Phosphorylation of MYPT1 by protein kinase C attenuates interaction with PP1 catalytic subunit and the 20 kDa light chain of myosin

Attila Tóth[a], Enikö Kiss[a], Pál Gergely[a], Michael P. Walsh[b], David J. Hartshorne[c], Ferenc Erdödi[a,*]

[a] *Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Bem tér 18/B, H-4012 Debrecen, Hungary*
[b] *Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alta., Canada T2N 4N1*
[c] *Muscle Biology Group, University of Arizona, Tucson, AZ 85721, USA*

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Abstract The effect of phosphorylation in the N-terminal region of myosin phosphatase target subunit 1 (MYPT1) on the interactions with protein phosphatase 1 catalytic subunit (PP1c) and with phosphorylated 20 kDa myosin light chain (P-MLC20) was studied. Protein kinase C (PKC) phosphorylated threonine-34 (1 mol/mol), the residue preceding the consensus PP1c-binding motif (^{35}KVKF^{38}) in MYPT1$^{1-38}$, but this did not affect binding of the peptide to PP1c. PKC incorporated 2 mol P_i into MYPT1$^{1-296}$ suggesting a second site of phosphorylation within the ankyrin repeats (residues 40–296). This phosphorylation diminished the stimulatory effect of MYPT1$^{1-296}$ on the P-MLC20 phosphatase activity of PP1c. Binding of PP1c or P-MLC20 to phosphorylated MYPT1$^{1-296}$ was also attenuated. It is concluded that phosphorylation of MYPT1 by PKC may therefore result in altered dephosphorylation of myosin.

Key words: Myosin phosphatase; Protein phosphatase 1; Myosin phosphatase target subunit 1; Protein kinase C; Ankyrin repeat

1. Introduction

Phosphorylation of the 20 kDa light chains of smooth muscle myosin (MLC20) has an essential regulatory function in the contractile activity of smooth muscle and motile events in non-muscle cells [1]. The phosphorylation level of myosin is mediated by the activities of MLC20 kinase (MLCK) [2] and myosin phosphatase (MP) [3]. There is renewed interest in the role of MP since it was realized that MP could be regulated. This occurs during Ca^{2+}-sensitization of many smooth muscles in which contraction at low intracellular [Ca^{2+}] reflects an inhibition of MP via a G-protein-linked mechanism [4]. Smooth muscle MP is composed of three subunits: the catalytic subunit of type 1 protein phosphatase (PP1c), the 110/133 kDa myosin binding (targeting) subunit, termed myosin phosphatase target subunit 1 (MYPT1), and a 20 kDa protein [5,6]. MYPT1 appears to be an important component for the regulation of MP and its interactions with proteins and lipids. MYPT1 interacts with PP1c, affecting substrate specificity [7,8], and it binds to myosin [9–11] localizing the enzyme to the contractile proteins. In addition, MYPT1 is phosphorylated on Thr-695 (in the 133 kDa isoform) by Rho-associated kinase and this results in the inhibition of MP activity [12]. Other putative phosphorylation sites in MYPT1 that increase MP activity [13] or affect interaction with phospholipids [14] have also been described.

The N-terminal half of MYPT1 binds to PP1c [7,8,15] and this region of the molecule is also involved in binding to myosin [9]. Residues 35–38 (KVKF) in MYPT1 correspond to a consensus PP1c-binding sequence (K/R-I/V-X-F/W) identified in many PP1c-binding proteins [16]. Binding assays with truncation mutants of MYPT1 established that the presence of the PP1c-binding motif is essential for the interactions between PP1c and MYPT1 [7,15]. Using the surface plasmon resonance (SPR) binding technique it was shown that the interaction of PP1c with the ^{35}KVKF^{38} sequence promoted the binding of other regions of MYPT1, including sequences 1–22, 40–296 (ankyrin repeats) and 304–511 [15]. These data suggested that subunit interactions between PP1c and MYPT1 develop in an ordered and cooperative manner. Interactions with the N-terminal region of MYPT1 also influence the enzymatic properties of PP1c in a substrate-dependent manner. The N-terminal peptide (residues 1–38) and the ankyrin repeats of MYPT1 stimulate the phosphorylated form of MLC20 (P-MLC20) phosphatase activity of PP1c due to an increase of V_{max} and a decrease of K_m, respectively [17]. P-MLC20 bound to truncation mutants of MYPT1 that included the ankyrin repeats in overlay assays suggesting a role for this region in increasing the affinity of MP for the myosin substrate [7]. In contrast, interaction of PP1c with the ankyrin repeats resulted in effective inhibition of its phosphorylase phosphatase activity [8,15].

The interactions of PP1c with the glycogen targeting subunit in skeletal muscle (G_M) [18], the nuclear inhibitor of PP1 (NIPP1) [19] and neurabin [20] are modulated via phosphorylation of Ser/Thr residues located close to or within the PP1c-binding motif. The present study was undertaken to examine whether phosphorylation of the N-terminal region

*Corresponding author. Fax: (36)-52-412566.
E-mail: erdodi@jaguar.dote.hu

Abbreviations: MC-LR, microcystin-LR; MLC20, 20 kDa light chain of smooth muscle myosin; MLCK, myosin light chain kinase; MP, myosin phosphatase; MYPT1, myosin phosphatase target subunit 1; PBS, phosphate-buffered saline; PKA, cAMP-dependent protein kinase; P-MLC20, phosphorylated form of MLC20; PP1, type 1 protein phosphatase; PP1c, catalytic subunit of type 1 protein phosphatase; rPP1c, recombinant PP1c (δ isoform) expressed as a hexahistidine-tagged protein in *Escherichia coli*; SPR, surface plasmon resonance; PKC, protein kinase C

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in MYPT1 influences binding to PP1c and affects PP1c activity with distinct substrates. Our results demonstrate that protein kinase C (PKC) phosphorylates the threonine residue (Thr-34) that precedes the PP1c-binding motif. However, this phosphorylation has no influence on the interaction of the N-terminal MYPT1 fragments with PP1c. PKC also phosphorylates the ankyrin repeats and this results in modification of the phosphatase activity and also reduced binding of P-MLC20 to this region of MYPT1.

2. Materials and methods

2.1. Materials

Chemicals and vendors were as follows: [γ-^{32}P]ATP (ICN Biomedicals), Q-Sepharose Fast Flow and chelate-Sepharose Fast Flow (Pharmacia). Peptides corresponding to the MYPT1 sequences of residues 1–38, 23–38 and 1–34 were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO, USA) coupling an N-terminal cysteine residue to each peptide. All of the MYPT peptides and mutants were based on the sequence of chicken gizzard MYPT1 isoform [6]. Other chemicals were of the highest grade commercially available.

2.2. Protein preparations

Rabbit skeletal muscle [^{32}P]phosphorylase [21], turkey gizzard [^{32}P]MLC20 [22], PKC (a mixture of Ca^{2+}-dependent α, β and γ isoforms) from rat brain [23] and PP1c from rabbit skeletal muscle [15] were prepared as described. Recombinant PP1cδ (rPP1c) was expressed in *Escherichia coli* with a hexahistidine tag (His-tag) [24] and, after renaturation [25], final purification was achieved by chromatography on Ni-agarose. His-tagged MYPT$^{1-296}$ was expressed [7], renatured and purified using the same procedure as for rPP1c [25]. It should be noted that the application of this renaturation process is necessary to obtain recombinant MYPT$^{1-296}$ that stimulates P-MLC20 phosphatase activity of PP1c and that can be phosphorylated by PKC.

2.3. Phosphorylation of MYPT1 peptides

MYPT1 peptides (2–10 μM) were phosphorylated by PKC (2 μg/ml) at 30°C in 20 mM HEPES/NaOH (pH 7.5) containing mixed micelles (0.3 mg/ml L-α-phosphatidyl-L-serine, 0.062 mg/ml 1,2-diolein and 0.03% Triton X-100), 0.2 mM $CaCl_2$, 10 mM DTT, 0.25 mM [γ-^{32}P]ATP (200–500 cpm/pmol) and 10 mM $MgCl_2$ (total volume 100 μl). Aliquots (10 μl) were removed at different intervals, spotted onto P81 phosphocellulose paper and washed three times in 500 ml 1% phosphoric acid, then with acetone. Incorporation of ^{32}P into the peptides was determined by counting the dried P81 papers in a scintillation counter. Alternatively, 10 μl aliquots were added to an equal volume of SDS–PAGE sample buffer, boiled for 5 min and the samples were electrophoresed on 10% (for proteins) or 20% (for peptides) SDS–PAGE mini-gels. The gels were dried and subjected to autoradiography to verify incorporation of $^{32}P_i$ into relevant proteins or peptides.

2.4. Purification of phosphorylated MYPT1 peptides

One volume of 30% acetic acid was added to the peptide phosphorylation medium (100 μl) and the mixture was loaded on a 1 ml Q-Sepharose Fast Flow column equilibrated with 30% acetic acid [27]. The column was eluted with 30% acetic acid and 0.5 ml fractions were collected. The majority of the peptide was eluted in the third fraction as judged by protein assays as well as by radioactivity determination. Chelate-Sepharose Fast Flow (0.5 ml) was charged with 2.5 ml 20 mM $FeCl_3$ and washed sequentially with 4 ml distilled water, 3 ml 0.5 M NH_4HCO_3 (pH 8.0) and 3 ml distilled water followed by equilibration with 50 mM MES (pH 5.5) plus 1 M NaCl. The peptide fraction was loaded on the chelate-Sepharose column, the column was washed with 2.5 ml 50 mM MES (pH 5.5) plus 1 M NaCl, then with 2.5 ml 20 mM MES (pH 6.0) and the phosphorylated peptides were eluted with 2.5 ml 0.5 M NH_4HCO_3 (pH 8.0). The eluted fractions (500 μl each) were dried in a Speed-Vac apparatus and dissolved in 100 μl distilled water. This procedure (drying–dissolving) was repeated three times and the dried sample was redissolved in the appropriate assay buffer.

2.5. Phosphatase assays

Protein phosphatase activity was assayed with 10 μM [^{32}P]phosphorylase or 5 μM [^{32}P]MLC20 at 30°C as described previously [22,26]. Effectors (MYPT1 peptides) were preincubated with PP1c for 10 min at 30°C. The reaction was initiated by the addition of substrate.

2.6. Overlay experiments

For dot blot overlay experiments unphosphorylated or phosphorylated MYPT1$^{1-296}$ was spotted on nitrocellulose membrane, then the membrane was blocked with phosphate-buffered saline (PBS) containing 5% non-fat dried milk plus 0.1% Tween-20. The blots were developed with anti-MYPT1$^{1-38}$ or overlaid with either 5 μM P-MLC20 or 2 μg/ml rPP1cδ in PBS containing 0.1% Tween-20. Bound P-MLC20 or bound PP1c was detected by a rabbit polyclonal antibody raised

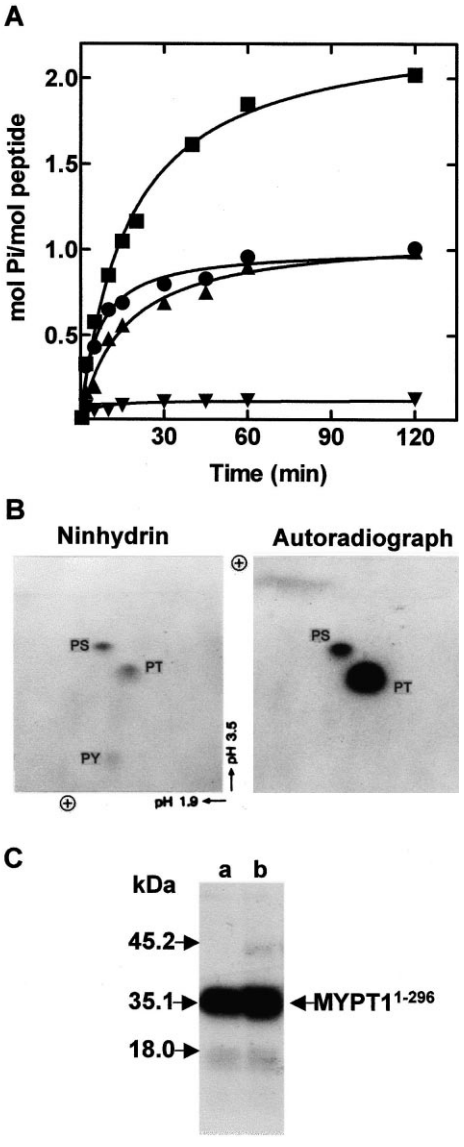

Fig. 1. A: Time course of phosphorylation of N-terminal MYPT1 fragments by PKC. 10 μM of MYPT1$^{1-38}$ (●), MYPT1$^{23-38}$ (▲), MYPT1$^{1-34}$ (▼) and MYPT1$^{1-296}$ (■) were incubated with PKC at 30°C and the extent of phosphorylation was determined as described in Section 2. The data represent the average of two determinations each carried out in duplicate. B: Phosphoamino acid analysis of phosphorylated MYPT1$^{1-38}$: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. C: Autoradiograms of dried SDS gels of MYPT1$^{1-296}$ (2 μM) phosphorylated by PKC for 120 min in the absence (a) or presence (b) of 2 μM rPP1cδ plus 2 μM MC-LR.

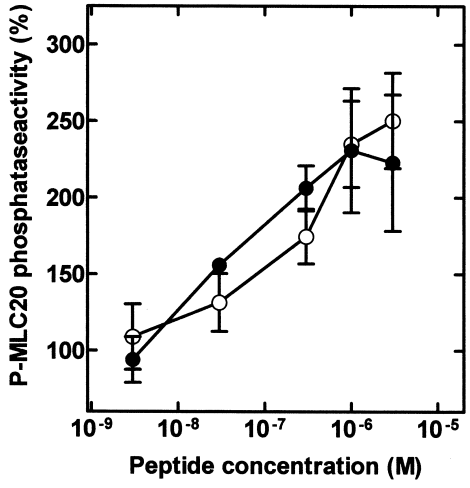

Fig. 2. Effect of non-phosphorylated (○) and phosphorylated (●) MYPT1$^{1-38}$ on the P-MLC20 phosphatase activity of PP1c. Phosphatase activity in the absence of peptides was taken as 100%. Values are mean ± S.E.M. ($n = 4$).

against smooth muscle MLC20 [28] or by a rabbit polyclonal antibody specific for the C-terminal peptide of PP1cδ [26]. Horseradish peroxidase-labeled anti-rabbit secondary antibody and enhanced chemiluminescence were used to detect bound primary antibodies.

2.7. Other procedures

Phosphoamino acid analysis was carried out as described [28]. Protein concentrations were determined with either the BCA (Pierce) or Bradford (BioRad) methods using bovine serum albumin as standard.

3. Results

3.1. Phosphorylation of MYPT1 peptides by PKC

Preliminary experiments suggested that both PKC and the catalytic subunit of cAMP-dependent protein kinase (PKA) could phosphorylate the N-terminal peptides of MYPT1. However, PKC appeared to be more effective and was used in this study. Fig. 1A shows the time course of phosphorylation of MYPT1 peptides by PKC. MYPT1$^{1-38}$ and MYPT1$^{23-38}$ were phosphorylated to 1 mol phosphate/mol peptide. In contrast, the extent of MYPT1$^{1-34}$ phosphorylation (including Ser-20, Thr-22 and Thr-34 as possible phosphorylation sites) was low. Phosphoamino acid analysis identified phosphothreonine in phosphorylated MYPT1$^{1-38}$ (Fig. 1B) and MYPT1$^{23-38}$ (results not shown). These data suggest that the site of phosphorylation is threonine-34, since MYPT1$^{23-38}$ contains only this phosphorylatable residue. The low level of phosphorylation in MYPT1$^{1-34}$ may reflect a requirement of residue(s) C-terminal of Thr-34 for effective substrate recognition by PKC. MYPT1$^{1-296}$ was phosphorylated by PKC to 2 mol/mol (Fig. 1A) suggesting additional phosphorylation site(s) in the ankyrin repeat regions (residues 40–296). The autoradiogram in Fig. 1C demonstrates that MYPT1$^{1-296}$ was phosphorylated to a similar extent in the absence (1.92 mol/mol) or in the presence (2.05 mol/mol) of equimolar rPP1cδ plus microcystin-LR (MC-LR). Initially, MC-LR was added to ensure that dephosphorylation of MYPT1$^{1-296}$ by PP1c did not occur. However, it was later found that dephosphorylation of phosphorylated MYPT1$^{1-38}$ and MYPT1$^{1-296}$ was not detected over a time course of 120 min at 30°C. These data suggested that PP1c was not effective with the phosphorylated peptides and thus the interactions of the peptides with PP1c could be studied in the absence of phosphatase inhibitors.

3.2. Effect of phosphorylation of the N-terminal MYPT1 peptide on its interaction with PP1c

Thr-34 precedes the consensus PP1c-binding motif of MYPT1. Phosphorylation of this residue therefore might affect interaction with PP1c. It was shown previously that MYPT1$^{1-38}$ activated P-MLC20 phosphatase activity of PP1c [8,16], whereas MYPT1$^{23-38}$ or MYPT1$^{1-34}$ had no effect [15]. These data suggested that binding of the ^{35}KVKF^{38} motif initiated interaction of PP1c with residues 1–22 and the latter resulted in the activation of P-MLC20 activity. Non-phosphorylated or phosphorylated MYPT1$^{1-38}$ stimulated the myosin light chain phosphatase activity of PP1c to a similar extent (Fig. 2). These data support the conclusion that phosphorylation of Thr-34 in the N-terminal peptide of MYPT1 does not affect interaction with PP1c.

3.3. Effect of phosphorylation of the ankyrin repeats in MYPT1 on interactions with PP1c and P-MLC20

The N-terminal region of MYPT1 (residues 1–296) has opposite effects on the activity of PP1c with phosphorylase or P-MLC20 as substrates [7,8,15]. The N-terminal peptides (MYPT1$^{1-38}$, MYPT1$^{1-34}$ or MYPT1$^{23-38}$) had no effect, whereas MYPT1$^{1-296}$ inhibited the phosphorylase phosphatase activity of PP1c [15]. In contrast, both the N-terminal peptide (residues 1–38) and the ankyrin repeats (residues 40–296) contributed to the stimulatory effect of MYPT1 (or N-terminal MYPT1 mutants) on the P-MLC20 phosphatase activity of PP1c [17]. The effects of non-phosphorylated and phosphorylated MYPT1$^{1-296}$ on the phosphorylase phosphatase and P-MLC20 phosphatase activities of PP1c were examined. Fig. 3 indicates that phosphorylated MYPT1$^{1-296}$ inhibited the phosphorylase phosphatase activity of PP1c less potently compared to the non-phosphorylated

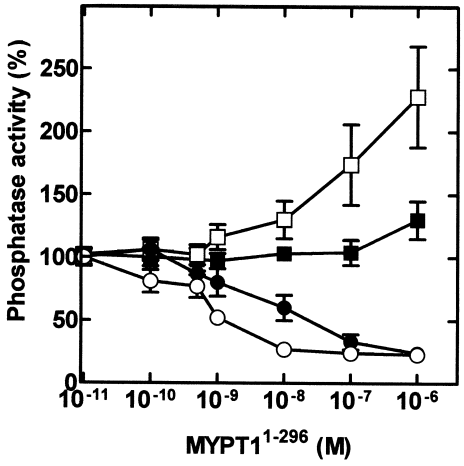

Fig. 3. Phosphorylation of the ankyrin repeats by PKC alters the influence of MYPT1$^{1-296}$ on the phosphorylase phosphatase and P-MLC20 phosphatase activities of PP1c. Phosphorylase phosphatase (○, ●) and P-MLC20 phosphatase (□, ■) activities of PP1c were determined in the presence of different concentrations of non-phosphorylated (○, □) or phosphorylated (●, ■) MYPT1$^{1-296}$. Phosphatase activity in the absence of MYPT1$^{1-296}$ was taken as 100%. Values are mean ± S.E.M. ($n = 3–5$).

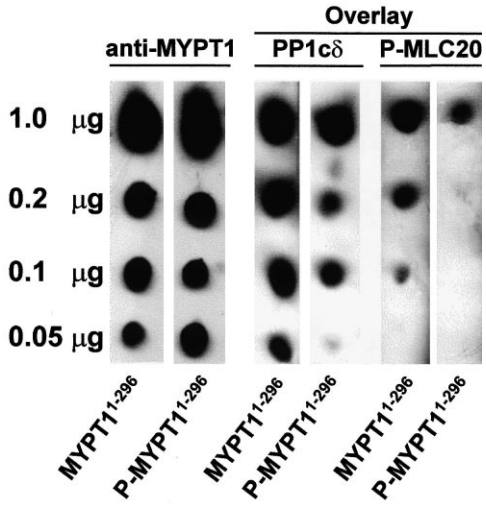

Fig. 4. Phosphorylation of MYPT1$^{1-296}$ affects its binding to PP1c and P-MLC20 as judged by overlay assays. Phosphorylated (P-MYPT1$^{1-296}$) or non-phosphorylated MYPT1$^{1-296}$ (0.05 to 1 μg) was applied to nitrocellulose membrane. After blocking the membrane, the blots were probed with anti-MYPT1 antibody or overlaid with rPP1cδ or P-MLC20 followed by detection of the bound proteins with anti-PP1c or anti-MLC20.

peptide. Non-phosphorylated MYPT1$^{1-296}$ stimulated dephosphorylation of P-MLC20 by PP1c in a concentration range from 0.01 to 1 μM. This stimulatory effect was abolished following phosphorylation of MYPT1$^{1-296}$ by PKC.

To examine whether these effects reflected altered binding of MYPT1$^{1-296}$ to PP1c and/or P-MLC20 upon phosphorylation, we carried out dot blot overlay assays. Non-phosphorylated or phosphorylated MYPT1$^{1-296}$ was bound to nitrocellulose membrane at different concentrations (Fig. 4). The blots were developed with anti-MYPT1 antibody specific for the N-terminal region, or overlaid with PP1c or P-MLC20. Binding of the latter proteins to MYPT1$^{1-296}$ was detected by anti-PP1c and anti-MLC20 antibodies. Overlay with rPP1cδ revealed that less PP1c bound to phosphorylated than to non-phosphorylated MYPT1$^{1-296}$. A more apparent difference was observed in the binding of P-MLC20. With non-phosphorylated MYPT1$^{1-296}$, binding of P-MLC20 was detected at 0.1 μg (MYPT fragment), whereas with phosphorylated MYPT1$^{1-296}$, binding was apparent only at a 10-fold higher concentration. The blot developed using anti-MYPT1 established that similar amounts of non-phosphorylated or phosphorylated MYPT1$^{1-296}$ were bound to the membrane, thus the above results probably reflect differences in the binding of PP1c or P-MLC20 as a result of phosphorylation of MYPT1$^{1-296}$.

4. Discussion

Our present data indicate that PKC phosphorylates MYPT1$^{1-296}$ at Thr-34 and within the ankyrin repeat region (residues 40–296), although it remains to be established that phosphorylation occurs at these sites in the phosphatase holoenzyme. Thr-34 is located immediately N-terminal to the PP1c-binding motif in the MYPT1 sequence and it was of particular interest to assess how phosphorylation of this residue might affect the interaction of the N-terminal peptide with PP1c. Recent binding studies using SPR [15] proved that in MYPT1$^{1-38}$ two regions bind to PP1c: the PP1c-binding motif (KVKF) corresponding to residues 35–38 plus an N-terminal sequence located in residues 1–22. Binding of this latter sequence (residues 1–22) activates the P-MLC20 phosphatase activity of PP1c. Kinetic assays with P-MLC20 as substrate suggested that phosphorylated and non-phosphorylated MYPT1$^{1-38}$ did not differ in their abilities to bind PP1c or to stimulate P-MLC20 phosphatase activity of PP1c (see Fig. 2). These data imply that phosphorylation of Thr-34 had no influence on the interaction with PP1c of either the binding motif or the binding sequence in residues 1–22. It remains possible, however, that phosphorylation of Thr-34 in intact MYPT1 may affect its interaction with PP1c.

Our results are in contrast to findings regarding the effect of phosphorylation on the interaction of other targeting subunits with PP1c. It was shown that phosphorylation of Ser-67 by PKA within the binding motif of G_M [8,16,18] inhibits its interaction with PP1c. Similar effects were observed when NIPP1 [19] or neurabin [20] were phosphorylated by PKA on residues preceding or following the binding motif. Interestingly, the glycogen binding subunit of rat liver also includes a Ser residue within the PP1c-binding motif, however, this is not phosphorylated by PKA [29]. In addition, phosphorylation of Ser-67 in G_M complexed with PP1c was not observed suggesting that subunit interactions may also affect the availability of Ser-67 for phosphorylation [8,16]. MYPT1$^{1-296}$ was also shown to interact with PP1c and both phosphorylatable regions (residues 1–38 and 40–296) are involved in this interaction [15,17]. However, the interaction with PP1c did not appear to influence the phosphorylation of either region by PKC (see Fig. 1) suggesting that differences exist between the phosphorylation characteristics of MYPT1 and the other PP1c-targeting molecules. It appears that in the PP1c-targeting proteins the binding motifs represent a general platform for interaction with PP1c, whereas individual binding profiles are reflected by differences in the residues flanking the motif and also in the effects of their phosphorylation.

The ankyrin repeats in MYPT1 represent an important 'secondary' binding site(s) for PP1c. Interaction with PP1c requires the binding motif and those mutants containing the ankyrin repeats but lacking the motif did not bind to PP1c [7,15,17]. However, the ankyrin repeat region exhibited facilitated binding to PP1c [15] in the presence of the N-terminal sequences (residues 23–28 or 1–38). Within the ankyrin repeats the C-terminal half was effective in increasing inhibition of phosphorylase phosphatase activity and also this region was thought to be involved in binding to P-MLC20 [7,8]. The site(s) of phosphorylation by PKC in the ankyrin repeats was not determined, but a search for PKC consensus phosphorylation sites predicted Thr-212 and/or Ser-292 as potential targets and these are located in the C-terminal half of the ankyrin repeats. This is consistent with the above observations that phosphorylation of MYPT1$^{1-296}$ altered its influence on PP1c activities with phosphorylase and P-MLC20 as substrates. Overlay assays indicated that phosphorylation also decreased the binding of MYPT1$^{1-296}$ to PP1c and this may underlie the observed enzymatic differences. In addition, overlay assays detected a dramatic decrease in the binding of P-MLC20 to phosphorylated MYPT1$^{1-296}$ and this could also contribute to the decreased dephosphorylation of this substrate. These data suggest that phosphorylation of the ankyrin repeats (if it occurs in vivo) might decrease the affinity of MP

toward myosin modifying the access of phosphorylated Ser-19 on MLC20 to the catalytic site of PP1c. Phosphorylation at both locations (i.e. Thr-34 and within the ankyrin repeats) might be required for inhibition of the stimulatory effect of $MYPT1^{1-296}$ on P-MLC20 phosphatase activity of PP1c. In this regard PKC catalyzed phosphorylation of MYPT1 could be involved in Ca^{2+}-sensitization of smooth muscle contraction [30], although exogenous PKC was shown to have no stimulatory effect on contraction of demembranated smooth muscle [30,31], whereas PKC activators triggered Ca^{2+}-sensitization of contraction of α-toxin permeabilized smooth muscle [30]. CPI-17, a 17 kDa cytosolic protein that becomes a potent inhibitor of PP1 when phosphorylated by PKC [32], is retained in α-toxin permeabilized, but not in demembranated, smooth muscle [30]. Nevertheless, direct phosphorylation of MYPT1 by PKC might also decrease activity of MP toward myosin and contribute to the above effect. It is also possible that this event is utilized in other cellular functions, not necessarily involved directly in the contractile mechanism.

In summary, we report here that phosphorylation of the N-terminal region in MYPT1 can be catalyzed by classical PKC isoenzymes. Phosphorylation of Thr-34 preceding the PP1c-binding motif in MYPT1 had no influence on the interaction with PP1c and, in light of opposite findings with other PP1c-binding proteins this was an unexpected result. In contrast, phosphorylation of the ankyrin repeats (in addition to Thr-34) reduced binding of both PP1c and P-MLC20 to the N-terminal MYPT1 fragment (residues 1–296) suggesting that this mechanism might be involved in modulation of myosin dephosphorylation by PKC or other yet unidentified protein kinases.

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