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Myotonic dystrophy protein kinase phosphorylates the myosin phosphatase targeting subunit and inhibits myosin phosphatase activity

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Abstract Myotonic dystrophy protein kinase (DMPK) and Rho-kinase are related. An important function of Rho-kinase is to phosphorylate the myosin-binding subunit of myosin phosphatase (MYPT1) and inhibit phosphatase activity. Experiments were carried out to determine if DMPK could function similarly. MYPT1 was phosphorylated by DMPK. The phosphorylation site(s) was in the C-terminal part of the molecule. DMPK was not inhibited by the Rho-kinase inhibitors, Y-27632 and HA-1077. Several approaches were taken to determine that a major site of phosphorylation was T654. Phosphorylation at T654 inhibited phosphatase activity. Thus both DMPK and Rho-kinase may regulate myosin II phosphorylation.

Key words: Myosin target subunit; Myosin phosphatase; Myotonic dystrophy protein kinase; Rho-kinase

1. Introduction

Myotonic dystrophy protein kinase (DMPK) is the protein encoded by the *DM-1* locus that is mutated in myotonic dystrophy [1,2]. The functional and structural characteristics of DMPK [3] are related to a group of serine-threonine protein kinases designated as the 'myotonic dystrophy family of protein kinases' (MDFPK) [4]. This group includes *Neurospora* Cot1, *Drosophila* Wts, rat ROKα, human $p160^{ROCK}$, human PK418 [4], *Caenorhabditis elegans* LET-502, *Drosophila* Genghis Khan, murine Citron Rho-interacting kinase (CRIK) and rat DM kinase-related Cdc42-binding kinase (MRCK) [5]. Several MDFPK kinases interact with members of the Rho family of small GTPases. The Rho family includes RhoA, Rac-1 and Cdc42; this family is functionally and structurally related to the Ras superfamily of regulatory GTPases [6]. We have recently shown that DMPK binds preferentially to Rac-1 of the Rho family and their coexpression results uniquely in GTP-sensitive activation of DMPK ([5]; see also [7]).

Myosin phosphatase (MP) is composed of three subunits [8]; the catalytic subunit, protein phosphatase type 1, delta isoform (PP1cδ); and two non-catalytic subunits, of 18–21 kDa and approximately 100 kDa. The function of the smaller subunit is not known but the larger subunit is thought to act as a targeting subunit and bind PP1cδ and myosin [8]. It is termed MP target subunit, MYPT. There are two human genes for MYPT [9]. MYPT1 is widely distributed and found in smooth muscle and most non-muscle cells [8], whereas MYPT2 is found in brain, skeletal and cardiac muscle [9]. In addition to a targeting role, MYPT1 also regulates the activity of PP1cδ. Phosphorylation of MYPT1 by an endogenous kinase in MP holoenzyme preparations [10] or by Rho-associated protein kinase (Rho-kinase) ([11,12] also termed ROKα and β, ROCK I and II and $p160^{ROCK}$ [13]) inhibits phosphatase activity. A consequence of MP inhibition is to increase the level of myosin phosphorylation and this leads to Ca^{2+} sensitization in smooth muscle [8] and cytoskeletal rearrangements (increase in stress fibers and focal adhesions) in non-muscle cells [13]. The 'inhibitory' site of phosphorylation for both the endogenous kinase [10] and Rho-kinase [12] is T654 (based on the sequence of the chick M130 isoform, or T695 for the M133 isoform [14]).

The overexpression of DMPK in cultured lens epithelial cells induced marked rearrangements of the actin cytoskeleton and plasma membranes [7]. These observations and the similarity of the sequences of the catalytic domains of DMPK and Rho-kinase [14] suggested that the two kinases may operate via a common pathway. One possibility being the phosphorylation of MYPT1 and the inhibition of MP activity. In this communication it is shown that DMPK phosphorylates MYPT1 in vitro at a site also phosphorylated by Rho-kinase and that this phosphorylation results in inhibition of PP1cδ activity.

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Abbreviations: DMPK, myotonic dystrophy protein kinase; MDFPK, myotonic dystrophy family of protein kinases; MP, myosin phosphatase; MYPT, myosin phosphatase target subunit; PP1cδ, delta isoform of the catalytic subunit of protein phosphatase type 1; Rho-kinase, Rho-associated protein kinase (ROK, ROCK, $p160^{ROCK}$); GST, glutathione *S*-transferase

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2. Materials and methods

2.1. Materials

Chemicals and vendors were as follows: [γ-^{32}P]ATP (NEN Life Science Products Inc.); ATP (Sigma); ATPγS (Calbiochem); HA-1077, microcystin-LR (LC Laboratories); Rho-kinase (ROKα/ROCK II), catalytic subunit of cAMP-dependent protein kinase (PKA), Y-27632 (Upstate); Talon metal affinity resin (Clontech); glutathione Sepharose 4B (Amersham Pharmacia Biotech); nitrocellulose membrane (0.2 μm, Bio-Rad). All other chemicals and reagents were of the highest grade commercially available.

2.2. Protein preparations

A catalytically active fragment of DMPK (residues 1–540) was expressed in Sf9 cells as an N-terminal polyhistidine fusion protein and purified as described [3,5]. This construct contained the N-terminal leucine-rich repeat, the catalytic domain and the α-helical coiled-coil leucine zipper at its C-terminus. The full-length MYPT1 (M133 chicken isoform [8]) was expressed in *Escherichia coli* BL-21 as a glutathione *S*-transferase (GST) fusion protein [15] and termed GST-MYPT1. The N-terminal fragment of MYPT1 (residues 1–633) was expressed in *E. coli* M15 as a hexahistidine-tagged protein [16]. The C-terminal fragment of MYPT1 (residues 514–963; M130 chicken isoform [8] and termed C130$^{514-963}$) was prepared by the following procedure. The cDNA for C130$^{514-963}$ was obtained by PCR amplification directly from a chicken gizzard Uni-Zap cDNA library [17] with *Taq* DNA polymerase. The forward primer was 5′-AGAAGTGGATCCTTTGGTAGA-3′ (underlined residues show the *Bam*HI site), the reverse primer was 5′-TTCTCGTCGACTTCAGATATCCTT-3′ (underlined residues show the *Sal*I site). The PCR product was digested with *Bam*HI and *Sal*I and ligated into pQE30 vector (Qiagen). The recombinant hexahistidine-tagged C-terminal fragment of MYPT1 was produced in the M15 strain of *E. coli* as described [16]. The T654 to A mutation (T654A) was created in the pQE30 C130$^{514-963}$ construct with Transformer TM site-directed mutagenesis kit (Clontech) using 5′-TCCAGAAGATCTGCGCAGGGTGTAACA-3′ (underline indicates the mutated codon for the residue 654). The mutant (termed C130$^{514-963}$ T654A) was selected by the concomitant conversion of the unique *Alf*III site to a *Bgl*II site and resistance to digestion with *Alf*III. The recombinant protein was expressed in *E. coli* JM109 and purified as described [16].

The polyclonal site- and phosphorylation-specific antibody was raised in rabbits and affinity-purified as described [12]. Other protein preparations were as follows: PP1c from rabbit skeletal muscle [18]; the 20 kDa myosin light chain (MLC20) from turkey gizzard myosin [19] and the ^{32}P-labeled MLC20 [20].

2.3. Phosphorylation assays

Phosphorylation of MYPT1 fragments (100 μg/ml) by either DMPK (see figure legends) or Rho-kinase (see figure legends) was carried out in 20 mM Tris–HCl (pH 7.5), 30 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol (DTT); 1 μM microcystin-LR, and 0.1 mM [γ-^{32}P]ATP (150–500 cpm/pmol). Total assay volumes ranged from 150 to 200 μl. Aliquots (20 μl) were removed at different intervals, spotted onto P81 Whatman phosphocellulose paper and washed three times in 300 ml 0.5% phosphoric acid, then with acetone. Incorporation of ^{32}P into the substrates was determined by counting the dried P81 papers in a scintillation counter. Alternatively, 20 μl aliquots were added to an equal volume of SDS–PAGE sample buffer, boiled for 5 min and the samples were electrophoresed on 10% SDS–PAGE mini-gels. The gels were stained, dried and subjected to autoradiography or blotted onto nitrocellulose membrane. To determine the effects of phosphorylation on phosphatase activity, phosphorylation of the full-length MYPT1 mutant was carried out under similar conditions without microcystin-LR and using ATPγS instead of ATP. The reactions were diluted (3–200-fold) with a buffer containing 20 mM Tris–HCl (pH 7.5), 30 mM KCl, 0.2 mM DTT, 0.4 mg/ml bovine serum albumin and assayed for phosphatase activity.

2.4. Phosphatase assays

Phosphatase assays were carried out at 30°C using ^{32}P-labeled MLC20 (5 μM) as substrate. The assay mixtures (30 μl total volume) contained approximately 3 nM PP1c prepared from rabbit skeletal muscle and various concentrations of phosphorylated MYPT1 in 20 mM Tris–HCl (pH 7.5), 30 mM KCl, 0.2 mM DTT, 0.4 mg/ml bovine serum albumin. Reactions were started after a 5 min incubation at 30°C by the addition of substrate and terminated by the addition of bovine serum albumin to 6 mg/ml and trichloroacetic acid to 10%. The precipitated proteins were sedimented at 15 000 × *g* for 2 min and the radioactivity of the supernatant was determined by Cerenkov counting.

2.5. Other procedures

SDS–PAGE was performed using the conditions of Laemmli [21] on Bio-Rad mini-gels of 10% polyacrylamide. Proteins from mini-gels were transferred to nitrocellulose for 60 min at 250 mA. Non-fat dried milk in TBS containing 0.05% Tween 20 was used for blocking the membrane (5%) and as a carrier (1%). After blocking, the membrane was washed and exposed to the primary antibody for 60 min at room temperature. For detection of primary antibody, peroxidase-coupled secondary antibody was used with enhanced chemiluminescence reagent (Pierce). Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) methods using bovine serum albumin as standard.

3. Results

3.1. Phosphorylation of MYPT1 by DMPK

The initial experiment was to determine if MYPT1 and its fragments are phosphorylated by DMPK. As shown in Fig. 1A the full-length MYPT1 and the C-terminal fragment of

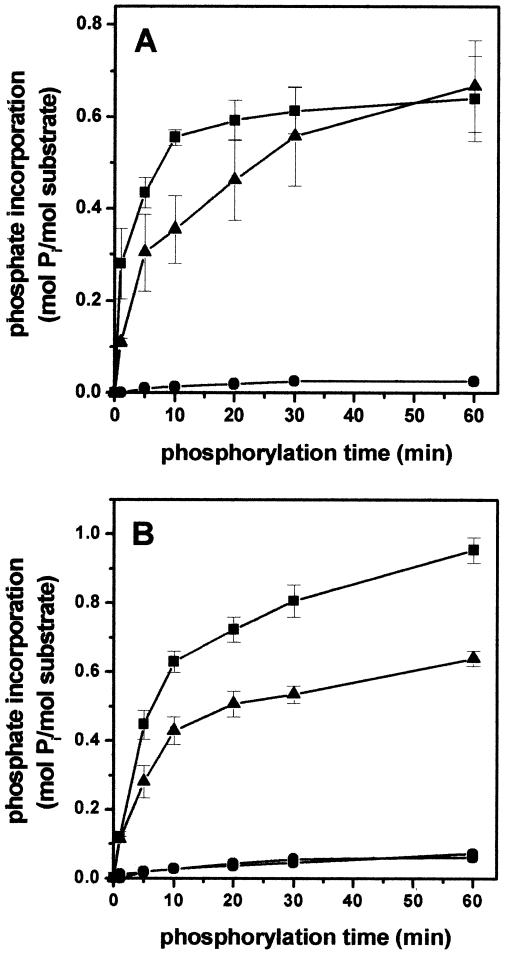

Fig. 1. Phosphorylation of MYPT1 and its fragments by DMPK (A) and by Rho-kinase (B). Substrates are each at 100 μg/ml. GST-MYPT1 (▲), N-terminal fragment, residues 1–633 (●); C-terminal fragment, C130$^{514-963}$ (■). DMPK, 10 μg/ml; Rho-kinase, 50 mU/ml. For other conditions see Section 2. Values are mean ± S.E.M. (n = 3–5).

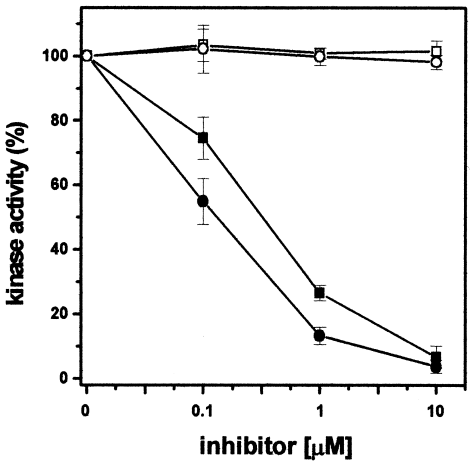

Fig. 2. Effect of Rho-kinase inhibitors, Y-27632 (○, ●) and HA-1077 (□, ■) on activities of DMPK and Rho-kinase. Conditions: DMPK, 1.5 μg/40 μl (○, □); Rho-kinase, 5 mU/40 μl (●, ■); substrate $C130^{514-963}$, 4 μg/40 μl. Other conditions see Section 2. Values are mean ± S.E.M. ($n = 3$).

MYPT1, $C130^{514-963}$, are phosphorylated by DMPK. The final stoichiometry of phosphorylation was about 0.6 mol P/mol substrate. In contrast, the N-terminal fragment of MYPT1 containing residues 1–633 was not phosphorylated by DMPK. Similar phosphorylation profiles were obtained for Rho-kinase and MYPT1 and its mutants (Fig. 1B). The level of phosphorylation of $C130^{514-963}$ by Rho-kinase was higher than for DMPK and approached 1 mol P/mol substrate. Again, the extent of phosphorylation of the N-terminal MYPT1 fragment by Rho-kinase was considerably reduced. These results indicate that MYPT1 is a substrate for DMPK and the major phosphorylation site(s) is located in the C-terminal part of the molecule, i.e. similar to the phosphorylation of MYPT1 by Rho-kinase.

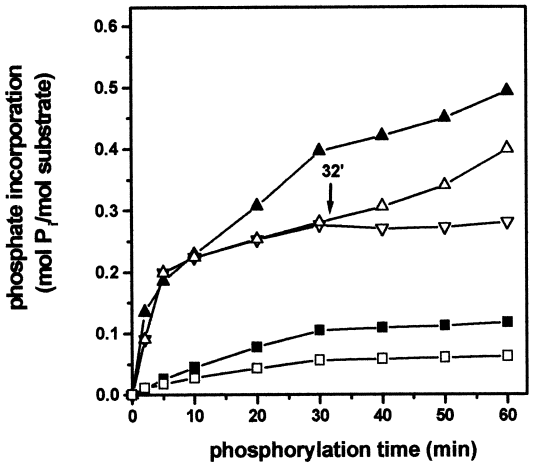

Fig. 3. Phosphorylation of GST-MYPT1 and $C130^{514-963}$ T654A by DMPK and Rho-kinase. GST-MYPT1 and DMPK, 1 μg/200 μl (▲). Sequential phosphorylation of GST-MYPT1 by Rho-kinase and DMPK. Initial phosphorylation by Rho-kinase, 5 mU/200 μl. At 32 min (arrow) either Y-27632, 12.7 μM, was added (▽) or Y-27632 plus DMPK, 1 μg/200 μl (△). Phosphorylation of $C130^{514-963}$ T654A by DMPK (■) or Rho-kinase (□). Other conditions see Section 2.

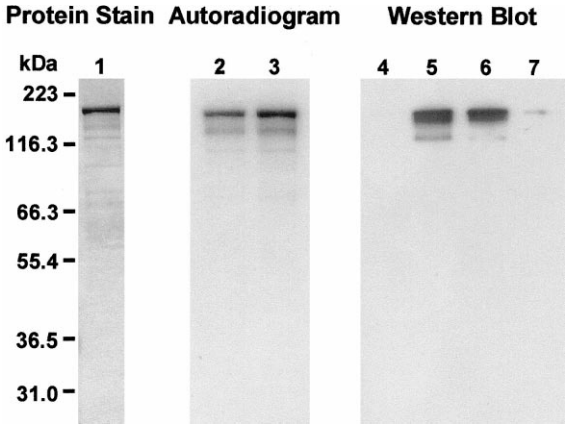

Fig. 4. Protein stain, autoradiograms and Western blots of GST-MYPT1 after phosphorylation for 1 h by DMPK, Rho-kinase, and PKA. Lane 1, Coomassie protein stain, molecular weight markers indicated. Autoradiograms after phosphorylation with [γ-^{32}P]ATP by Rho-kinase, lane 2, and DMPK, lane 3. Western blots using site- and phosphorylation-specific antibody after thiophosphorylation with ATPγS by Rho-kinase, lane 5, DMPK, lane 6, and PKA, lane 7. Unphosphorylated GST-MYPT1, lane 4. Approximately 2 μg protein was loaded per lane. Other conditions see Section 2.

Rho-kinase can phosphorylate the regulatory light chain of smooth muscle myosin [22] and because of the similarity between Rho-kinase and DMPK it was important to screen MLC20 as a substrate for DMPK. It was found that DMPK does not phosphorylate smooth muscle MLC20 (data not shown). It was shown previously [23] that a DMPK construct does not phosphorylate the light chain of skeletal muscle myosin.

3.2. Effect of Rho-kinase inhibitors on DMPK activity

Two widely used inhibitors of Rho-kinase are Y-27632 and HA-1077 [24] although these are not specific for Rho-kinase [25]. Because of the sequence similarity in the catalytic domains of DMPK and Rho-kinase the effect of these inhibitors on DMPK activity was assayed. The results shown in Fig. 2 indicate that DMPK is not inhibited by either Y-27632 or HA-1077 at concentrations up to 10 μM. Even at higher concentrations of both inhibitors (100 μM) DMPK activity was reduced by only approximately 10% (data not shown). In contrast, Rho-kinase is effectively inhibited by these compounds. Reported K_i values for Y-27632 and HA-1077 with Rho-kinase are 0.14 and 0.33 μM, respectively [24]. These results were obtained with $C130^{514-963}$, but full-length MYPT1 yielded similar results (data not shown).

3.3. Site(s) of phosphorylation on MYPT1 for DMPK

It is established [12,22] that the sites of phosphorylation for Rho-kinase on MYPT1 are the inhibitory site, T654 (T695 in the longer chicken M133 isoform) and T809 (T850 in M133). Various procedures were used to determine if the inhibitory site in MYPT1 was also phosphorylated by DMPK.

Fig. 3 shows the effect of DMPK following phosphorylation of MYPT1 with Rho-kinase. Phosphorylation with full-length GST-MYPT1 was initiated by Rho-kinase and at 32 min either Y-27632 (12.7 μM) or Y-27632 plus DMPK were added. The increase in phosphorylation by DMPK following inhibition of Rho-kinase paralleled the phosphorylation profile obtained with DMPK alone. This is consistent with phos-

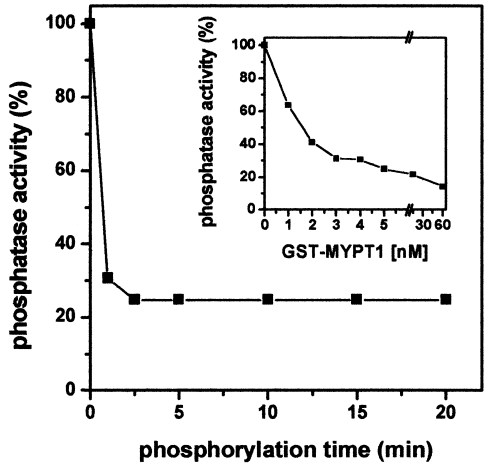

Fig. 5. Inhibition of PP1c activity following phosphorylation of GST-MYPT1 by DMPK. The samples obtained in Fig. 1 were used, diluted 125-fold (to 5 nM) and used in phosphatase assays with PP1c and [^{32}P]MLC20 (see Section 2). Inset, the 20 min time point (from Fig. 1) was used at different dilutions in phosphatase assays. For these assays PP1c was approximately 3 nM.

phorylation of the same site by Rho-kinase and DMPK. To determine whether T654 was a major site the T654A mutant (C130$^{514-965}$ T654A, see Section 2) was used. As shown in Fig. 3 the phosphorylation of the T654A mutant by both DMPK and Rho-kinase was considerably reduced. To confirm that T654 was a major site of phosphorylation by DMPK, the full-length GST-MYPT1 was phosphorylated using [^{32}P]ATP and ATPγS (Fig. 4), applied to SDS–PAGE and transferred to nitrocellulose membrane. The protein staining pattern (Coomassie blue) is shown in Fig. 4, lane 1, and the major band for GST-MYPT1 runs at approximately 160 kDa. Proteolysis products of GST-MYPT1 are also visible. The autoradiograms following phosphorylation by Rho-kinase and DMPK are shown in lanes 2 and 3 and align with the 160 kDa band. Western blots using the T654 site- and phosphorylation-specific antibody are shown in Fig. 4, lanes 5 and 6, and indicate that T654 is phosphorylated by both Rho-kinase and DMPK. As a control, phosphorylation of GST-MYPT1 by the catalytic subunit of the cAMP-dependent protein kinase was monitored. This showed phosphorylation via the autoradiogram but the signal with the specific antibody was considerably reduced (Fig. 4, lane 7).

These data establish that T654 is a major site of phosphorylation for DMPK on MYPT1. In fact, from the results obtained with the T654A mutant there is little evidence for a second major site of phosphorylation.

3.4. Effect of phosphorylation of MYPT1 by DMPK on phosphatase activity

A critical point was to determine if phosphorylation of T654 by DMPK inhibited PP1c activity. GST-MYPT1 was phosphorylated by DMPK for various times (the time points indicated in Fig. 5 correspond to those shown in Fig. 1), mixed with PP1c and phosphatase activity was assayed using [^{32}P]MLC20. As shown in Fig. 5 the phosphorylated GST-MYPT1 inhibited PP1c activity. The maximum extent of inhibition was about 80%. The marked sensitivity of the phosphorylation dependence of inhibition is not understood, but may reflect a lower concentration of active PP1c, compared to total protein concentration. For these experiments the stoichiometry of PP1c:GST-MYPT1 was constant at about 1.5. The inset in Fig. 5 shows inhibition of PP1c with varying amounts of phosphorylated GST-MYPT1. At an apparent stoichiometry of about 1 the inhibition was close to maximum.

4. Discussion

The data presented above show that MYPT1 is a substrate for DMPK and its phosphorylation at T654 inhibits PP1c activity. Designation of the inhibitory phosphorylation site depends on the MYPT1 isoform, particularly on the presence or absence of a central insert. For example, in the shorter chicken MYPT1 isoform the site is T654 and for the longer (plus sequence insert) isoform it is T695 [8]. In human MYPT1 it is T696 [26]. However, in each isoform the sequence around this site is highly conserved. The sequence G653 to T747 in the longer chicken isoform (G612 to T706 in the shorter isoform) is identical to human and porcine MYPT1 [26,27] and is 97% identical to rat MYPT1 [28]. Even in MYPT2, the product of a different gene, the sequence R632 to T660 is 93% identical to the chicken isoforms [9]. In MYPT2 the phosphorylation and putative inhibitory site is T646. Conservation of this sequence suggests that this site has an important function and the finding that several kinases (endogenous kinase [10], Rho-kinase [12], DMPK (this study)) can phosphorylate MYPT1 and inhibit PP1c activity may indicate a general regulatory mechanism, i.e. not confined to one signal transduction pathway. Activation of RhoA obviously is important for Rho-kinase activity and via inhibition of MP is thought to be involved in Ca^{2+} sensitization of smooth muscle [8]. On the other hand it has been suggested that DMPK is regulated by Rac-1 and Raf-1 kinase [5] and a novel member of the small heat shock protein family [29] and may reflect a convergence of signals to regulate a common mechanism, namely phosphorylation of myosin II. Alternatively, the differential localization of Rac-1 and RhoA to specific regions of the cytoskeleton (6) may suggest that myosin II phosphorylation by DMPK and Rho-kinase might be localized similarly.

In order for DMPK to influence MP activity it is reasonable to expect that both DMPK and MYPT1 are expressed in the same cell. Recently it was found that the DMPK protein is detected mainly in skeletal and cardiac muscle and to a lesser extent in smooth muscle [30]. Northern blot analyses also indicate the presence of DMPK in brain [31]. For the myosin targeting subunit both MYPT1 and MYPT2 should be considered. Northern blots for MYPT2 mRNA detected abundant signals in adult human heart and skeletal muscle and a weaker signal in brain [9]. At the protein level both MYPT1 and MYPT2 were identified in brain and heart, although in the latter MYPT2 was the major component [9]. Full-length MYPT1 and MYPT2 were not detected in skeletal muscle but fragments of MYPT2 have been identified [32,33]. Thus, the DMPK/MYPT1 pairing seems to be satisfied in smooth muscle, brain and possibly cardiac muscle. In adult skeletal muscle it is thought that the major isoform is MYPT2 and although MYPT2 contains a conserved sequence flanking a putative phosphorylation site it is not known if MYPT2 is phosphorylated. Another possibility is that expression of MYPT1 and MYPT2 varies at different stages of striated muscle development, or differentiation. In support of this

idea it was found that MYPT1 was detected at relatively high levels in embryonic and neonatal rat cardiomyocytes [34] and also MYPT1 was present in C2C12 cells (Y. Wu and D.J. Hartshorne, personal communication). The importance of myosin light chain kinase (and hence myosin phosphorylation) in cardiac sarcomere organization has recently been documented [35].

In summary, it is shown that MYPT1 is phosphorylated by DMPK and that phosphorylation at T654 inhibits PP1c activity. This situation is similar to that observed for Rho-kinase and for the endogenous kinase present in MP holoenzyme preparations. Inhibition of MP via phosphorylation by different kinases raises the possibility that signal transduction pathways may converge on one mechanism controlling the cell level of myosin phosphorylation.

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