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MptpB, a virulence factor from *Mycobacterium tuberculosis* exhibits triple-specificity phosphatase activity

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Abbreviations: DSP, dual-specificity protein phosphatase; TSP, triple-specificity phosphatase; MAPK, Mitogen-activated protein kinases; IFN- γ , interferon gamma; MTM, myotubularin; MTMR2, myotubularin-related protein 2; PTP, protein tyrosine phosphatase; PI(3)P, phosphatidylinositol-3-phosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI(5)P, phosphatidylinositol-5-phosphate; PI(3,5)P₂, phosphatidylinositol-3,5-phosphate; PI(3,4)P₂, phosphatidylinositol-3,4-phosphate; PI(4,5)P₂, phosphatidylinositol-4,5-phosphate; PI(3,4,5)P₃, phosphatidylinositol-3,4,5-phosphate; PI, phosphoinositide; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenol phosphate .

Keywords: protein phosphatase, signalling, dual-specificity, lipid phosphatase, bacterial phosphatase.

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Abstract

Bacterial pathogens have developed sophisticated mechanisms of evading the immune system to survive in the infected host cells. Central to pathogenesis of *Mycobacterium tuberculosis* is the arrest of phagosome maturation partly through interference with phosphatidylinositol signalling. The protein phosphatase MptpB is an essential secreted virulence factor in *M. tuberculosis*. A combination of bioinformatics analysis, enzyme kinetics and substrate specificity characterization revealed that MptpB exhibits both dual-specificity protein phosphatase activity and, importantly, phosphoinositide phosphatase activity. Mutagenesis of conserved residues in the active site signature indicates a cysteine-based mechanism of dephosphorylation and identifies two new catalytic residues, Asp165, essential in catalysis and Lys164, apparently involved in substrate specificity. Sequence similarities with mammalian lipid phosphatases and preference for phosphoinositide substrates, suggests a potential novel role of MptpB in phosphatidylinositol metabolism in the host and opens new perspectives for the role of this phosphatase in mycobacteria pathogenicity.

INTRODUCTION

Mycobacterium tuberculosis is the causative agent of tuberculosis, a decimating disease affecting one third of the human population and causing around two million deaths every year (according to the World Health Organisation, www.who.int). This intracellular pathogen survives and replicates primarily in host macrophages despite the antimicrobial functions of the macrophage. The primary defence against intracellular microbial pathogens is the phagosomal maturation into phagolysosomes, leading to the acidification of lysosome contents and destruction of bacterial particles. Many pathogens have evolved sophisticated ways of evading this innate response to extend their survival in host cells. *M. tuberculosis* is a landmark example of such strategy [1], since it can persist in phagocytes preventing the normal maturation to phagolysosomes and survive inside the host lungs in a latent asymptomatic state. In recent years, a number of studies have been directed to understand the molecular mechanisms that allow *M. tuberculosis* survival in infected macrophages (reviewed in [2-4]). One proposed mechanism involves the host phosphoinositide PI3P responsible for recruitment of Rab5 effectors, hVPS34 and EEA1 to endocytic organelles, an essential step in phagosome maturation [5]. Manipulation of phosphoinositide metabolism is emerging now as a common theme in bacterial pathogenesis [6].

Other mechanisms correlate infection of *M. tuberculosis* with disruption of several normal host signalling pathways involving MAPKs, interferon- γ (IFN- γ), calcium signalling and apoptosis (reviewed in [4]). As phosphorylation and dephosphorylation of host proteins regulate these intracellular processes, it is not surprising that protein kinases and phosphatases play a role in pathogen infection and survival [3-4]. In fact phosphatases are major virulence factors in other pathogenic bacteria such as *Yersinia pseudotuberculosis*, and *Salmonella typhimurium* [7-8]. Likewise, the *M. tuberculosis* secreted protein phosphatases MtpA and MtpB have been reported to be important for persistence of mycobacterial infection [9-10]. Disruption of the *mtpB* gene severely impairs the ability of the mutant strain to survive in both IFN- γ activated macrophages and in guinea pigs [10]. The function of MtpB remains unclear, although a role in dephosphorylation of protein targets in the IFN- γ pathway has been suggested [10], the biological substrates of MtpB have not yet been elucidated. Therefore, a true understanding of its enzymatic and substrate specificity is essential to dissect the actual biological role of MtpB.

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Bioinformatics analysis of MptpB reveals a large family of related proteins in bacterial and fungal species, including many pathogens. In depth sequence analysis highlights common features to dual-specificity protein phosphatases and surprisingly, a singular active site signature motif, reminiscent of eukaryotic lipid phosphatases. These observations prompted us to further investigate the biochemical and kinetic properties of this enzyme to define its substrate specificity. We present here experimental evidence that MptpB dephosphorylates, phosphotyrosine and phosphoserine/threonine substrates, and importantly, phosphoinositides, thus exhibiting triple-specificity phosphatase activity (TSP). The report of a new, completely unexpected catalytic specificity for MptpB should aid in defining novel ways to address its mechanism of action *in vivo*, its potential role in host phosphoinositide metabolism, and to identify host targets for MptpB. In addition, this catalytic versatility provides new insights for future rational design of specific inhibitors of MptpB.

EXPERIMENTAL PROCEDURES

Bioinformatics analysis

Multiple sequence alignments were done with ClustalX [11], after initial identification of MptpB-related sequences using PSI-BLAST searches of GenBank [12]. Analysis of the active site motif was performed using a ScanProsite search in SwissProt and TrEMBL (<http://www.expasy.org/tools/scanprosite/>).

Cloning and mutagenesis of MptpB

The open reading frame of Rv0153c, encoding MptpB, was amplified from *M. tuberculosis* H37Rv DNA. The oligonucleotide primers, forward (5'-CATATGGCTGTCCGTGAACTGCCGGGCGCG-3') and reverse (5'-CTCGAGTCATCCGAGCAGCACCCCGCGCATC-3') contained *Nde*I and *Xho*I restriction sites and the resulting MptpB coding sequence was cloned into pET28a (Novagen) to generate an N-terminal His₆-tagged expression construct. Site directed mutagenesis of the following residues; D82A, C160S, K164A, D165N and R166A were carried out using the "QuikChange Kit" (Stratagene).

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Over-expression and purification of recombinant proteins

Each construct (wild type and mutants) was transformed into *Escherichia coli* strain BL21 (DE3) and grown in LB broth at 37°C to mid-log phase. Expression was induced at 18°C with 0.5mM IPTG and the cells were harvested by centrifugation 16 hours later. The expressed His₆-MptpB (wt and mutants) was purified by nickel affinity chromatography. The supernatant from the bacterial lysate was loaded onto a 5ml HiTrap column (Amersham Bioscience) in binding buffer (50mM Hepes, 500mM NaCl, pH 7) and the protein was recovered following on-column cleavage of the His₆-tag with thrombin (Sigma) by washing with binding buffer. The recovered fractions were further purified on a MonoQ column using 20mM Tris-HCl pH 8 and eluted with 200mM NaCl in the same buffer. The phosphatase from *Trypanosoma brucei* TbPTP1 has been recently characterised by us as a tyrosine specific phosphatase [13]. We used TbPTP1 in this study as a control. TbPTP1 was amplified from *T. brucei* DNA and cloned into pET28a (Novagen). Recombinant TbPTP1 was expressed in *E. coli* strain BL21 DE3 Codon+ RIPL, grown in LB broth at 37°C and induced at 30°C with 0.4 mM IPTG. Purification of His-tagged TbPTP1 was done by nickel affinity chromatography using the same method as for MptpB.

Phosphatase activity assays

The malachite green assay (Sigma) was used to determine the amount of free phosphate during the dephosphorylation assays with a range of substrates: phospho-Tyr peptides from EGFR (DADEpYLIPQQG) and insulin receptor (TRDIpYETDYRK), phospho-Ser peptide (RRApSVA), phospho-Thr peptide (KRpTIRR) (Alta Bioscience, University of Birmingham, www.altabioscience.bham.ac.uk), phospho-serine, phospho-threonine, phospho-tyrosine (Sigma), IMP, AMP, pNPP (Sigma), diC8-PI(3)P, diC8-PI(3,4)P₂, diC8-PI(3,5)P₂, diC8-PI(4)P, diC8-PI(4,5)P₂, diC8-PI(5)P and diC8-PI(3,4,5)P₃ (Echelon Bioscience). Each reaction was prepared in triplicates in a 96-well microplate, containing 50µl of reaction mix with 5µg enzyme (MptpB wt, mutants and TbPTP1), 0.05-2mM substrate (3-500µM range for phosphoinositides) and buffer (50mM Tris, 50mM BisTris, 100mM sodium acetate, pH 6). The reactions were incubated for 15 minutes at 37°C prior to the addition of 50µl malachite green reagent (Sigma) and further incubated for 10 minutes at room temperature. The absorbance was subsequently read at 620 nm and the mean values calculated. Control reactions

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containing no enzyme were included to measure the background level of phosphate. A phosphate standard curve was produced using known amounts of phosphate (25-3000 pmoles of Sigma phosphate standard solution). Experimental points were interpolated in the standard curve to calculate the amount of phosphate released.

Kinetic analysis

The kinetic parameters V_{\max} and K_m for each substrate were determined using non-linear regression fit of the initial velocity versus substrate concentration curve using Hyper32 (developed by Dr John Easterby, University of Liverpool). The k_{cat} was determined by dividing the V_{\max} by the molar enzyme concentration and standard errors were calculated.

RESULTS AND DISCUSSION

MptpB-related bacterial and fungal phosphatases

A PSI-BLAST search with the full-length MptpB sequence identified orthologues in other mycobacterium species (*M. bovis*, *M. flavescens*, *M. vanbaalenii* and *M.sp.MCS*) and returned more than 150 related sequences in bacteria and fungi, most of which are currently annotated as hypothetical proteins. The multiple sequence alignment highlights the existence of a characteristic active site signature motif CX₅R, present in the protein tyrosine phosphatase (PTP) superfamily, which forms the phosphate-binding loop (P-loop) (Figure 1A). The presence of the CX₅R motif suggests a classification of these sequences as predicted phosphatases. However, the analysis failed to identify landmark motifs conserved only in the tyrosine-specific phosphatases, such as the WPD motif that contains the catalytic aspartic acid, or the Q-loop involved in catalysis [14]. Instead, the active site region of MptpB and related proteins resembles the extended signature motif described for a subfamily of PTPs, the dual-specificity phosphatases (DSPs) that can efficiently dephosphorylate phosphotyrosine and phosphoserine/threonine substrates [15]. The extended signature comprises the P-loop motif and flanking hydrophobic residues (Fig.1A). Consistent with this observation, the overall sequence similarity of MptpB with tyrosine-specific PTPs like human PTP1B is only 6%, whereas similarity with known DSPs is 24% with VHR, 25.8% with MKP3 and 33.5% with IphP. Therefore, although MptpB is annotated as a tyrosine phosphatase in the genome databases, sequence analysis suggest otherwise.

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Furthermore, all bacterial and fungal MptpB related sequences contain in the P-loop two strictly conserved residues: Lys164 and Asp165 (MptpB numbering). Such a feature is unusual in both classical tyrosine-specific PTPs and DSPs. A ScanProsite search using the regular expression pattern H₂CX₂GKDR[T/A] found in the active site signature, returned eukaryotic orthologues of the lipid phosphatases PTEN, TPTE, TPTE2 [16], myotubularins (MTMs), and FYVE-DSP1/2 [17-18]. The alignment of the P-loop motif from these lipid phosphatases underlines the similarities with MptpB in this region (Figure 1B). The conserved Lys is also found in PTEN, and PTEN-related phosphatases and it is important in substrate binding [19-20]. The conserved Asp, found in MTMs and FYVE-DSP1/2 acts as the general acid in catalysis [21-22]. The combination of PTEN and myotubularin features in the active site suggests that MptpB may also share similar enzymatic properties. MTMs, FYVE-DSP1/2 and PTEN display both dual-specificity protein phosphatase and phosphoinositide phosphatase activity [17-19, 23]. To test this we carried out a full investigation of the kinetic properties and substrate specificity of MptpB.

MptpB exhibits dual-specificity protein phosphatase activity

Recombinant MptpB was used for kinetic analyses towards a panel of phosphosubstrates (see methods). The catalytic activity of MptpB towards pNPP and EGFR peptide was optimal at pH 6 both in the absence and presence of DTT (data not shown). MptpB dephosphorylates phosphotyrosine (P-Tyr) and phosphoserine/threonine (P-Ser/P-Thr) substrates, with higher catalytic efficiency towards phosphopeptides (P-peptides) as indicated by higher $k_{\text{cat}}/K_{\text{m}}$ values (Table 1). A higher catalytic rate, k_{cat} is observed for P-Tyr peptide substrates whereas lower K_{m} values are found for P-Ser/P-Thr peptides, indicating enhanced binding affinity (Table 1). Dephosphorylation of P-Tyr peptides is approximately 3.2 times more efficient than dephosphorylation of P-Ser/P-Thr peptides. This is in good agreement with the ratio observed for other DSPs [24], and in contrast with the rates of dephosphorylation for Tyr-specific PTPs commonly up to 10^5 times higher for P-Tyr substrates than for P-Ser/P-Thr substrates [24]. Overall, the catalytic profile obtained from our kinetic analysis shows that MptpB has dual-specificity protein phosphatase activity with a preference for P-Tyr substrates.

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MptpB exhibits lipid phosphatase activity

The similarity between the active site signature from MptpB with those of MTMs and PTEN suggested that MptpB might also display lipid phosphatase activity. To test this the activity of the enzyme was assayed against different phosphoinositides (PIs) (Table 2). MptpB readily dephosphorylated PI(3)P, PI(3,5)P₂, PI(4)P and PI(5)P, with higher efficiency for PI(3)P and PI(3,5)P₂, but showed comparable K_m values for the four PI substrates indicating similar binding affinity. PI substrates exhibited lower K_m values than the P-amino acid or P-peptide substrates tested (Table 1, 2), thus indicating that MptpB has higher affinity for lipid substrates. The k_{cat}/K_m values for PI dephosphorylation are also higher than for peptidic substrates. Interestingly, MptpB appears to have a broad specificity as it dephosphorylates single phosphate substitutions at the D3, D4 and D5 position. However, the enzyme exhibited no activity against PI(3,4,5)P₃, PI(3,4)P₂ or PI(4,5)P₂, showing an apparent selectivity against phosphates at the D4 position of the inositol head when in combination with another phosphate group at D3 or D5. This broad PI activity is in contrast with mammalian lipid phosphatases that exhibit a much more restricted specificity [20, 23].

Mutagenesis of the active site signature residues

Next, we wanted to explore the potential implication of conserved P-loop residues (C160, K164, D165 and R166) in catalysis or substrate binding. As expected, the C160S and R166A mutated enzymes exhibit less than 1% of the WT activity for any of the substrates tested, including pNPP and PIs. This confirms that these residues are essential for enzymatic activity presumably in a Cys-based mechanism of catalysis similar to PTPs and DSPs [15], where the Cys is the nucleophile and the Arg contributes to substrate binding.

Enzymatic assays with the D165N mutant showed that it displays less than 0.1% of WT activity for pNPP or peptide substrates (Figure 1C). Furthermore, the D165N mutant showed < 3% activity towards phosphoinositides (Figure 1D). On the basis of this new experimental evidence we propose Asp165 as a putative catalytic residue for MptpB. Supporting this, the analogous conserved Asp residue in MTMs and FYVE-DSP1/2 (Fig. 1B) was found to be essential for catalysis in MTMR2 [21]. Interestingly, mutation of Asp82 to alanine showed similar activity to the WT enzyme towards all substrates tested (Fig. 1C, D). This residue was previously proposed as the

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general acid in catalysis [25], although experimental evidence has not been reported to date. We have established that Asp82 is not the general acid in catalysis.

The presence of Lys164 in the P-loop is intriguing. Typically, only one basic residue, the conserved Arg, is necessary in the active site to direct binding of the negatively charged phosphosubstrates. Additional basic residues (e.g. Lys residues) are uncommon, and only found in the lipid phosphatases PTEN and TPTE (Fig 1B), which bind substrates with more than one negatively charged phosphate group. To investigate the functional role of Lys164, we mutated it to alanine and tested its activity towards substrates (Table 2, Fig. 1C, D). The activity of K164A is severely impaired towards P-Tyr peptides, with less than 10% of the wild-type activity (Table 2, Fig. 1C), but remains unaltered towards pNPP and P-Ser/P-Thr peptides. Dephosphorylation of lipids is also reduced in a substrate-dependent manner. The largest reduction is observed for PI(3,5)P₂, with a 70% decrease in activity respect to WT, in contrast, there is little effect on dephosphorylation of PI(4)P (Table 2, Fig. 1D). Kinetic analysis with K164A shows that k_{cat}/K_m values decrease by more than one order of magnitude for all PIs with respect to the WT enzyme, but K_m values remain similar. On the other hand, a significant reduction in the catalytic rate is observed for PI(3)P and PI(3,5)P₂ (Table 2). These results indicate a potential role for Lys164 in catalysis, albeit in a substrate-dependent manner. The analogous Lys residue in the lipid phosphatase PTEN, Lys128, is important in lipid binding [20]. Docking of PI(3,5)P₂ to the active site of MptpB suggests that the wide active site could easily accommodate PI(3,5)P₂ (Figure 1E), and that binding might be favoured through interactions of the D5 phosphate with a basic residue like Lys164. In contrast, PI(3,4,5)P₃ would not bind in the active site because the D4-phosphate would clash with the catalytic Asp165 and Lys164 side chains. New insights on the role of this residue should come from the three-dimensional structure of MptpB in complex with a lipid substrate.

The enzymatic activity towards PI(3)P, PI(4)P and PI(5)P indicates that all three monophosphate substrates bind in the active site, although different binding modes or alternative binding orientations of the phosphoinositol head must occur. The lack of activity for PI(3,4)P₂, PI(4,5)P₂ and PI(3,4,5)P₃ could be rationalised by the fact that contiguous biphosphate substitutions (3,4; 4,5; or 3,4,5) would likely result in steric

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clashes with Asp165, while non-contiguous substitutions (3,5), may be favoured by interactions with Lys164 (Fig. 1E).

CONCLUSIONS

In this study we describe the new findings on the triple-specificity phosphatase activity of MptpB, a virulence factor from *M. tuberculosis*. Our kinetic characterisation provides experimental evidence that MptpB exhibits dual-specificity protein phosphatase activity and unexpected phosphoinositide phosphatase activity for a broad range of lipids. This is the first report of PI phosphatase activity for MptpB. Previously, a partial characterisation of MptpB detected tyrosine phosphatase activity [26], although lipid substrates were not tested in that study. The broad specificity towards PIs observed for MptpB could be important for *in vivo* targeting of a wide range of host phosphoinositides or as part of a more sophisticated pleiotropic effect coupling PI turnover with protein mediated cell-signalling events. The different enzymatic activities observed *in vitro* may not reflect true relative activities *in vivo*, where other factors may modulate the physiological function of MptpB. Hence, the biologically relevant substrates of MptpB *in vivo* will still need to be investigated considering both protein and lipid substrates. The molecular factors responsible for controlling PI levels in mycobacterium-infected macrophages have not yet been fully defined, and from the evidence presented in this study MptpB may play an important role. Future investigations will be directed to study the potential targeting of MptpB to membrane phosphoinositides and to define the molecular basis for phosphoinositide binding. The newly identified enzymatic activity of MptpB opens new horizons to test its potential role in host phosphoinositide metabolism and phagosome maturation arrest, and provides exciting new directions for pharmacological intervention.

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Table 1. Kinetic parameters of MptpB towards different phosphorylated substrates (all values are mean \pm S.E.M.)

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$) $\times 10^5$
p-Y aa.	0.291 \pm 0.01	60.00 \pm 1.26	2.06 \pm 0.05
p-S aa.	0.447 \pm 0.08	18.32 \pm 0.93	0.41 \pm 0.04
p-T aa.	0.253 \pm 0.01	12.50 \pm 0.47	0.50 \pm 0.04
p-Y Insulin	0.217 \pm 0.04	159.57 \pm 9.22	7.36 \pm 0.74
p-Y EGFR	0.327 \pm 0.03	158.31 \pm 4.27	4.84 \pm 0.24
p-S peptide	0.078 \pm 0.01	18.08 \pm 0.11	2.32 \pm 0.30
p-T peptide	0.076 \pm 0.01	9.12 \pm 0.11	1.20 \pm 0.34
5'AMP	1.749 \pm 0.07	60.97 \pm 1.55	0.35 \pm 0.01
5'IMP	1.196 \pm 0.05	88.40 \pm 2.38	0.74 \pm 0.02
pNPP	0.170 \pm 0.01	134.70 \pm 4.88	7.91 \pm 0.10

Table 2. Kinetic parameters for MptpB wt and K164A dephosphorylation of phosphoinositides (all values are mean \pm S.E.M.)

Substrate	WT			K164A mutant		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$) $\times 10^5$	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$) $\times 10^5$
PI(3)P	0.018 \pm 0.002	38.5 \pm 0.93	21.48 \pm 1.27	0.055 \pm 0.003	14.6 \pm 0.40	2.64 \pm 1.13
PI(3,5)P	0.028 \pm 0.001	75.4 \pm 1.00	27.23 \pm 0.65	0.022 \pm 0.003	12.7 \pm 0.62	5.79 \pm 0.56
PI(3,4)P	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PI(3,4,5)P	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PI(4)P	0.074 \pm 0.004	100.3 \pm 2.74	13.50 \pm 0.48	0.103 \pm 0.013	82.6 \pm 6.07	8.00 \pm 0.56
PI(4,5)P	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PI(5)P	0.064 \pm 0.004	80.5 \pm 2.13	12.66 \pm 0.48	0.086 \pm 0.016	35.9 \pm 2.81	4.16 \pm 0.70
pNPP	0.170 \pm 0.010	134.7 \pm 4.88	7.91 \pm 0.10	0.087 \pm 0.009	163.9 \pm 4.30	18.8 \pm 0.87

n.a., no detectable activity

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FIGURE LEGENDS

Figure 1.

A. Alignment of a subset of MptpB-related sequences. Alignments prepared with ClustalX. *Mycobacterium tuberculosis* (MYTU0, MptpB), *Mycobacterium bovis* (MYBO0), *Mycobacterium flavescens* (MYCFV), *Mycobacterium vanbaalenii* (MYCVN), *Mycobacterium species* (MYCSS), *Photorhabdus luminescens* (PHLU0), *Erwinia carotovora* (ERWCT), *Bdellovibrio bacteriovorus* (BDEBA), *Clostridium perfringens* (CLOPE), *Lactobacillus plantarum* (LAPL0), *Lactococcus lactis* (LALA0), *Bradyrhizobium japonicum* (BRJA0), *Listeria monocytogenes* (LISMO), *Bacteroides fragilis* (BACFN). **B.** Alignment of P-loop motifs in lipid phosphatases. Indicated are the conserved “AGK” motif found in PTEN and the “DRT” from MTMs (bold), where K and D (italic) are catalytic residues. MptpB has a unique combination of both signatures, with a conserved “AGKDRT” motif (underlined). The DSP IphP from cyanobacteria [27] also contains the conserved “AGKDRT” motif. **C.** The graph shows specific activity (SA) for MptpB wt and mutants towards phosphorylated substrates. Each assay was done in triplicates and standard errors are shown as bars. The activity of the Tyr-specific phosphatase from *T. brucei* (TbPTP1) is shown as a control. **D.** Enzyme activity of MptpB wt and mutants towards PI substrates. **E.** Stick representation of MptpB (purple) [25] superimposed into the active site of MTMR2 (pink) with bound PI(3,5)P2 [22]. Catalytic residues and D82 are labelled and interactions are displayed as dashed lines. Generated with PyMol (DeLano Scientific LLC.).

