# Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase

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**Abstract Phosphorylation of CPI-17 and PHI-1 by the MYPT1-associated kinase (M110 kinase) was investigated. M110 kinase is a recently identified serine/threonine kinase with a catalytic domain that is homologous to that of ZIP kinase (ZIPK. GST-rN-ZIPK, a constitutively active GST fusion fragment, phosphorylates CPI-17 (but not PHI-1) to a stoichiometry of 1.7 mol/mol. Phosphoamino acid analysis revealed phosphorylation of both Ser and Thr residues. Phosphorylation sites in CPI-17 were identified as Thr 38 and Ser 12 using Edman sequencing with $^{32}P$ release and a point mutant of Thr 38.** 

*Key words:* CPI-17; M110 kinase; Myosin phosphatase; Calcium sensitization; Smooth muscle

## 1. Introduction

Contractile activity in smooth muscle is linked to transients in $[Ca^{2+}]_i$ that activate the phosphorylation of the 20 kDa myosin light chain (MLC20) [1,2]. The level of phosphorylated MLC20 is determined by a balance of the activity of two counteracting enzymes: a $Ca^{2+}$-calmodulin-dependent myosin light chain kinase and a myosin light chain phosphatase (SMPP-1M) [3,4]. However, at fixed $Ca^{2+}$ levels contraction also can be induced by agonist stimulation or by activation of G-proteins [5,6]. This leads to so-called $Ca^{2+}$ sensitization [6–8] and is correlated with an inhibition of SMPP-1M activity [5,9]. The inhibition of SMPP-1M following agonist stimulation was proposed to occur via the small GTPase RhoA and one of its downstream targets, Rho kinase [9,10].

Protein phosphatase 1 (PP-1) is one of the major Ser/Thr protein phosphatases in eukaryotic cells, and different forms of PP-1 are composed of a catalytic subunit and different regulatory subunits that target the phosphatase to specific locations and particular substrates [11,12]. The smooth muscle myosin targeted phosphatase, SMPP-1M, is composed of three subunits: the 37 kDa catalytic subunit of PP-1 (PP-1Cδ), a 110–130 kDa regulatory myosin phosphatase targeting subunit (MYPT1) and a 20 kDa subunit of undetermined function [13]. SMPP-1M inhibition occurs in response to phosphorylation of the MYPT1 subunit by Rho kinase [10], or an endogenously associated MYPT1 kinase [14]. A recent report presented the previously unidentified SMPP-1M-associated kinase [15] as a close relative to ZIP kinase (ZIPK). This newly identified M110 kinase was shown to associate with MYPT1 and phosphorylate an inhibitory site on MYPT1. It was suggested that the M110 kinase could act downstream of RhoA and Rho kinase in the $Ca^{2+}$ sensitization mechanism in the smooth muscle and that M110 kinase may be the terminal member of a $Ca^{2+}$ sensitizing kinase cascade.

The myosin phosphatase activity of SMPP-1M also can be regulated by a smooth muscle specific 17 kDa inhibitory phosphoprotein, CPI-17 [16]. Phosphorylation of CPI-17 at Thr 38 by PKC [17] or an unidentified endogenous kinase [16] enhanced the inhibitory potency toward SMPP-1M by about $10^3$. Agonist-induced CPI-17 phosphorylation was demonstrated in intact smooth muscle and about half this response was due to a kinase sensitive to Y-27632, i.e. Rho kinase and/or PKN [18]. Subsequently, CPI-17 was shown to be an in vitro substrate of both Rho kinase [19] and PKN [20]. Here we examined whether CPI-17 is a substrate for the recently identified M110 kinase.

## 2. Materials and methods

### 2.1. Materials

Constitutively active recombinant GST-ZIPK encoding the N-terminal$^{(1-320)}$ portion of ZIPK (GST-rN-ZIPK) was produced from I.M.A.G.E. cDNA clone AI660136 (Genome Systems Inc., MO, USA) [15]. The cDNA clone was in-frame inserted into vector pGEX-4T-1 (Amersham Pharmacia Biotech) in order to express the glutathione *S*-transferase (GST) fusion protein. *Escherichia coli* cells were cultured in LB broth, 50 μg/ml ampicillin, overnight at 37°C. Cells were induced with 100 μM isopropyl-β-D-thiogalactopyranoside, and GST-rN-ZIPK was isolated using glutathione-Sepharose 4B beads according to the manufacturer's instructions. HtCPI-17, HtCPI-17$^{T38A}$, and HtPHI-1 recombinant fusion proteins were prepared and purified as described previously [21]. [γ-$^{32}$P]ATP was obtained from ICN. All other chemicals were of reagent grade.

### 2.2. Protein phosphorylation

Phosphorylation of HtCPI-17 and HtCPI-17$^{T38A}$ proteins (50 μg) by GST-rN-ZIPK (5 μg) was carried out at 23°C in 25 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT) with 0.2 mM [γ-$^{32}$P]ATP. The reactions were initiated by addition of [γ-$^{32}$P]ATP and the stoichiometry of CPI-17 phosphorylation was measured by terminating the kinase reactions with 25% trichloroacetic acid (TCA) after pre-determined time intervals. The reactions were centrifuged for 5 min at 20 000 × *g*; the protein precipitate was washed three times

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with 25% TCA, and the radioactivity incorporated into the precipitated proteins was assessed by Cerenkov counting.

*2.3. Phosphorylation site analysis*

Recombinant CPI-17 proteins were phosphorylated for 3 h at 25°C in a 100 μl volume containing 20 μg recombinant CPI-17, 25 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 0.1 mM DTT, 0.5 mM [γ-$^{32}$P]ATP (250 cpm/nmol) and 2 μg GST-rN-ZIPK. $^{32}$P-labeled CPI-17 samples from in vitro phosphorylation reactions were incubated with 0.5 μg of endoproteinase Lys-C at 37°C for 15 h. The digests were acidified by the addition of trifluoroacetic acid (TFA). Peptides were purified away from contaminating [$^{32}$P]ATP by repetitively passing the sample over a C18 Zip-Tip (Millipore) followed by repetitive washes in 0.1% TFA. Peptides were eluted in a 10 μl of 80% acetonitrile/0.1% TFA. Peptides were immobilized to Immobilon membrane (Millipore) following the manufacturer's instructions. The membrane was placed into a 494 Procise sequencing cartridge (Applied Biosystems). Phosphorylated residues within phosphopeptides were located by determining the cycles in which $^{32}$P was released when samples were subjected to sequential Edman degradation under conditions that optimized recovery of $^{32}$P [22]. The cleaved radioactive protein (CRP) analysis program was used to assign a phosphorylation site to the $^{32}$P released in a specific cycle. The CRP analysis program is available at http://wrpx1.bioch.virginia.edu/crap/.

*2.4. Other procedures*

Phosphoamino acid analysis of TCA-precipitated CPI-17 was performed as described previously [23]. SDS–PAGE was carried out with the discontinuous buffer system of Laemmli [24]. Protein concentrations were determined using the Bradford (Bio-Rad) procedure with bovine serum albumin as a standard.

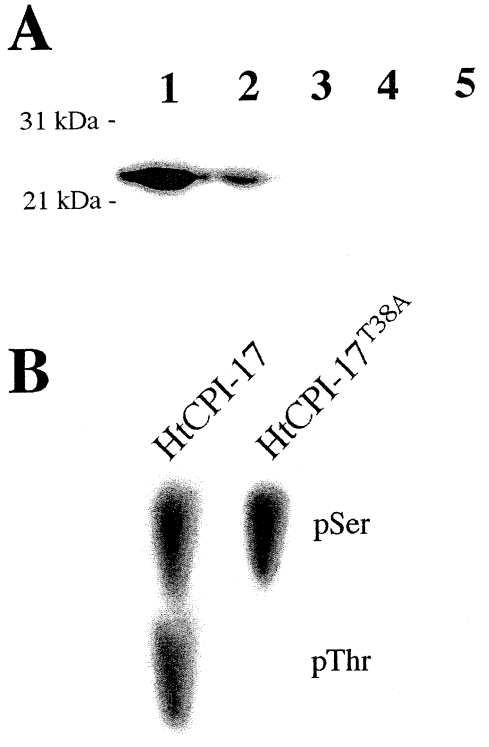



Fig. 1. A: Phosphorylation of CPI-17 and PHI-1 by GST-rN-ZIPK. HtCPI-17 (lane 1), HtCPI-17$^{T38A}$ (lane 2), HtPHI-1 (lane 3), and HtPHI-1$^{T57A}$ (lane 4) were phosphorylated with GST-rN-ZIPK for 15 min. GST-rN-ZIPK was incubated alone as a control (lane 5). Each sample (20 μg) was separated by SDS–PAGE and subjected to autoradiography. B: Phosphoamino acid analysis of HtCPI-17 and HtCPI-17$^{T38A}$ phosphorylated by GST-rN-ZIPK.

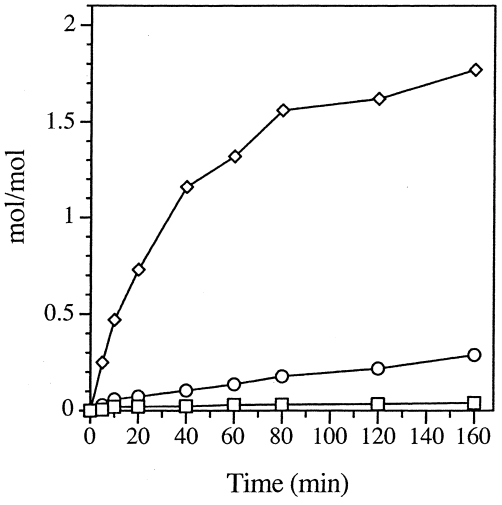



Fig. 2. Stoichiometry of phosphorylation of CPI-17 by GST-rN-ZIPK. Wild-type HtCPI-17 (◇) and HtCPI-17$^{T38A}$ (○) were phosphorylated by GST-rN-ZIPK for the indicated time under the conditions described. GST-rN-ZIPK (□) was also incubated alone as a control.

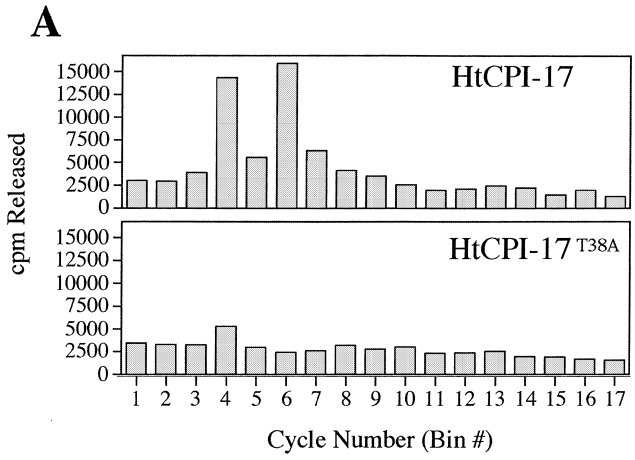
## 3. Results

*3.1. Phosphorylation of CPI-17 by GST-rN-ZIPK*

The phosphorylation of recombinant HtCPI-17 and recombinant HtPHI-1 by GST-rN-ZIPK was examined. As shown in Fig. 1A, HtCPI-17 was a good substrate for GST-rN-ZIPK. PHI-1, a widely expressed analog protein of CPI-17 with nearly identical sequence at the primary site of phosphorylation, was not phosphorylated to any extent by GST-rN-ZIPK. Mutation of the known critical phosphorylation site by substitution of Ala for Thr 38 (HtCPI-17$^{T38A}$) in CPI-17 reduced $^{32}$P incorporation. Mutation of Thr 57 to Ala in PHI-1 had no effect on phosphorylation. The time course and stoichiometry of phosphorylation by GST-rN-ZIPK for HtCPI-17 were examined (Fig. 2). HtCPI-17 was phosphorylated to 1.7 mol phosphate/mol of protein while HtCPI-17$^{T38A}$ was phosphorylated to 0.3 mol of phosphate/mol of protein over a 160 min incubation. Phosphorylation of Thr 38 makes CPI-17 a high potency inhibitor of the myosin phosphatase [16,17], and our results indicate that recombinant GST-rN-ZIPK can efficiently and specifically phosphorylate Thr 38 in CPI-17 in vitro.

*3.2. Identification of Thr 38 and Ser 12 as phosphorylation sites by Edman sequencing*

Phosphoamino acid analysis revealed phosphorylation of both Ser and Thr residues in HtCPI-17 (Fig. 1B). When HtCPI-17$^{T38A}$ phosphorylation was analyzed, there was nearly complete reduction of phospho-Thr while phospho-Ser was still present. Phosphorylation of HtCPI-17 at Thr 38 was confirmed by Edman sequencing with $^{32}$P release (Fig. 3A) and the CRP analysis program (Fig. 3B). Endoproteinase Lys-C digestion of phosphorylated HtCPI-17 followed by solid phase Edman sequencing indicated that the incorporated $^{32}$P was released in the fourth and sixth cycles. CRP analysis of HtCPI-17 revealed that the only residue that could yield the release of $^{32}$P in the fourth cycle was Ser 12. Both Thr 38 and Thr 104 could yield the release of $^{32}$P in the sixth cycle. However, when HtCPI-17$^{T38A}$ was digested with endoproteinase




B

**Histogram of expected "Hot Spots"**

| *Bin #* | *Potential Phosphorylation Sites* | | *# in bin* | *Coverage* |
|---|---|---|---|---|
| 1 | Y041 | S099 | 2 | 11% |
| 2 | | | | |
| 3 | S016 | T101 | 2 | 23% |
| 4 | S012 | | 1 | 29% |
| 5 | S018 | | 1 | 35% |
| 6 | T038 | T104 | 2 | 47% |
| 7 | | | | |
| 8 | S128 | | 1 | 52% |
| 9 | | | | |
| 10 | Y064 | S130 | 2 | 64% |
| 11 | | | | |
| 12 | | | | |
| 13 | S026 | | 1 | 70% |
| 14 | S134 | | 1 | 76% |
| 15 | | | | |
| 16 | S136 | | 1 | 82% |
| 17 | | | | |

Fig. 3. Phosphorylation site analysis of CPI-17. HtCPI-17 and HtCPI-17$^{T38A}$ were phosphorylated by GST-rN-ZIPK. Samples were treated with endoproteinase Lys-C and applied to an Edman sequencer. Radioactivity was collected after each cycle (A) and compared with theoretical results obtained from the CRP analysis program (B). The CRP analysis data include: the Edman sequence cycle number (*Bin #*); any Ser, Thr or Tyr residues present in the protein (*Potential Phosphorylation Sites*); the number of phosphorylatable residues present in a particular Bin# (*# in bin*); and the percentage of phosphorylatable residues included within the number of Edman sequencing cycles (*Coverage*).

Lys-C and subjected to Edman sequencing, $^{32}P$ was only detected in the fourth cycle. CRP analysis of HtCPI-17$^{T38A}$ indicated the phosphorylation of only Ser 12. In addition, CRP analysis and $^{32}P$ cycle data of endoproteinase Lys-C-derived peptides that were further digested with endoproteinase Arg-C produced results (data not shown) which were consistent with phosphorylation of Thr 38 and Ser 12 in HtCPI-17 by GST-rN-ZIPK. The HtCPI-17$^{T38A}$ protein was a poorer substrate for phosphorylation of Ser 12. The amount of $^{32}P$ incorporation into Ser 12 in HtCPI-17$^{T38A}$ was only 25% of that recovered from the wild-type HtCPI-17. This observation may indicate an ordered phosphorylation process in which prior phosphorylation of Thr 38 enhances the phosphorylation of Ser 12.

## 4. Discussion

CPI-17 was identified as a 17 kDa phosphorylation-dependent inhibitory protein of myosin light chain phosphatase [16]. CPI-17 expression was found to be specific to smooth muscle tissues such as aorta and bladder[17], and the protein was implicated in PKC-mediated $Ca^{2+}$ sensitization of smooth muscle contraction [16,25]. Studies have shown that agonist stimulation of intact and permeabilized smooth muscle results in CPI-17 phosphorylation of Thr 38, a site that enhances inhibitory potency 1000-fold [18]. Presumably, this phosphorylation occurs via a G-coupled mechanism via RhoA and its downstream partner Rho kinase, although other kinases including PKN and PKC may be involved. When smooth muscle was stimulated by the addition of agonists in the presence of PKC and Rho kinase inhibitors, the effect varied with the agonist used; thus it was suggested that some combination of signal transduction pathways is in operation to contribute to the SMPP-1M inhibition that is witnessed during $Ca^{2+}$ sensitization. As well, Rho kinase can phosphorylate the MYPT1 subunit of myosin phosphatase in vitro and inhibit SMPP-1M activity [10].

M110 kinase, the previously unidentified SMPP-1M-associated kinase, is a recently identified serine/threonine kinase [15] with a catalytic domain in its N-terminal region that is homologous to that of ZIPK. Associated with SMPP-1M, M110 kinase can phosphorylate the inhibitory site of MYPT1 in intact smooth muscle and, as demonstrated in this study, the potentiating site on CPI-17. Many reports have documented the important role played by RhoA in $Ca^{2+}$ sensitization (review [3]) and its downstream partner Rho kinase has also been implicated [26,27]. One potential problem with Rho kinase being the final link to MYPT1 and CPI-17 is the localization of the active enzyme. It has been demonstrated that $Ca^{2+}$ sensitization of smooth muscle involves translocation of RhoA to the membrane [28] where it presumably targets Rho kinase. The activated Rho kinase at the membrane and its substrates on myofibrils might be linked by M110 kinase. In this hypothesis the M110 kinase would act as a shuttle between Rho kinase and the contractile apparatus.

GST-rN-ZIPK is a constitutively active, recombinant N-terminal fragment of ZIPK that has an identical sequence to the smooth muscle M110 kinase (M. Borman, unpublished data). Because the catalytic characteristics of GST-rN-ZIPK are essentially the same as the purified native M110 kinase [15], we used GST-rN-ZIPK to analyze the phosphorylation of CPI-17 by M110 kinase. Using a point mutant of CPI-17 and the CRP analysis program with data obtained from the sequential Edman degradation under conditions that optimized recovery of $^{32}P$, we were able to identify Thr 38 as the primary and stoichiometric phosphorylation site. This is the previously identified inhibitory site that is phosphorylated by PKC, PKN, Rho kinase and an unidentified endogenous kinase.

We have identified Ser 12 as a novel site of phosphorylation in CPI-17. It has been shown that native CPI-17 is phosphorylated by PKN to a stoichiometry approximating 1.5 mol/mol [20] and recombinant CPI-17 is phosphorylated by both PKN [20] and Rho kinase [19] to a stoichiometry greater than 1.5 mol/mol. These previous results suggested the presence of an additional site of phosphorylation on CPI-17. It is possible, but yet unproven, that the secondary site of phosphorylation by all of these kinases is Ser 12. It is interesting that the second site phosphorylation tends to be less than stoichiometric, and phosphorylation of T38A versions of CPI-17 is much less efficient. Thus, it seems that CPI-17 undergoes ordered or hierarchical phosphorylation at Thr 38 followed by Ser 12. This observation is consistent with a conformational change

upon phosphorylation of Thr 38 that exposes Ser 12 for reaction with kinases.

PHI-1 is a phosphatase holoenzyme inhibitor protein with sequence similarity to CPI-17 and is phosphorylated in both Thr and Ser residues [29]. Phosphorylation of PHI-1 and enhancement of inhibitory potency by PKC have been reported [29] though it is not yet known if the protein is also a substrate for Rho kinase. Interestingly, GST-rN-ZIPK had no reactivity with PHI-1 as a substrate even though the sequence at the phosphorylation site of CPI-17 is RVTVKY compared to KVTVKY for PHI-1. Whether M110 kinase is a smooth muscle specific isozyme remains to be determined; however, it is interesting that the GST-rN-ZIPK specifically phosphorylates a PP-1 inhibitory protein found exclusively in smooth muscle.

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