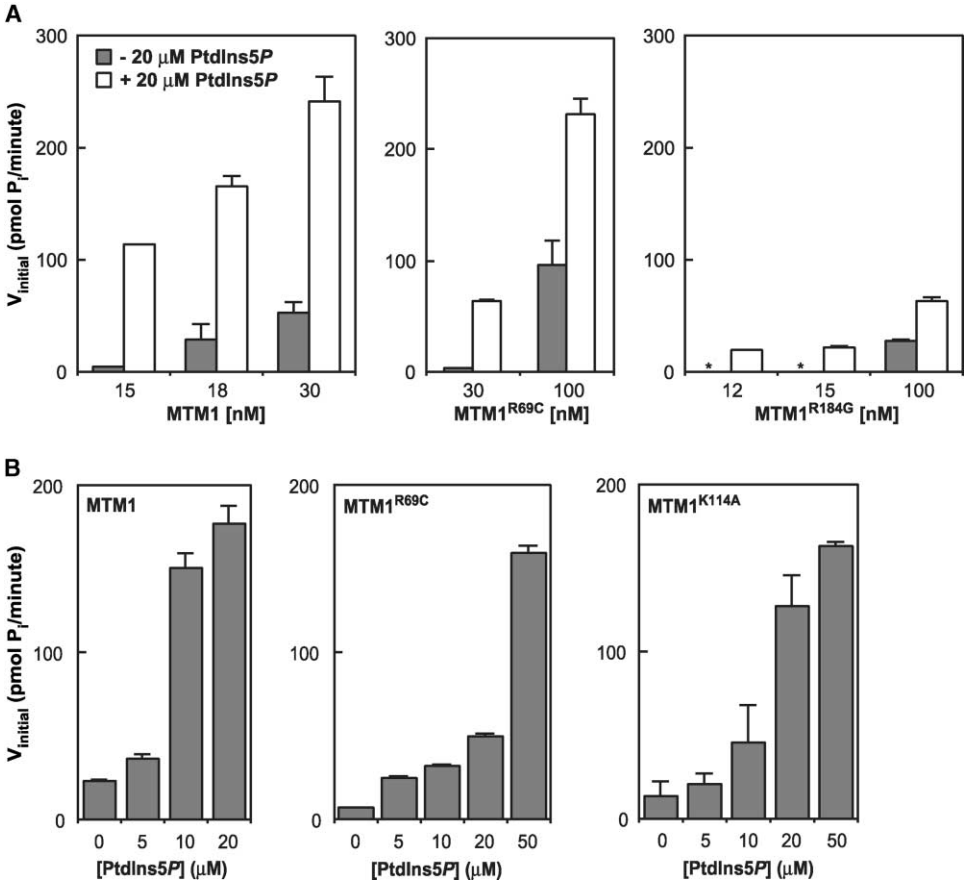

Figure 4. Characterization of MTM1 Mutants Associated with Disease

(A) The activity of MTM1, MTM1^{R69C}, and MTM1^{R184G} toward 50 μM PtdIns3*P* was determined as a function of time at the indicated enzyme concentrations in the presence and absence of 20 μM PtdIns5*P*. Initial reaction velocities were calculated from these time courses and are plotted against the respective enzyme concentration.

(B) The activities of 30 nM MTM1, MTM1^{R69C}, and MTM1^{K114A} toward PtdIns3*P* were determined as a function of time in the presence of the indicated concentrations of PtdIns5*P* and in its absence. Initial reaction velocities were calculated from these time courses and are plotted against the respective PtdIns5*P* concentration.

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